SUPPRESSION SUBTRACTIVE HYBRIDIZATION TO INVESTIGATE VIRUSES IN THE LYMPHOID ORGAN OF *Penaeus merguiensis* AND THE GILLS OF *Cherax quadricarinatus*

VOLUME 1: CHAPTERS

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R U S A I N I

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I, the undersigned, the author of this thesis was an awardee of Australian Development Scholarship (ADS) Program from the Australian Agency for International Development (AusAID). Partly this work supported by Graduate Research Scheme (GRS) Grant 2010, 2011 and 2012 of Faculty of Medicine, Health and Molecular Sciences, James Cook University. In attending international conferences to present parts of this work, financial support was received from the ADS and School of Veterinary and Biomedical Sciences, JCU.

This work is under supervision of A/Prof. Leigh Owens. Editorial assistance and critical feedback also provided by my Co-supervisors Dr. Ellen Ariel and Dr. Graham W. Burgess. Suggestion on the bench work and proof reading some of the chapters was offered by Dr. Kathy A. LaFauce and Dr. Jennifer Elliman. Northern Queensland farmers contributed in supplying Australian banana prawn *Penaeus merguiensis* and freshwater redclaw crayfish *Cherax quadricarinatus* for this study. Extracted DNA from the gills and cuticular epithelium of crayfish was received from Kelly M.L. Condon and Dr. Rachel O. Bowater. The QPF1/QPR1 primer sequences were provided by Dr. Jeff A. Cowley.

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DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted in compliance with the National and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animal for Scientific Purposes, 7th Edition, 2004 and the Qld Animal Care and Protection Act, 2001. The proposed research methodology received animal ethics approval from the James Cook University Animal Ethics Committee Approval Number A1475 and A1881.

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DEDICATION

In the name of Allah, the compassionate, the merciful
Praise be to Allah, Lord of the universe
Peace and prayers be upon His final prophet and messenger

Allah has let free the two bodies of flowing water, meeting together. Between them is a barrier which they do not transgress. Then which of the favours of your Lord will ye deny? Out of them come pearls and coral. Then which of the favours of your Lord will ye deny? And His are the ships, sailing smoothly through the seas, lofty as mountains. Then which of the favours of your Lord will ye deny? All that is on earth will perish. But will abide (forever) the face of thy Lord-full of majesty, bounty and honour. Then which of the favours of your Lord will ye deny? Of Him seeks every creature in the heavens and on earth, every day in (new) splendour doth He (shine). Then which of the favours of your Lord will ye deny?
(The Holy Qur'an, 55: 19 – 40).

ABSTRACT

A polymerase chain reaction (PCR)-based cDNA subtraction technique termed suppression subtractive hybridization (SSH) was used to investigate the possible viral aetiology of cellular changes in decapod crustacea. These included hypertrophied nuclei with marginated chromatin (signet rings)but without Cowdry type A (CA) intranuclear inclusion bodies in the gills of freshwater redclaw crayfish *Cherax quadricarinatus* (Chapter 4), spheroid cells in the lymphoid organ of banana prawn *Penaeus merguiensis* (Chapter 6) and to identify differential gene expression which were associated with these two cellular changes. In both cases, viral genomes were not detected in SSH cDNA libraries, but multiple-transcripts were identified being induced in the hypertrophied nuclei population of redclaw crayfish and the hatchery population of banana prawn. These transcripts represented protein related to immunity, proteases and inhibitors, synthesis, processing and regulation-related proteins, structural and cytoskeletal related proteins, energy and metabolism factors, and ribosomal proteins, which are all known to be involved in biological process and defence mechanisms against infectious pathogens, in particular viral diseases.

To investigate the probability of the viral aetiology of the lesions in *C. quadricarinatus* (Chapter 5) and *P. merguiensis* (Chapter 6) due to virus with no poly(A) tail, reverse transcriptase (RT)-PCR using primers designed from Bunyaviridae were performed. However, the results showed those were not the case. The PCR amplification using HPV140F/HPV140R primers revealed the absence of *Penaeus merguiensis* densovirus (PmergDNV) or related sequences in the redclaw populations (Chapter 5), but suggested the presence of PmergDNV in the hatchery population, while it was undetected in the wild population (Chapter 6). This suggested that these spheroid cells may be formed as defensive response against the viral infection.

Further studies were conducted to uncover the cause of the signet ring changes in the gills of redclaw crayfish using several parvovirus primers for PCR amplifications (Chapter 5). Instead of identifying the exogenous viral sequences, the presence of

endogenous Brevidensovirus-like elements (EBreVEs) were detected for the first time in *C. quadricarinatus*. Nine fragments that can be assembled into four consensus sequences were found from different sources of crayfish suggesting the widespread nature of these elements in *C. quadricarinatus* populations in northern Queensland, Australia. The most remarkable feature of these elements is that they are located in the same region relative to IHNNV sequences and most likely have originated from the non-structural protein of ancestral virus. Even though definitive insertion sites could not be determined, probably most of these elements, if not all, are randomly inserted within the mobile elements (microsatellites) of the host genomes. In addition, the presence of these endogenous virus-like elements may have immunological function for the host through RNAi pathway against infection of the more closely related exogenous viruses. Taken together, these studies have provided an insight into the host-viral interaction at the molecular level. This knowledge may contribute to future research on crustacean immunity into establishing a holistic approach to combat the devastating impact of infectious diseases, in particular viral pathogens, in order to maintain production in crustacean aquaculture.

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LIST OF ABBREVIATION

ADP	Adenosine diphosphate
Agmo	Alkylglycerol monooxygenase
Ago	Argonaute
ALF	Anti-lipopolysaccharide factor
Alix	*A*poptosis-*l*inked-gene-2-*i*nteracting protein *X*
AMP	Anti-microbial peptide
APP	Acute phase protein
ATP	Adenosine triphosphate
β-D-GBP	Beta-D-glucan binding protein
BLAST	Basic local alignment search tool
CA	Cowdry type A (intranuclear inclusion body)
Caspase	Cystein aspartate protease
CCLS	Chemical cross linking subtraction
cDNA	Complementary DNA
CdSPV	*Cherax destructor* systemic parvo-like virus
CHK	Checkpoint homolog and nucleoside diphosphate kinase
cMnSOD	Cytosolic MnSOD
CENP	Centromere protein
Cnf	Connective tissue fibre
CO	Cytochrome oxidase
CP	Capsid protein
CqPV	*Cherax quadricarinatus* parvo-like virus
CRT	Calreticulin
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DAFF	Department of Agriculture, Fisheries and Forestry
ddH_2O	Double distilled H_2O
Dfsl	Direct forward subtractive library
DIG	Digoxigenin
DMC	Disrupted meiotic cDNA

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dpi	Day post-injection
DROP	Direct random oligonucleotide prime
ds cDNA	Double stranded cDNA
EBreVE	Endogenous Brevidensovirus-like element
EDTA	Ethylenediaminetetraacetic acid
EF	Elongation factor
eEF	Eukaryotic (translation) elongation factor
efMOSl	Enriched forward mirror orientation selectionlibrary
eIF	Eukaryotic (translation) initiation factor
EPstDNVE	Endogenous *Penaeus stylirostris* densovirus-like element
ER	Endoplasmic reticulum
EST	Expressed sequence tag
EtBr	Ethidium bromide
EVE	Endogenous viral element
EWSSVE	Endogenous white spot syndrome virus-like element
FaMeT	Farnesoic acid *0*-methytransferase
FAO	Food and agriculture organisation of the united nation
FBSF	Foetal bovine serum-F
Fc	*Fenneropenaeus chinensis*
FITC	Fluorescein isothiocyanate
FRDC	Fisheries Research and Development Corporation
G6PDH	Glucose-6-phosphate dehydrogenase
GAV	Gill associated virus
GHF	Glycosyl hydrolase family
GILT	Gamma-interferon-inducible lysosomal thiol reductase
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
H & E	Haematoxylin and eosin
HEL	Hen egg lysozyme
H-L(3)MBT-LIKE	Human-lethal (3) malignant brain tumor-like 2 protein

HH	Hyaline haemocyte
hpi	Hours post-injection
HPV	Hepatopancreatic parvo-like virus
HSP	Heat shock protein
ICDH	Isocitrate dehydrogenase
IGFBP	Insulin growth factor binding protein
IFA	Immunofluorescence assay
IFN	Interferon
IgG	Immuno-globulin G
IHC	Immunohistochemistry
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
IMNV	Infectious myonecrosis virus
IN	Integrase
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRF	Interferon regulatory factors
ISH	*In situ* hybridization
JCU	James Cook University
KGDH	Ketoglutarate dehydrogenase
LB agar	Lauria-Bertani agar
LGH	Large granular haemocyte
LINE	Long interspersed elements
LO	Lymphoid organ
LOS	Lymphoid organ spheroid
LOV	Lymphoid organ virus
LOVV	Lymphoid organ vacuolization virus
LPS	Lipopolysaccharides
LPV	Lymphoidal parvo-like virus
LRR	Leucine-rich repeat
LSNV	Laem-Singh virus
LT	Lymphoid tubule
LTR	Long terminal repeat
Lum	Lumen

Lv	*Litopenaeus vannamei (Penaeus vannamei)*
MAb	Monoclonal antibody
MARFU	Marine and Aquaculture Research Facilities Unit
MAVS	Mitochondrial antiviral signalling protein
MCL	Myeloid cell leukaemia
MCMS	Midcrop mortality syndrome
MDH	Malate dehydrogenase
MHC	Major histocompatibility complex
MIH	Moult inhibiting hormone
Mj	*Marsupenaeus japonicus (Penaeus japonicus)*
MMP	Mitochondrial membrane permeabilization
MMT	Million metric tones
MnSOD	Manganese superoxide dismutase
MOS	Mirror orientation selection
MoV	Mourilyan virus
mRNA	Messenger RNA
MT	Metallothionein
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide
ND	NADH dehydrogenase
nDNA	Nuclear DNA
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NCBI	National centre for biotechnology information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptors
NLS	Nuclear localization signal
NOD	Nuclear oligomerization domain
NS	Non-structural protein
NTC	Non-template control
OHM	Oka organ hypertrophy and metastasis syndrome
ORF	Open reading frame
PBS	Phosphate buffer saline

PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
Pen	Penaeidin
PG	Peptidoglycan
PGMRC	Progestin membrane receptor component
Pm	*Penaeus monodon*
PmergDNV	*Penaeus merguiensis* densovirus
PO	Phenoloxidase
POD	Peroxidase
Poly(A) RNA	Polyadenylation RNA
proPO	Prophenoloxidase
PRR	Pattern recognition receptor
Prx	Peroxiredoxin
PTC	Peptidyl-transferase centre
qRT-PCR	Quantitative RT-PCR
RACE	Rapid amplification of cDNA end
RACK	Receptor of activated C kinase
Ran	Ras-related nuclear protein
RDA	Representational difference analysis
RIG	Retinoic acid inducible gene
RING	Really interesting new gene
RISC	RNA-induced silencing complex
RLO	Rickettsia-like organisms
RLR	RIG-like receptor
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RPCH	Red pigment concentrating hormone
RPS	Rhabdovirus of penaeid prawn
rRNA	Ribosamal RNA
RT-PCR	Reverse transcriptase-polymerase chain reaction
RWA	RNA wash solution

SAA	Serum amyloid A
SAGE	Serial analysis of gene expression
SA-PMP	Streptavidin magnesphere paramagnetic particle
SD	Standard deviation
SDH	Succinate dehydrogenase
SEM	Scanning electron microscope
SEP	Secretory eggshell protein precursor
Serpin	Serine protease inhibitor
SGH	Small granular haemocyte
SIAH	Seven in absentia homolog
Sin	Haemal sinuses
Sina	Seven in absentia
Smc	Stromal matrix cells
SMV	Spawner-isolated mortality virus
SPF	Specific pathogen free
SPR	Specific pathogen resistance
SOC medium	Superoptimal broth with catabolite repression medium
SOD	Superoxidase dismutase
SOP	Shrimp ovarian peritrophin
SPC	Signal peptidase complex
ss cDNA	Single stranded cDNA
ss RNA	Single stranded RNA
SSH	Suppression subtractive hybridisation
STING	Stimulator of interferon gene
SVBMS	School of Veterinary and Biomedical Sciences
TAE	Tris-acetate-EDTA
TCA	Tricarboxylc acid
TCTP	Translationally controlled tumour protein
TEM	Transmission electron microscope
TGase	Transglutaminase
TIF	Translation initiation factor
TLR	Toll-like receptor

tmRNA	Transfer-messenger RNA
tRNA	Transfer RNA
TOM	Translocase of the mitochondrial outer membrane
TRBP	Transactivating response RNA-binding protein
Trx	Thioredoxin
TSL	Tumour suppressor-like protein
TSP	Thrombospondin
TSV	Taura syndrome virus
TUNEL	Terminal deoxynucleotidyl transferase (TdT) – mediated dUTP nick-end labelling
Ubc	Ubiquitin
VADC	Voltage-dependent anion channel
Vn	Vitellin
VP	Viral protein
WAP	Whey acidic protein
WSSV	White spot syndrome virus
X-gal	5-bromo-4-choloro-3-indodyl-β-D-galactopyranoside
YHV	Yellow head virus

CHAPTER 1

GENERAL INTRODUCTION

1.1. Overview

The world fish production reached 148.5 million metric tonnes (MMT) in 2010. While production of capture fisheries remained steady at 90 MMT since 2001, aquaculture production (excluding aquatic plants and non-food products) has continued to increase with annual growth rate around 6.3% between 2001 (34.6 MMT) to 2010 (59.9 MMT). It was estimated that in 2011 the aquaculture industry made up 63.6 MMT from 154 MMT of global fish production. If aquatic plants and non-food products were included, then in 2010 global aquaculture produced approximately 78.9 MMT at a value of US $125.2 billion from total world fisheries (FAO, 2012a; FAO, 2012d).

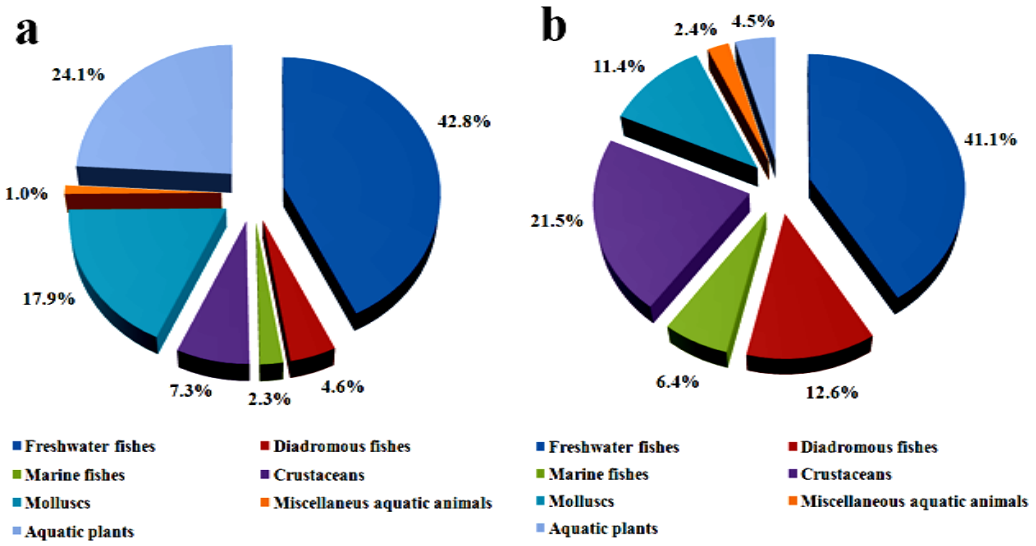


Figure 1.1. Estimated world aquaculture production based on the major species group in terms of total volume, 78.9 MMT (a) and value,US $125.2 billion (b)in 2010 (FAO, 2012a; FAO, 2012d; FAO, 2012b).

Crustaceans accounted for 7.3% of the total quantity of world aquaculture production, molluscs 17.9% and freshwater fishes 42.8% (Figure 1.1). However in terms of value, crustaceans bring a higher price per unit than the other species groups. Crustaceans contributed 21.5% to the total value of world aquaculture production in 2010, with freshwater fishes accounting for 41.1% (FAO, 2012a; FAO, 2012d). Of the 5.7 MMT of crustacean production which is valued at US $26.9 billion, prawns make a major contribution, around 66.2% of the total cultured crustacean production in total volume (3.8 MMT) and 62.1% in value (US $16.7

billion) (Figure 1.2). Two species dominate the prawn aquaculture industry (Figure 1.3), the whiteleg prawn, *Penaeus vannamei* (71.8%) and the giant black tiger prawn, *P. monodon* (20.6%), while the banana prawn *P. merguiensis* also makes a significant contribution (0.5%) (FAO, 2012b; FAO, 2012c).

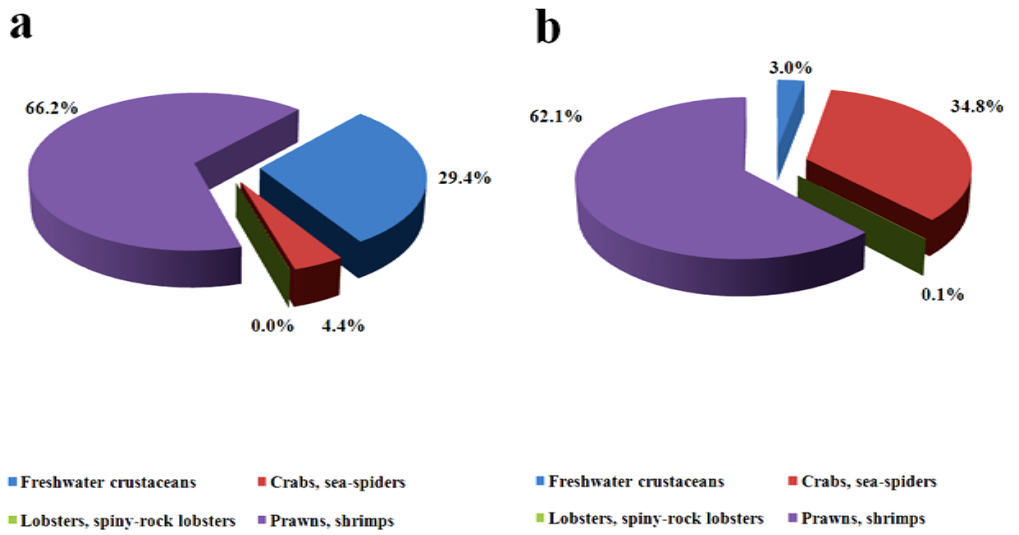


Figure 1.2. Estimated world crustacean aquaculture production in terms of total volume, 5.7 MMT (a) and value, US $26.9 billion (b) in 2012(FAO, 2012a; FAO, 2012d; FAO, 2012b).

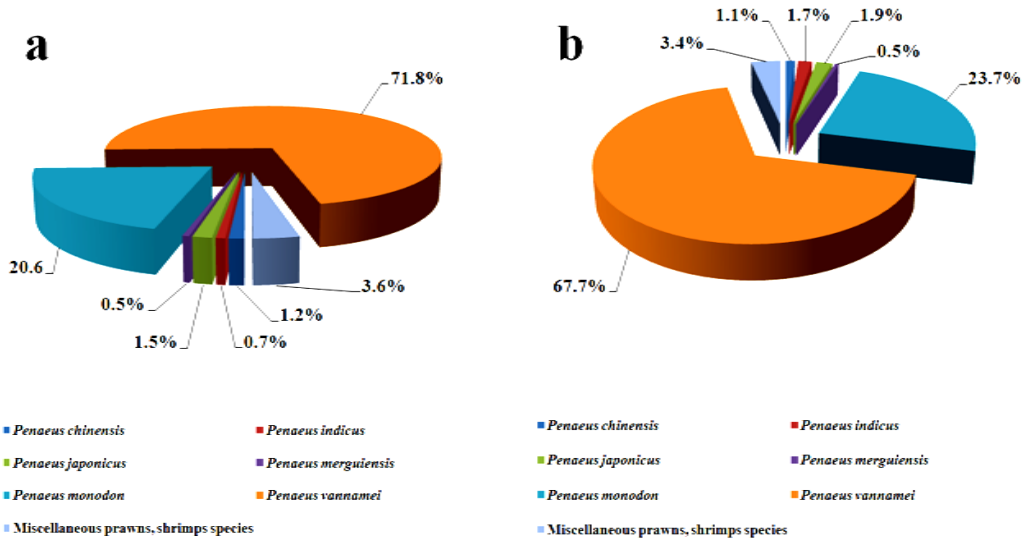


Figure 1.3. Estimated world prawn aquaculture production in terms of total volume, 3.8 MMT (a) and value, US $16.7 billion (b)in 2010 (FAO, 2012b; FAO, 2012c).

The rapid development of the prawn aquaculture industry in the last three decades has been hampered by emerging infectious pathogens. It was predicted that in the

last 15 years around US $15 billion loss in this industry was due to infectious diseases (Flegel *et al.*, 2008; Flegel, 2012; Lightner *et al.*, 2012), but a previous estimation revealed that this figure may reach US $3 billion annually (Lundin, 1997). Viruses are the main cause of catastrophic effects to cultured prawn industry worldwide. Approximately 60% of disease loses in this industry was associated with viral diseases (Flegel *et al.*, 2008; Flegel, 2012). More than 20 viral related diseases in penaeid prawns have been listed (Bonami *et al.*, 2008) and the number has increased in recent years as new viruses are identified. Recently, infectious myonecrosis virus (IMNV), Mourilyan virus (MoV) and Laem-Singh virus (LSNV) have emerged as disease agents of penaeid prawns (Cowley *et al.*, 2005b; Tang *et al.*, 2005; Rajendran *et al.*, 2006; Sritunyalucksana *et al.*, 2006). Meanwhile, white spot syndrome virus (WSSV), yellow head virus (YHV), Taura syndrome virus (TSV), and infectious hypodermal and haematopoietic necrosis virus (IHHNV) are still the main threats to the penaeid prawn industry. Viral infection can not only cause mortality, but also slow growth (stunting and runting), produce abnormalities (deformity) and decrease the quality of carcasses; thereby causing great economic loses.

Many attempts have been made to deal with the devastating impact of infectious pathogens in prawn culture. These include the applications of antibiotics, vaccines and immunostimulants. However, it is more likely that most of these efforts produce more controversies than solutions. For example, the use of antibiotics or other therapeutic agents in ponds can create high production costs, contamination of the environment and carcasses, andproduce abnormalities and mortality (Smith *et al.*, 2003). Recently, administration of antibiotics has been reported to suppress or down regulate the immune components of the prawn (Fagutao *et al.*, 2009). In addition, the long term application of these chemical substances results in pathogens becoming resistant to antibiotics (Smith *et al.*, 2003). More worrying is the wide spread antibiotic resistant bacteria in the aquaculture industry that may transfer resistance plasmids to bacteria associated with human health problems (Kesarcodi-Watson *et al.*, 2008).

Improvement of the immune system of the stock is an alternative method to control infectious pathogens and to reduce the application of antibiotics.As it has been successfully applied in humans, livestock and cultured fish, the administration of vaccines, immunostimulants and probiotics have also been considered as a means to protect prawn stock. However the real efficacy of these methods is still open to question due to the temporary nature of the effects (Smith *et al.*, 2003; Hauton and Smith, 2007). If the efficacy cannot last the life span of penaeids, for instance 1.7 years for *P. stylirostris*(Vogt, 2012), at least it should give protection for about 4 months which is the cultivation period of prawns in ponds. In addition, most of these tests were conducted in laboratory trials without proving the efficacy in a field trial (Smith *et al.*, 2003; Flegel *et al.*, 2008). Furthermore, inappropriate or repeated application of vaccines or immunostimulants can also have a detrimental effect on the prawn immune system itself (Smith *et al.*, 2003; Hauton and Smith, 2007).

RNA interference has also been considered as a therapeutic tool to combat viral infection in cultured prawns (Robalino *et al.*, 2004; Robalino *et al.*, 2007b; La Fauce and Owens, 2009; La Fauce and Owens, 2012). Even though this technique seems very promising, once again questions arise about the longevity of the protection and efficacy of this technique in the field trial. Furthermore, several drawbacks including off target effect, activation of RNAi suppressor proteins, cost and safetyissues, and ethically acceptable and commercially viable products still remain to be resolved (Flegel *et al.*, 2008; Flegel and Sritunyalucksana, 2011; La Fauce and Owens, 2012).

Breeding programs to produce specific pathogen free (SPF), specific pathogen resistant (SPR) or genetically modified strains to some extent are able to minimise the impact of viral infection (Smith *et al.*, 2003; Flegel, 2012). However, devastating impacts of broad range of viral diseases to the industry cannot be unravelled relying only on these programs since free or resistant (tolerant) to one particular strain of virus does not mean freedom or tolerance to another (Moss *et al.*, 2005; Flegel *et al.*, 2008). These methods are still awaiting further development to maintain prawn production (Smith *et al.*, 2003). Therefore, understanding penaeid

prawn immunity has been seen as essential to eliminating the catastrophic effects of infectious pathogens. This may lead to the development of immunointervention strategies that can in turn increase production and the profit margin of prawn aquaculture industry.

Like other invertebrates, crustaceans such as crabs, lobsters, crayfish, and prawns do not possess antibody based immunity. Their defence mechanisms depend on an innate immunity consisting of cellular and humoral components that are remarkably effective in recognising and sequestering invading pathogens (Roch, 1999; Bachere, 2003; Loker *et al.*, 2004). These immune responses are mainly based on the activities of the blood cells or the haemocytes, where the immunoreactive factors are stored in an inactive state and then released when stimulated by the presence of foreign invaders (Soderhall and Cerenius, 1992; Rodriguez and Le Moullac, 2000; Smith *et al.*, 2003).

Apart from the haemocytes, the lymphoid organ (LO) of penaeid prawns is thought to have an important role in defence against invading pathogens (Nakamura, 1987; Kondo *et al.*, 1994; Martin *et al.*, 1996; Hasson *et al.*, 1999b; Anggraeni and Owens, 2000; van de Braak *et al.*, 2002b). Currently, the discovery of defence-related genes within the LO confirmed the protective function of this organ in penaeid prawns (Pongsomboon *et al.*, 2008). Further studies on genes related to immunity within the lymphoid organ may lead to a better understanding of the immunological role of this organ.

Recently, the application of the high throughput molecular method, subtractive-polymerase chain reaction (subtractive-PCR), also called suppression subtractive hybridization (SSH) in identifying differential gene expression has become an important tool to gain a better understanding of prawn immunology. The suppression subtractive hybridization has been used in identifying differential gene expression of penaeid prawns in viral(Bangrak *et al.*, 2002; James *et al.*, 2010; Junkunlo *et al.*, 2010; Prapavorarat *et al.*, 2010), bacterial (de Lorgeril *et al.*, 2005; Nayak *et al.*, 2010)and environmental challenges(de la Vega *et al.*, 2007a). These gene transcripts were classified according to their functional classes (Pan *et al.*,

2005; de la Vega *et al.*, 2007a; de la Vega *et al.*, 2007b; Robalino *et al.*, 2007a; Zhao *et al.*, 2007) based on the sequences having significant homology in the GenBank database. Some of these genes are unknown because they have no significant matches with genes in GenBank (Pan *et al.*, 2005; Robalino *et al.*, 2007a). Even though some of these genes match with the Genbank database, some of them have an unknown function (Pan *et al.*, 2005; de la Vega *et al.*, 2007b; Zhao *et al.*, 2007). The levels of transcription of these genes have also been claimed to undergo up or down regulation (Bangrak *et al.*, 2002; He *et al.*, 2004; de Lorgeril *et al.*, 2005; Pan *et al.*, 2005; Wang *et al.*, 2006; de la Vega *et al.*, 2007a; de la Vega *et al.*, 2007b; Reyes *et al.*, 2007; Zhao *et al.*, 2007; García *et al.*, 2009; Lu *et al.*, 2009). However, the regulatory mechanism and the exact role of genes in prawns' immunity are poorly investigated.

These SSH cDNA libraries of prawn species including *P. monodon* (Bangrak *et al.*, 2002; de la Vega *et al.*, 2007a; de la Vega *et al.*, 2007b; Leelatanawit *et al.*, 2008), *P. vannamei* (O'Leary *et al.*, 2006; Reyes *et al.*, 2007; Robalino *et al.*, 2007a; Zhao *et al.*, 2007; García *et al.*, 2009), *P. japonicas* (He *et al.*, 2004; He *et al.*, 2005; Pan *et al.*, 2005), *P. stylirostris* (de Lorgeril *et al.*, 2005), *P. chinensis* (Wang *et al.*, 2006) and *Macrobrachium rosenbergii* (Lu *et al.*, 2009), were constructed from haemocytes, hepatopancreas, gills, subcuticular epithelium of cephalothorax and testis samples. However, the SSH cDNA library from *Penaeus merguiensis* and the lymphoid organ of penaeid prawns are unexplored. Viral genomes have also been elucidated by the SSH method in haemocytes, hepatopancreas, gills and subcuticular epithelium of cephalothorax of penaeid species (Reyes *et al.*, 2007; Robalino *et al.*, 2007a; García *et al.*, 2009), but the expression of viral genomes in the lymphoid organ of viral infected penaeid prawns has not been investigated using SSH.

What is more, around 75 – 100% of the banana prawn, *P. merguiensis* population from different family lines in northern Queensland has massive amounts of spheroid development, comprising over 40% of the lymphoid organ (Owens, unpublished data). Despite this, no candidate virus has been identified in causing these changes and differential gene transcripts of *P. merguiensis* are unstudied. Therefore, a study

using SSH on the lymphoid organ of banana prawn infected with viral pathogens was conducted to determine the viral genomes within the LO. Any differentially expressed genes induced during a viral infection were also documented.

As a pilot study of suppression subtractive hybridization technique, an investigation was conducted to characterise possible viral genomes and differentially expressed genes from the gills of two different populations of redclaw crayfish *Cherax quadricarinatus*. Hypertrophic nuclei with rarefied chromatin without Cowdry type A (CA) intranuclear inclusion bodies in the gills were identified in a population of redclaw crayfish (hypertrophied nuclei population), while these changes were not observed in another crayfish population (non-hypertrophied nuclei population) in aquaculture facilities of the School of Veterinary and Biomedical Sciences, James Cook University. These nuclear changes resemble those associated with parvovirus infection previously reported in this species (Edgerton *et al.*, 2000). However, since this study became more challenging and interesting, then more time was allocated to uncover the viral aetiology of the nuclear changes.

1.2. Hypothesis

The working hypothesis for this research is that an unknown virus has caused spheroid formation in the lymphoid organ of the Australian banana prawn *Penaeus merguiensis*. In addition, that the immune related genes within the lymphoid organ are expressed differently during a viral infection.

1.3. Research Aims

The main objective of this study is to identify the viral genes and differential host gene expression in the lymphoid organ of viral infected *P. merguiensis*using suppression subtractive hybridization. The identification of viral genomes and differential transcripts of genes in the lymphoid organ of penaeid prawns is important in understanding the mechanism of host-virus interaction at a molecular level that may help to circumvent the catastrophic effect of viral diseases on the penaeid prawn industry. To test the technique, a pilot study on using SSH on the gills of redclaw crayfish, *Cherax quadricarinatus* was undertaken.

CHAPTER 2

REVIEW OF LITERATURE

Partly published. Rusaini and Owens, L. (2010). Insight into the lymphoid organ of penaeid prawns: A review. *Fish Shellfish Immunol **29***: 367 – 377 (Appendix 4.1)

2.1. Introduction

The rapid development of the cultured prawn industry is associated with environmental and sociological disturbances in land use, the ecology of the aquatic organisms and global trade patterns. A major consequence of these changes is the emergence and the spread of infectious diseases. In the late 1980s, previously unknown diseases emerged in the cultured prawns both in Asia and Americas, spread rapidly to all countries farming prawns and brought catastrophe to this industry around the world. Even though some progress has been made to deal with these challenges and recovery has taken place in recent years, infectious diseases, in particular viral diseasesstill remain a major problem to the prawn aquaculture industry (Flegel *et al.*, 2008; Walker and Mohan, 2009). Recently, knowledge on immune response of the prawn has been considered to play an important part in establishing a holistic approach to deal with this problem.This review will present the state of knowledge of the penaeid lymphoid organ. It will then go on to explain suppression subtractive hybridization as a method in identifying prawn genes or gene products.

2.2. Lymphoid Organ of Penaeid Prawns

In crustacea, the lymphoid organ has only been identified in penaeid species. Other crustacea such as crabs, lobsters, and crayfish do not possess the lymphoid organ. The presence of this organ in penaeids was first noticed in the koroi prawn (*Penaeus orientalis* Kishinouye). Afterward, it was also found in the ginger prawn (*P. japonicus* Bate), the giant tiger prawn (*P. monodon* Fabricius), the white prawn (*P. indicus* de Man) and the banana prawn (*P. merguiensis*de Man) (Oka, 1969). Later on, the lymphoid organ was discovered in the ridgeback prawn (*Sicyonia ingentis*) but it was incorrectly identified as haematopoietic tissue (Martin *et al.*, 1987; Hose *et al.*, 1992; Martin *et al.*, 1996). Meanwhile, the development of the lymphoid organwas described in *P. japonicus* larvae (Nakamura, 1987). Sometimes this organ was called Oka or Nakamura organ (Lightner and Brock, 1987; Lightner *et al.*, 1987; Nadala *et al.*, 1992; Lu *et al.*, 1995). However, the more usual term, the lymphoid organ, will be used throughout this review.

2.2.1. Localisation and anatomical features of the lymphoid organ

There are divergent views about the location of lymphoid organ in penaeid prawns.These differences in the LO positions described by the authors (Oka, 1969; Martin *et al.*, 1987; van de Braak *et al.*, 2002b; Rusaini, 2006; Duangsuwan *et al.*, 2008a) are probably due to the differences in histological planes, either sagittal or cross-sectional, and also the cutting position of the cephalothorax: anterior, posterior or lateral. However, Bell and Lightner (1988) indicated that the LO lay ventro-lateral to the junction of the anterior and posterior stomach chamber and opposed slightly dorso-anterior to the ventral hepatopancreas.

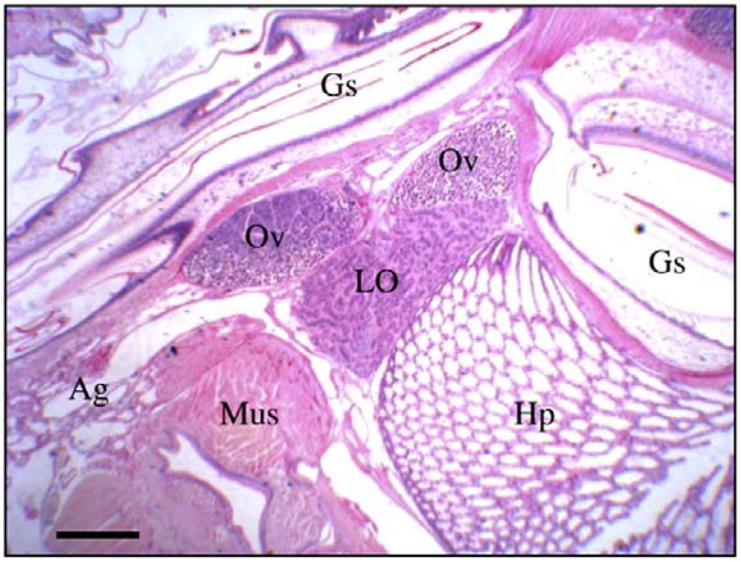


Figure 2.1. Overall longitudinal view of the lymphoid organ and surrounding tissue of *Penaeus monodon* female, H & E stain, scale bar = 200 µm. Ag, antennal gland; Gs, gastric sieve; Hp: hepatopancreas; Mus, muscle; Ov, ovary; Sin, haemal sinuses (Rusaini, 2006).

Sex and gonad maturation may also contribute to the differences in the LO position. In males, the LO lies between the hepatopancreas and the stomach (gastric sieve). In female prawns, this organ is positioned between the ovary and the hepatopancreas (Figure 2.1); the ovary seems to press this organ onto the upper part of the hepatopancreas (Oka, 1969). Moreover, the position of the LO possibly changes with the life cycle of the prawns (Nakamura, 1987).

The lymphoid organ changes in size during the developmental stages of the prawn. At mysis 3, the LO was relatively the same size as in postlarvae 1, 2 and 3. Between postlarvae 4 – 20, the LO decreased in relative size. However, from postlarvae 20, the size of the LO increased significantly. The LO was smallest in the postlarvae 4 and 10 which suggests that at these stages the larvae are most vulnerable to infectious pathogens (Nakamura, 1987).

Animal size, species and health status may also affect the LO size. The bigger the prawn, the larger the size of the LO. The LO sizewas less than 2 mm in diameter in *P. chinensis* with a body length of 12 – 13 cm (Shao *et al.*, 2004). In the ridgeback prawn, S*icyonia ingentis* with an average body weight of 14.5 g, the LO had an oval shape and ranged between 1 × 1.5 mm to 1.5 × 4 mm in size (Martin *et al.*, 1987). The lymphoid organ of the black tiger prawn, *P. monodon* with a body weight of 150 – 170 g (body length of 18 – 20 cm) was ovoid in shape and measured around 3 – 4 mm in length, 2 – 3 mm in width, and 1 – 2 mm in thickness (Duangsuwan *et al.*, 2008a). In addition, viral infection may cause enlargement of the lymphoid organ. In*P. chinensis*(body length of 16 – 17 cm) with tissue changes (lymphoid organ spheroid cells) within the LO probably due to viral infection, the LO size was more than 2 mm in diameter(Shao *et al.*, 2004). By weighing the LO in *P. stylirostris* with an average weight of 5 – 6 g, the LO was 15 ± 5 mg in the control prawns compared to 110 ± 10 mg in prawns infected with rhabdovirus of penaeid shrimp (RPS). Similarly, in prawns with average weight of 15 – 16 g, the LO was 17.8 ± 0.3 mg in an uninfected control group and 107 ± 11 mg in RPS injected prawns (Nadala *et al.*, 1992).

Structurally, the penaeid lymphoid organ comprises a pair of lobes (Figure 2.2) (Bell and Lightner, 1988; van de Braak *et al.*, 2002b; Rusaini, 2006; Duangsuwan *et al.*, 2008a). These two lobes are similar, surrounded by connective tissue capsules and connected directly to the heart via the subgastric artery. Valves on the front of the heart ensure that the blood flow is unidirectional into the central lumen of the LO. Each lobe consists of tubules with a central haemal lumen, stromal matrix cells and interstitial sinuses. Tubules are encircled with connective tissue fibres. Haemal

sinuses occupy the spaces between tubules. The lumen sometimes appears occluded, often with haemocytes and lined with flattened endothelial cells (Bell and Lightner, 1988; van de Braak et al., 2002b). From the lumen, haemocytes may enter the layer of endothelial cells, move into the stromalmatrix and penetrate the open circulatory system, the haemal sinuses (Anggraeni and Owens, 2000; van de Braak et al., 2002b; Duangsuwan et al., 2008a).

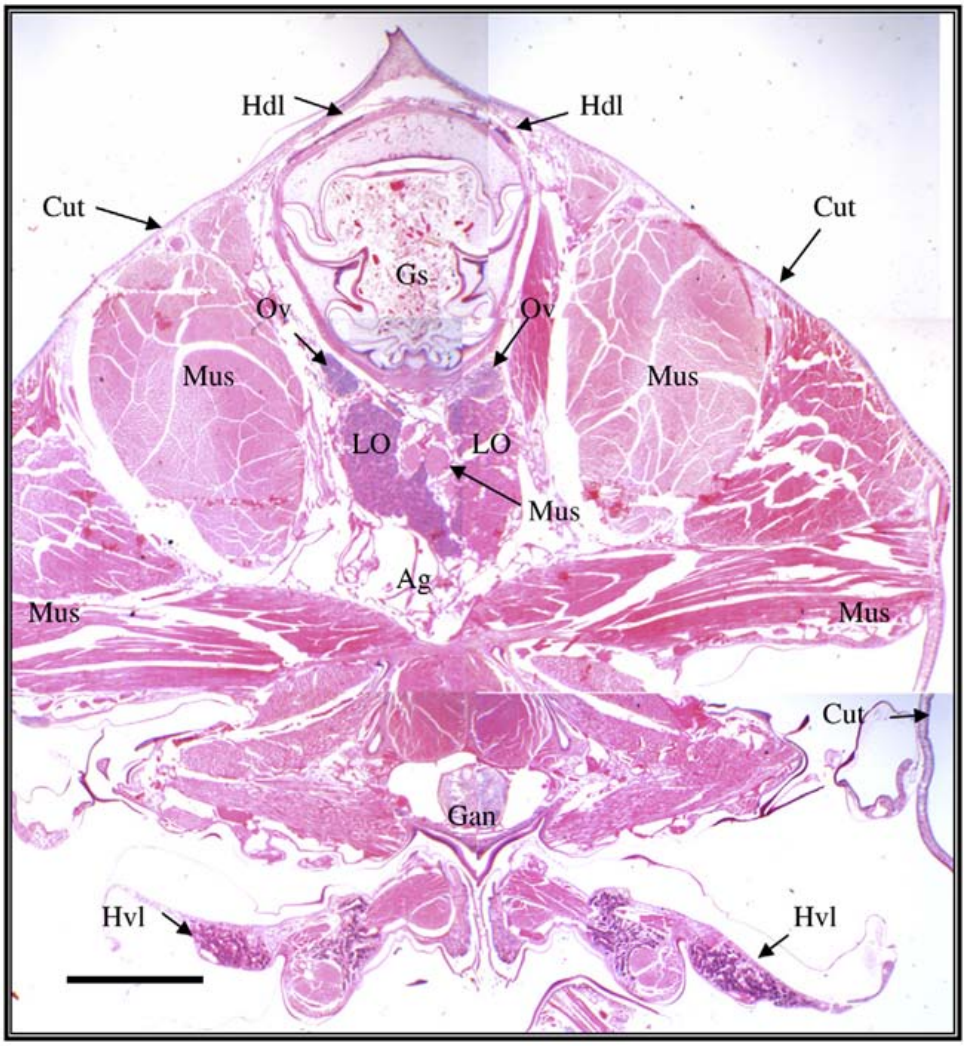


Figure 2.2. Transverse section of the lymphoid organ (LO) and surrounding tissue of *Penaeus monodon*. The LO consists of two lobes located ventro-lateral of the gastric sieve. H & E stain. Scale bar = 100 µm. Ag, antennal gland; Cut, cuticle; Gan, ganglion; Gs, gastric sieve, Hdl, haematopoietic dorsal lobules; Hvl, haematopoietic ventral lobules; Mus, muscle; LO, lymphoid organ; Ov, ovary (Rusaini, 2006).

Vascular branches in the lymphoid organ have been described differently by various authors. It has been claimed that lymphoid tubules were afferent vessels branching

from the ophthalmic artery (Martin *et al.*, 1987). However, these tubules branch from the subgastric artery. The anterior aorta from the heart branches into ophthalmic and subgastric arteries. The ophthalmic artery divides further and terminates in the eyestalk. Meanwhile, the subgastric artery enters the lymphoid organ and branches several times forming a vascular plexus within this organ. Finally, the subgastric artery branches further to form terminal capillaries, the lumen of the LO tubules (Bell and Lightner, 1988; Duangsuwan *et al.*, 2008a).Because of this branching pattern, some authors (Oka, 1969; Martin *et al.*, 1987; Shao *et al.*, 2004; Duangsuwan *et al.*, 2008a) believe that this organ is a part of the vascular system. However, there is evidence suggesting this organ mostly has a filtering function removing foreign materials from the haemolymph (Kondo *et al.*, 1994; Martin *et al.*, 1996; van de Braak *et al.*, 2002b; Duangsuwan *et al.*, 2008a) but this does not preclude other as yet unknown functions.

2.2.2. The function of the lymphoid organ

Several immune reactivities have been attributed to the lymphoid organ. The lymphoid organ is probably a major phagocytic organ in penaeid prawns (Kondo *et al.*, 1994; Martin *et al.*, 1996; van de Braak *et al.*, 2002b). This organ was more efficient at phagocytosing foreign materials than the other organs evaluated such as gills, heart, digestive gland, abdominal muscle and connective tissue as demonstrated with biotic (Martin *et al.*, 1996; van de Braak *et al.*, 2002b) and abiotic substances (Kondo *et al.*, 1994). Bacterial phagocytosis within the lymphoid organ has been observed in *S. ingentis* injected with *Bacillus subtilis*, Carolina 15-4921 (Martin *et al.*, 1996) and in *P. monodon* injected with *Vibrio anguillarum*, serotype O2 (van de Braak *et al.*, 2002b). Phagocytic activity of the LO for abiotic materials has been studied in *P. japonicus* injected with FITC-latex, colloidal carbon and horseradish peroxidase (Kondo *et al.*, 1994).

The LO is proposed to have a bacteriostatic effect (Burgents *et al.*, 2005) and trapping ability to immobilise foreign material from the haemolymph before this material enters the open circulatory system (Kondo *et al.*, 1994; van de Braak *et al.*, 2002b). This organ also appears as a primary site for accumulation of foreign

substances (Kondo *et al.*, 1994; Burgents *et al.*, 2005). In addition, the lymphoid organ is suggested to be a major site for viral degradation by forming spheroid cells (Anggraeni and Owens, 2000). This supposition was supported by the work of Cowley *et al.*(2005a) and Rajendran *et al.*(2006) in that viral load in the lymphoid organ of penaeid prawns infected with Mourilyan virus is higher compared to other organs.

It has been stated that the lymphoid organ produced haemocytes to be released into the circulation, because in the stromal matrix and occasionally in the lumen, mitotic activity occurred (Hose *et al.*, 1992; Martin *et al.*, 1996). The possibility of cell division (mitosis) within the LO had also been reported by the other researchers (Martin *et al.*, 1987). However, there was not sufficient evidence to support the theory of mitosis within the LO, either in normal LO tubules or in the LOS cells (Anggraeni and Owens, 2000; van de Braak *et al.*, 2002a; van de Braak *et al.*, 2002b). In addition, the published figures of Bell and Lightner (1988) showed that numerous mitotic spindles can be seen in the haematopoietic tissue, some in the E-cells of the hepatopancreas, but they are very rare in the LO. The very small size of the LO and the very large number of haemocytes plus their turnover rate suggests the anatomy of the LO is not compatible with a role in producing haemocytes. Therefore, the supposition that this organ was a haematopoietic organ as claimed by some earlier workers (Martin *et al.*, 1987; Hose *et al.*, 1992; Martin *et al.*, 1996) is incorrect.

The penaeid lymphoid organ has also been considered to be a site for haemocyte maturation. According to Hose *et al*. (1992) hyaline and granular haemocytes matured from two different cell lines. The precursor of hyaline haemocytes (HH) was the hyaline stem cells which matured in the stromal matrix tubules and were released into the lumen, while granulocyte stem cells matured into small granular haemocytes (SGH) then into large granular haemocytes (LGH) also in the LO tubules and then released into the lumen. However, in contrast, van de Braak *et al.*(2002b) believed that production SGH and LGH occurred in the haematopietic

tissue as hyaline cells (HH) and were then released into the haemolymph. The HH matured into SGH, migrated and matured into LGH in connective tissue.

2.2.3. Diseases related to the lymphoid organ spheroid cells

The presence of spheroid cells within the lymphoid organ has been observed in many naturally or experimentally infected penaeid species. Diseases associated with lymphoid organ spheroids have been found in *P. penicillatus* (Lightner *et al.*, 1987), *P. monodon* (Lightner *et al.*, 1987; Owens *et al.*, 1991; Turnbull *et al.*, 1994; Fraser and Owens, 1996), *P. merguiensis* and *P. esculentus* (Owens *et al.*, 1991). These abnormal cells were also observed in *P. vannamei* (Bonami *et al.*, 1992; Hasson *et al.*, 1995), *P. japonicus* (Kondo *et al.*, 1994), *P. stylirostris* (Nadala *et al.*, 1992) and *P. chinensis* (Shao *et al.*, 2004).

The formation of spheroids in the lymphoid organ has been reported in many infectious diseases of penaeid prawns. These abnormal cells were first described as Oka organ hypertrophic and metastasis (OHM) syndrome (Lightner *et al.*, 1987). These cellular changes were also considered to be associated with midcrop mortality syndrome (MCMS) in Australian penaeid prawns (Anggraeni and Owens, 2000) and monodon slow growth syndrome (MSGS) (Anantasomboon *et al.*, 2006). Furthermore, many viral diseases of penaeid species (Table 2.1) were reported to be related to this spheroid formation.

Since this abnormal histopathological feature is mostly found in the lymphoid organ of prawns infected with either RNA or DNA viruses, it is generally believed that this is a non-specific immunodefense reaction of penaeid prawns to viral infection (Anggraeni and Owens, 2000). The spheroid formation might be related to the tolerance of penaeid prawn to viral infection. In the case of a survivor or in chronic infection, the spheroids were formed when animals controlled the infectious agents or at least reacted to them (Rodríguez *et al.*, 2003; Anantasomboon *et al.*, 2008).

Table 2.1. Viral diseases of penaeids associated with the formation of spheroid cells within the lymphoid organ.

Virus type/family	Viral diseases	Species	Authors
DNA viruses			
ssDNA- Parvoviridae	Lymphoidal parvo-like virus (LPV)	*P. monodon* *P. merguiensis* *P. esculentus*	(Owens *et al.*, 1991)
	Spawner-isolated mortality virus (SMV)	*P. monodon*	(Fraser and Owens, 1996)
dsDNA-Nimaviridae	White spot syndrome virus (WSSV)	*P. vannamei*	(Rodríguez *et al.*, 2003; Rodríguez *et al.*, 2012)
		P. japonicus	(Wu and Muroga, 2004)
RNA viruses			
ssRNA- Bunyaviridae	Mourilyan virus (MoV)	*P. monodon*	(Cowley *et al.*, 2005a)
		P. japonicus	(Rajendran *et al.*, 2006)
ssRNA-Dicistroviridae	Taura syndrome virus (TSV)	*P. vannamei*	(Hasson *et al.*, 1995)
ssRNA-Luteoviridae	Laem-Singh virus (LSNV)	*P. monodon*	(Sritunyalucksana *et al.*, 2006)
ssRNA-Rhabdoviridae	Rhabdovirus of penaeid shrimp (RPS)	*P. stylirostris*	(Nadala *et al.*, 1992)
ssRNA-Roniviridae	Yellowhead virus (YHV)	*P. monodon*	(Boonyaratpalin *et al.*, 1993; Chantanachookin *et al.*, 1993)
	Lymphoid organ virus (LOV)/gill associated virus (GAV)	*P. monodon*	(Spann *et al.*, 1995; Spann *et al.*, 1997)
ssRNA-Togaviridae	Lymphoid organ vacuolization virus (LOVV)	*P. vannamei*	(Bonami *et al.*, 1992)
dsRNA-Totiviridae	Infectious myonecrosis virus (IMNV)	*P. vannamei* *P. stylirostris* *P. monodon*	(Tang *et al.*, 2005)

Even though there was no report on a natural bacterial infection related to the LOS formation, there were several publications reporting the presence of spheroids in experimental bacterial infections in penaeid prawns (Alday-Sanz *et al.*, 2002; van de Braak *et al.*, 2002b; Pongsomboon *et al.*, 2008). However, these findings created more questions on spheroids than they solved. Firstly, the lack of statistical analysis questions the validity of the results. Also, comparative histological examination between challenged and control groups was absent. In addition, the absence of any tests to establish viral status of prawns in these experiments undermined these claims. Furthermore, similar to the study of Martin *et al*. (1996), most of these

studies clearly showed that bacterial antigens were generally observed within the lymphoid organ tubules (Alday-Sanz *et al.*, 2002; van de Braak *et al.*, 2002b) not the spheroids.

Another problem with bacterial associated LOS studies is the inconsistency of the results. For example, a study showed that the majority of the spheroid cells were found in prawns from field rearing but LOS cells were seldom found in prawnsmaintainedin recirculation systems (van de Braak *et al.*, 2002b).In addition, a recent study revealed that not only animals that were injected with *Vibrio harveyi* and white spot syndrome virus (WSSV) had spheroid cells but spheroids were also in control animals injected with lobster haemolymph medium (Pongsomboon *et al.*, 2008). These results suggest a predisposition of animals to having spheroids before being used. In addition, another experiment (Alday-Sanz *et al.*, 2002) found spheroids in prawns infected with bacteria by injection only, while in bacterial exposure by immersion and oral intubation, spheroids were never observed adding an inconsistency to the studies.

Studies on the effect of bacteria *V. harveyi* 642 and MCMS viral infection on the formation of spheroid cells in the lymphoid organ of *P. monodon* revealed that the area of spheroids was significantly higher in viral infected or combined viral and bacterial infected groups compared to control or bacterial treatment alone (Anggraeni, 1998). This author suggested that spheroid formation was only caused by viral infection that may be intensified by bacterial infection. Indeed, in most cases if not all, the presence of spheroid cells in the lymphoid organ is associated with viral diseases.

Furthermore, itseems more likely that the most prominent feature of histological changes due to bacterial infection is the formation of melanised nodules within the infected tissues. This inflammatory type of reaction has been observed in the lymphoid organ of penaeid prawns mostly infected with *Vibrio* spp. (Egusa *et al.*, 1988; Jiravanichpaisal and Miyazaki, 1994; Zhixun *et al.*, 2004; Rusaini, 2006; de la Vega *et al.*, 2008). These nodules are produced as a protective response to bacterial infection, to confine the bacteria to the infected tubules and to prevent bacteria from

spreading (Egusa *et al.*, 1988; Jiravanichpaisal and Miyazaki, 1994). Severe necrosis and melanisation of stromal matrix tubules, and haemocytic infiltration within the LO were also observed in prawns infected with *Vibrio* (Soto-Rodriguez *et al.*, 2010). The presence of these cellular changes within the lymphoid organ also supports the supposition that spheroid formation results from viral infection (Anggraeni, 1998; Anggraeni and Owens, 2000) not by bacterial infection as claimed by other authors (Alday-Sanz *et al.*, 2002; van de Braak *et al.*, 2002b; Pongsomboon *et al.*, 2008). Furthermore, Soto-Rodriguez *et al.*(2010) confirmed that there was no evidence of the existence of spheroid cells in *Vibrio* infected prawns.

The formation of spheroid cells within the LO of kuruma prawns due to injection with foreign material including, horseradish peroxidase, FITC-latex and colloidal carbon has also been reported (Kondo *et al.*, 1994). However, this claim is also doubtful. The absence of a control, a non-injected group limits its validation and confounded the results on whether the formation of the spheroid was a result of these foreign substances or previous pathogens. In addition, the formation of the spheroid cells such a long time (1 – 3 months) after injection was also worrying. This long period increases the probability of something else producing spheroids which was then attributed to the injection of the foreign substances. In addition, for a comparison, an increase in the number of spheroids was detected at 48 – 72 hours post-infection (hpi) in *P. japonicus* injected with Mourilyan virus (MoV) (Rajendran *et al.*, 2006) and much faster at 24 hpi in WSSV infected *P. vannamei*(Rodríguez *et al.*, 2012). Similarly, enlargement of the lymphoid organ of *P. monodon* due to spheroid formation occurred within 24 hpi with injection of yellow head virus (YHV) (Soowannayan *et al.*, 2002). This implied that if these previously mentioned substances led to lymphoid organ changes then spheroids would be formed very quickly upon the injection.

The lymphoid organ probably was a prime target and site for replication of most systemic viruses (see Table 2.1) (Nadala *et al.*, 1992; Lu *et al.*, 1995; Spann *et al.*, 1995; Spann *et al.*, 1997; Soowannayan *et al.*, 2002; Wu and Muroga, 2004; Rajendran *et al.*, 2006; Bourchookarn *et al.*, 2008). If it is the case, the presence of

virogenic material or viral components (viral particles, viral nucleic acid and viral nucleocapsid protein) should be visualised within this organ, in particular within the spheroid cells. However, there are reported discrepancies in the presence of viral components in the LO whether in stromal matrix cells or spheroid cells.

Viral genomes have been detected within the lymphoid organ by biomolecular techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) (Soowannayan *et al.*, 2003; Cowley *et al.*, 2005a; Anantasomboon *et al.*, 2008) or real time quantitative RT-PCR (Rajendran *et al.*, 2006). However, these methods cannot distinguish whether the nucleic acid was within the normal lymphoid tubule or the spheroid cells, since the whole LO was used without any separation between stromal matrix cells and spheroid cells. Application of laser microdissection may help to separate the normal lymphoid tubules and spheroids before using biomolecular techniques. Similar results were described when immunofluoresence assay (Nadala *et al.*, 1992), transmission electron microscope (TEM) (Chantanachookin *et al.*, 1993; Wang *et al.*, 1997; Rodríguez *et al.*, 2003) and *in situ* hybridization (ISH) (Wu and Muroga, 2004) were applied to investigate viruses in the lymphoid organ of penaeid prawns. These authors also did not confirm whether these virogenic materials were in the spheroid cells or in the normal tubule stromal matrix cells.

Other studies presented conflicting results. Using TEM, virions were only observed within the lymphoid organ tubules, but not in the spheroids (Spann *et al.*, 1997; Park *et al.*, 1998; Sritunyalucksana *et al.*, 2006). In contrast, using the same method, the presence of virions was illustrated within the spheroids but not in normal tubules (Owens *et al.*, 1991; Bonami *et al.*, 1992). Similarly, viral proteins were found only within the spheroid cells by immunohistochemistry (IHC) (Anantasomboon *et al.*, 2008) and viral nucleic acid by *in situ* hybridization (Andrade *et al.*, 2008). Other investigations found that ISH positive signal was detected both in normal lymphoid tubules and LOS cells (Cowley *et al.*, 2005a; Tang *et al.*, 2005)

The variability in localisation of viral components within the lymphoid organ may be a sign of viral infection stages. It was found that in *P. vannamei* infected with Taura

syndrome virus (TSV), probe-positive signal with *in situ* hybridization was only observed within the normal LO tubules of prawns in the acute phase of the infection. In the late transition and early chronic phases of TSV infection, diffuse positive signal was observed in the stromal matrix and some positive signals in the spheroids. Whilst in chronic stage, only LOS cells were positive for TSV-probe signal (Hasson *et al.*, 1999a; Hasson *et al.*, 1999b).

In transition from subacute to chronic phases of *P. vannamei* infected with infectious myonecrosis virus (IMNV), ISH signal was also predominantly observed in the lymphoid organ spheroid cells (Andrade *et al.*, 2008). Moreover, in penaeid prawns infected with yellow head virus complex, reacted signals of monoclonal antibody (MAb) for IHC and digoxigenin (DIG)-labeled DNA probe of ISH detected only in the lymphoid tubules of moribund prawns (acute phase) and only in the LOS of the surviving animals (chronic phase) (Soowannayan *et al.*, 2003; Spann *et al.*, 2003; Anantasomboon *et al.*, 2008). Using TEM, a similar result was observed in healthy *P. monodon* infected with Australian strain of yellow head virus, gill associated virus (GAV, previously named lymphoid organ virus, LOV) where the localization of this virus was only detected in the spheroid cells (Spann *et al.*, 1995).

The positive reaction during the early stage (acute phase) of viral infection may result from ongoing viral replication and viable virions in the LO tubules where phagocytosing by haemocytes was initiated and then in the later stage (chronic phase) the phagocytic haemocytes migrate into the haemal sinuses where they aggregate to form spheroids (Hasson *et al.*, 1999b; Anggraeni and Owens, 2000). In other words, in the early phase of viral infection, viral components would be in the stromal matrix cells and be gradually moved to end up in the spheroid cells in the later or chronic stage of the infection (Hasson *et al.*, 1999a; Hasson *et al.*, 1999b). If so, the distribution of virions by TEM, viral nucleic acid by ISH and PCR, and viral protein by IHC, most probably can only be detected in the normal lymphoid tubules during the acute phase of infection and in the spheroid cells during the chronic infection. Therefore, using different methods in elucidating the presence of viral components in the LO will give the same result as it depends on the infection stage.

This same phenomenon may occur in Laem-Singh virus (LSNV)-infected *P. monodon* (Sritunyalucksana *et al.*, 2006) where viral-like particles were only observed by TEM in the lymphoid tubules where the *in situ* hybridization reaction was positive.

2.2.4. Ontogeny of the lymphoid organ spheroid cells

The spheroid cells have had different terminology according to its histological appearance as described by various authors. These cellular forms have been described as possible multinucleate giant cells (Owens *et al.*, 1991), proliferative centre (Nadala *et al.*, 1992), nodular structure (Kondo *et al.*, 1994), lobular hyperplastic proliferation and degeneration of lymphoid organ cells (Turnbull *et al.*, 1994) and abnormal cell foci (Spann *et al.*, 1995). However, the terms of lymphoid organ spheroid cells, spheroid cells or spheroids have been used in most of the published papers (Lightner *et al.*, 1987; Bonami *et al.*, 1992; Fraser and Owens, 1996; Anggraeni and Owens, 2000; Shao *et al.*, 2004; Anantasomboon *et al.*, 2008) and will also be used throughout this review.

Even though there are slight differences in the histological features of the spheroids, similarities have also been described by different researchers. The most obvious characteristics of the spheroids (Figure 2.3) were the lack of central lumen, more basophilic cytoplasm with H & E staining compared to the normal lymphoid organ tubules and being surrounded by fibrous connective tissue or elongated flattened cells (Owens *et al.*, 1991; Bonami *et al.*, 1992; Kondo *et al.*, 1994; Turnbull *et al.*, 1994; Anggraeni and Owens, 2000; van de Braak *et al.*, 2002b; Rusaini and Owens, 2010). Cytoplasmic vacuolisation sometimes was observed within these abnormal cells (Owens *et al.*, 1991; Bonami *et al.*, 1992; Kondo *et al.*, 1994; Turnbull *et al.*, 1994; Hasson *et al.*, 1995; Anantasomboon *et al.*, 2008; Rusaini and Owens, 2010). The spheroid also underwent anaplasia (Owens *et al.*, 1991) and an increased cytoplasm to nuclear ratio. The nucleus was hypertrophied with marginated chromatin. Pyknotic or karyorrhectic nuclei were frequently observed in older infections (Owens *et al.*, 1991; Bonami *et al.*, 1992; van de Braak *et al.*, 2002b; Anantasomboon *et al.*, 2008). In addition, basophilic to magenta cytoplasmic or

intranuclear inclusion bodies might also be observed (Owens *et al.*, 1991; Turnbull *et al.*, 1994), depending on the infectious agents. Furthermore, the LOS size ranged from 50 – 150 µm (Hasson *et al.*, 1999b).

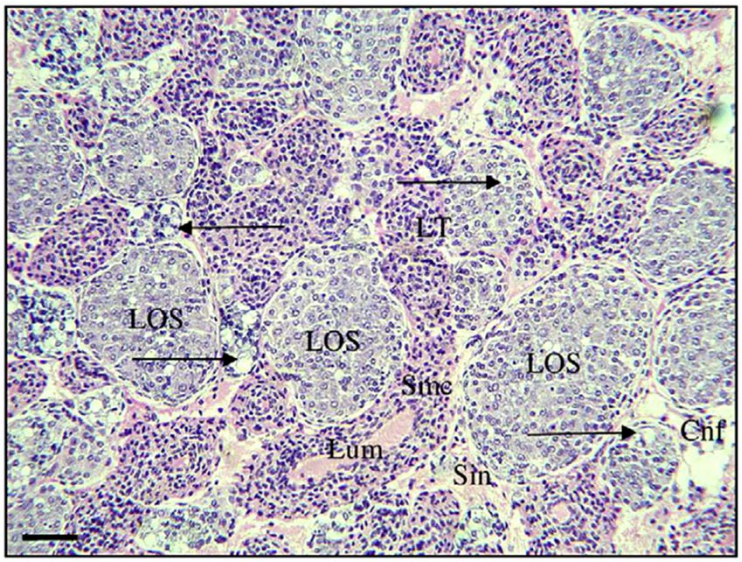


Figure 2.3. Light micrograph of longitudinal section of the LO of *Penaeus monodon*. LOS cells are formed in the haemal sinuses (Sin) and appear to have a more basophilic cytoplasm and lack of a central lumen (Lum) compare to the normal lymphoid tubule (LT). Some spheroids had cytoplasmic vacuoles (arrow). H & E stain. Scale bar: 50 µm. Cnf, connective tissue fibre; Smc, stromal matrix cells (Rusaini and Owens, 2010).

Lymphoid organ spheroids undergo morphological changes that represent developmental stages of LOS formation. These developmental stages have been divided into three distinct phases: formation, encapsulation and degeneration (Owens, unpubl. data) which corresponded to morphotypes A, B and C (Hasson *et al.*, 1999b) and spheroid types 1, 2 and 3 (Duangsuwan *et al.*, 2008b). Spheroid type 1 appeared lightly basophilic, homogeneous cell masses with few or no necrotic cells and cytoplasmic vacuoles. Spheroid type 2 was characterised by being more basophilic and highly encapsulated with fibrous connective tissue. Necrotic and apoptotic cells and cytoplasmic vacuoles were more frequently observed. The final stage, type 3 was characterised by highly basophilic and vacuolated cells with apoptotic nuclei and marginated chromatin (Hasson *et al.*, 1999b; Duangsuwan *et al.*, 2008b). These three stages have been modified with an intermediary between each major phase (Littik, 2003).Later on, the spheroid was classified into two

different types of cells based on its ratio of cytoplasm to nuclear volume (Shao *et al.*, 2004) that simplify the previous categories.

Controversies about the lymphoid organ lie mainly in the origin of the spheroid cells. Some authors suggested that spheroid cells had stromal matrix origin (Kondo *et al.*, 1994; Hasson *et al.*, 1999b; Duangsuwan *et al.*, 2008b). By *in situ* hybridization analysis, the probe signal showed that the number of TSV positive lymphoid tubules decreased while the number of spheroid type A increased during the chronic phase of TSV infection (Hasson *et al.*, 1999b). A similar finding was reported in recent work which used a vascular-corrosion cast to visualise the three dimensional structure of the LO by scanning electron microscopy (Duangsuwan *et al.*, 2008b). According to these authors, in chronically yellow head virus-infected *P. monodon*, the subgastric artery branched into shorter, smaller number of branches which were less convoluted with blind ending terminals due to breakdown of terminal part of the tubules to form spheroid cells. Therefore, it was proposed that the spheroid cells were morphological transformations of the LO tubule cells (Hasson *et al.*, 1999b; Duangsuwan *et al.*, 2008b).

However, a decreased number of the normal tubules due to spheroid formation might be just a perception, because tubule dispersion occurred during spheroid formation. The presence of the spheroids surrounding the subgastric artery supported this supposition (Hasson *et al.*, 1999b). This implied that the formation of LOS cell in the haemal sinuses did not result in decreased numbers of normal tubules, but caused hypertrophy of the lymphoid organ (Owens *et al.*, 1991; Nadala *et al.*, 1992; Turnbull *et al.*, 1994; Hasson *et al.*, 1999b). Unfortunately, this phenomenon was not considered by Shao *et al.* (2004) when they found that spheroids were only present in prawns with LO larger than 2 mm.

In addition, if the spheroid cells are the degeneration phase of the LO tubules during viral infection, then a lumen should be observed in the spheroid. With histological examination,the stromal matrix tubule cells will stain basophilic in the developmental stage of the LOS cells. However, these two observations have never been reported in the published papers. Furthermore, only LOS cells should be

observed when all the LO tubules have undergone morphological change to spheroid cells. Again there is no report on the total absence of stromal matrix in the LO even in the advancedstate of LOS formation. The occurrence of spheroid cells always comes with the presence of the normal tubules. Contrarily, in some prawns, lymphoid tubules are the only cells found in the LO without LOS cells, showing this state can exist by itself (Rusaini, 2006).

In contrast, it was proposed that lymphoid organ spheroids might be exocytosed granular haemocytes (Anggraeni and Owens, 2000). A cytochemical study revealed that spheroids stained positive for phenoloxidase (PO) and peroxidase activity, whilst the normal LO tubules were negative. As a result, spheroid cells shared features common to small granular haemocytes (SGH) and large granular haemocytes (LGH). However, LOS cells had no granularity. Therefore, LOS cells were probably degranulated (exocytosed) haemocytes from the central lumen that migrated through the stromal matrix cells as phagocytic cells and coalesced in the haemal sinuses of the lymphoid organ (Anggraeni and Owens, 2000). This process is consistent with the haemolymph flow but contrary to the pattern of haemocyte release incorrectly hypothesized by the other authors (Hose *et al.*, 1992).

In spite of Duangsuwan*et al.*(2008b) rejecting the haemocytic origin of the spheroids as proposed by Anggraeni and Owens(2000), the work of Duangsuwan *et al.*(2008b) supports this hypothesis. Even though these researchers (Duangsuwan *et al.*, 2008b) believed in the stromal matrix origin of spheroids, they proposed that it was more likely that most spheroids were disconnected from the normal tubules because they lacked a lumen. This could be interpreted that if spheroids detached from stromal matrix cells, then the spheroids are not a morphological transformation of the normal tubules and most probably they have another origin.In addition, these authors stated that probably spheroid cells were washed out during the vascular corrosion casting process. The main solutions for vascular washing werean alkaline solution, phosphate buffered saline, Tyroide's solution and Ringer's solution to flush the haemolymph from the circulatory system (Hossler, 2003; Verli *et al.*, 2007). It is not surprising that spheroids were removed from the LO during the casting process if

these cells are spent haemocytes (Anggraeni and Owens, 2000). As a result, the spheroid cells could not be visualised together with stromal matrix tubules in the lymphoid organ by this method as can be seen in their published figures (Duangsuwan *et al.*, 2008b). In addition, the phenomenon of decreased number and shortening of the lymphoid tubules (subgastric artery) during the formation of spheroids (Duangsuwan *et al.*, 2008b) may be an artefact rather than reality. Commonly, vascular corrosion analyses are descriptive with subjective and qualitative terms (Verli *et al.*, 2007).

The spheroid cells were formed in the haemal sinuses (Anggraeni and Owens, 2000). If the spheroid accumulated in the haemal sinuses during the life of the prawns, the area of the LOS should increase with the increase in the size of the animal (Anggraeni and Owens, 2000). However, the number of LOS cellsoscillated during the life span of the prawns and there was no association between the number of LOS cells and prawn size or sex (Anggraeni and Owens, 2000; Rusaini and Owens, 2010). It seemed that there was a process that avoids the accumulation i.e. removal of spheroids. Since many immunological factors of prawns are associated with moulting stages (Hose *et al.*, 1992; Sequeira *et al.*, 1995; Le Moullac *et al.*, 1997; Cheng and Chen, 2001; Liu *et al.*, 2004), then it was hypothesised that the spheroid cells might be disposed of during ecdysis, the actual shedding of old exoskeleton (Anggraeni and Owens, 2000; Rusaini, 2006). However, there was no statistically significant moulting effect on the ratio of spheroid to lymphoid organ total tissue (Rusaini and Owens, 2010).

Lunar phases might be the major factor influencing the fluctuation of LOS cells during the animals' life. Changes in the number of spheroids occurred during the lunar periodicity both in laboratory maintained and in farmed prawns (Rusaini and Owens, 2010). The lunar related pattern on spheroid cells in laboratory-held animals implied that this effect was endogenous and hereditary, without environmental cues (Withers, 1992; Griffith and Wigglesworth, 1993). In farmed animals, the presence of light intensity of dark-light moon and hydrostatical effect of spring-neap tide might provide environmental cues that were absent in the laboratory maintained

animals. However, the factor that drives the fluctuation of the LOS cells within the lunar phases is still to be investigated.

The mechanism of spheroid elimination within the LO is stilldebatable. It has been proposed that two concurrent processes might occur during the chronic phase of TSV infection, depending on nutritional and health status of the animals (Hasson *et al.*, 1999b). Firstly, viral replication might continue within LOS type B; virus might escape from the cells, and return to the lymphoid organ where phagocytosis occurs. As a consequence, the cyclic phase of LOS and chronic phase of TSV infection might persist. Secondly, the LOS type C might develop to eliminate the virus by self-destruction (apoptosis) of the cells and return the lymphoid organ back to normal (Hasson *et al.*, 1999b).

According to Spann *et al*. (2003) the former process was more likely the major mechanism occurring in chronic gill associated virus-infected penaeid prawns. However, if the first hypothesis occurs, the spheroid cells will persist and increase in number within the LO as the animal grew and aged, as a result of continued viral replication. In addition, there was a fluctuation in the number of spheroids and it was not related to the size and age of animals (Anggraeni and Owens, 2000; Rusaini and Owens, 2010). This suggested that the first supposition (Hasson *et al.*, 1999b) was unlikely. Furthermore, if apoptosis took place, by histopathology examination, transitional stage of spheroids should be observed. Since LOS cells have been classified into three distinct morphotypes (Hasson *et al.*, 1999b; Duangsuwan *et al.*, 2008b), a further (transition) stage after the third type (more vacuolated and necrotic spheroids) before LOS disappeared is needed to fill the gap. However, there is no report illustrating this last morphotype. In addition, *insitu* necrotic destruction of spheroids was non-existent or rare (Rusaini and Owens, 2010). Therefore, it was hypothesised that the LOS cells were disposed of during the life span of the animals (Anggraeni and Owens, 2000; Rusaini and Owens, 2010) and it was stimulated by lunar rhythmicity (Rusaini and Owens, 2010).

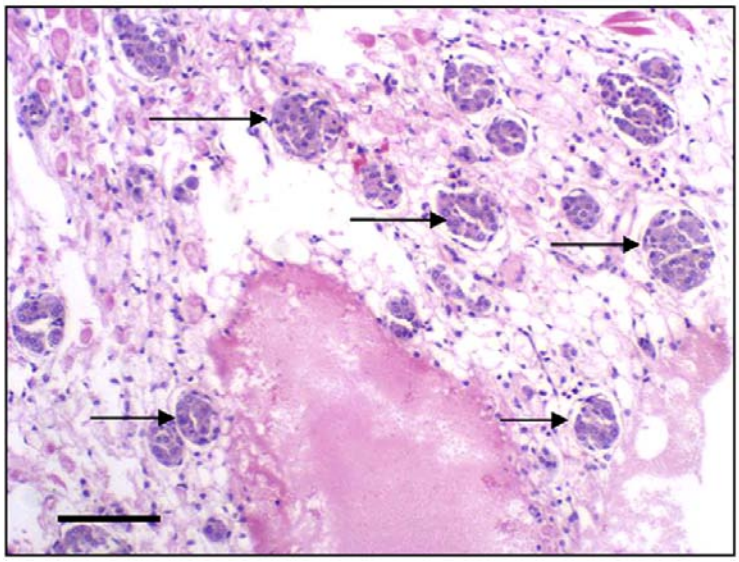


Figure 2.4. Numerous ectopic spheroids (arrow) in the connective tissue of *Penaeus monodon*, longitudinal section, H & E stain, scale bar = 100 µm (Rusaini, 2006).

If the spheroids are shed from the LO into the environment, then the presence of spheroid cells in the other organs should be visualised before these abnormal cells were removed to the environment, especially in organs which have direct contact with the environment such as excretory organs. The appearance of spheroids in the other organs is called ectopic spheroids (Figure 2.4) and these have been observed within the antennal gland, tegmental gland, heart, hepatopancreas, gills, connective tissue, gonad, muscle and appendages (Lightner *et al.*, 1987; Owens *et al.*, 1991; Turnbull *et al.*, 1994; Hasson *et al.*, 1999b; Littik, 2003; Soowannayan *et al.*, 2003; Rusaini, 2006). However, whether these cells were produced *in situ* from exocytosed haemocytes in those tissues, disseminated through the haemolymph or diapedised from the LO is unknown. It has been suggested that these ectopic cells were metastatic spheroid cells(Lightner *et al.*, 1987). However, the limited evidence of LOS metastasis from the LO to the other organs (Hasson *et al.*, 1999b) does not support the supposition. Furthermore, the lack of mitotic activity within the LO also repudiates the possibility of cellular division and proliferation. Therefore, the origin of these ectopic spheroids is another gaping hole in lymphoid organ studies that needs further investigation.

It seems posibble that LOS cells are disposed of into the environment via the antennal gland. The antennal gland is an excretory organ in prawns (Andersen and Baatrup, 1988; Bell and Lightner, 1988) and it connects directly to the lymphoid organ and the environment. This organ consists of a coelomosac, which communicates with the outer surface via the labyrinth. Coelomosac epithelium is composed of a single cell-type, the podocyte that is similar to invertebrate branchial podocyte and vertebrate podocytes of the glomerular nephrons (Andersen and Baatrup, 1988; Bell and Lightner, 1988). The coelomosac has been proposed to have a filtering function and the primary site for urine formation. In addition, coelomosac podocytes might play a role in removing substances from the ultrafiltrate by endocytosis along intercellular channels and sequester material within the cells (Andersen and Baatrup, 1988). The physiological function of the antennal gland in prawns has also been suggested to be the main site for ionic and osmotic regulatory mechanism, involved in the detoxification and excretion of toxic material, and the control of haemolymph volume (Dall and Smith, 1981; Doughtie and Rao, 1983; Doughtie and Rao, 1984; Andersen and Baatrup, 1988; Lin *et al.*, 2000). However, further work is needed to determine the functional role of the antennal gland in the elimination of spheroid cells.

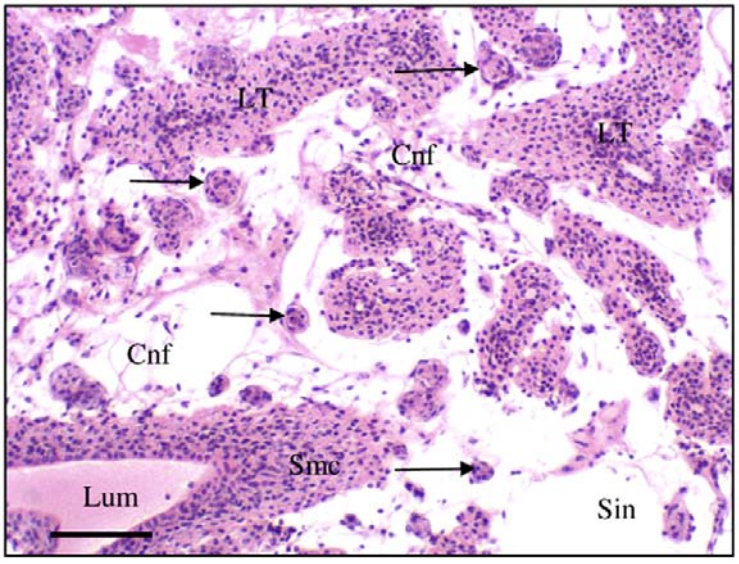


Figure 2.5. Light micrograph of longitudinal section of *Penaeus monodon* lymphoid organ with H & E stain. Abnormal interstitial space (haemal sinuses)/gapping between tubules, note the LOS cells (arrow), scale bar = 100 µm (Rusaini, 2006).

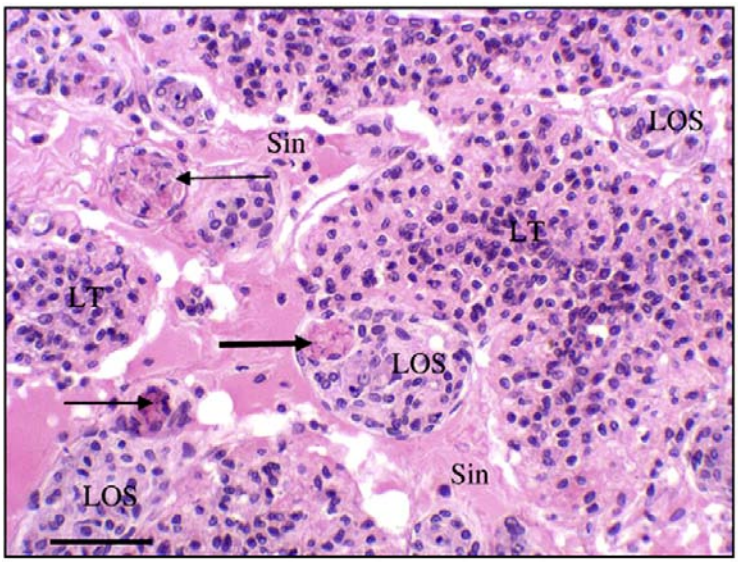


Figure 2.6. Light micrograph of longitudinal section of *Penaeus monodon* lymphoid organ with H & E stain. Eosinophilic foci (arrow) and one focus inside the LOS cell (bold arrow) suggesting that originally these foci have spheroid origin, scale bar = 50 µm (Rusaini, 2006).

The reticular connective tissue and haemal sinuses extensively expand during spheroid production because these abnormal cells are formed in the haemal sinuses (Anggraeni and Owens, 2000; Rusaini, 2006). Therefore, gapping between tubules might appear before they return to the normal form, if the spheroids were eliminated. The abnormal interstitial space or gapping (Figure 2.5) between tubules has been observed in prawns infected with YHV (Boonyaratpalin *et al.*, 1993) and GAV (Spann *et al.*, 1997; Spann *et al.*, 2003; Rusaini, 2006). This gapping has been assumed to result from the heavy accumulation of infected cells leading to necrosis of haemal sinuses and connective tissue (Spann *et al.*, 2003). However, this abnormal interstitial space appears to be due to LOS elimination within the lymphoid organ because it becomes more prominent when the spheroid cells decline (Rusaini, 2006).

Another histopathological feature that may be related to the lymphoid organ spheroid cells is necrotic eosinophilic foci (Spann *et al.*, 1997; Anggraeni, 1998; Rusaini, 2006). These eosinophilic foci were generally observed in viral infections of penaeids associated with spheroid formation (Owens, pers. commun.). These foci were often found in one tubule with LOS cells (Figure 2.6) and they were never

found within the normal stromal matrix tubules (Rusaini, 2006). Therefore, these foci were claimed to be the transformation type of spheroids with strong fibrocytic encapsulation (Anggraeni, 1998; Rusaini, 2006). However, what mechanism is driving the transformation of spheroids to necrotic eosinophilic foci is unknown.As with the spheroid cells, these foci might also be disposed of during the life of the prawns.

2.2.5. Methodology in the lymphoid organ studies

Various methods have been applied to lymphoid organ studies. In the early work, histology was the method used most frequently to illustrate the normal structure and the role of this organ (Oka, 1969; Martin *et al.*, 1987; Nakamura, 1987; Bell and Lightner, 1988). This technique still remains popular for scientists when they describe the tissue changes and the spheroid cells within the LO (Lightner *et al.*, 1987; Owens *et al.*, 1991; Bonami *et al.*, 1992; Nadala *et al.*, 1992; Turnbull *et al.*, 1994; Spann *et al.*, 1995; Fraser and Owens, 1996; Spann *et al.*, 1997; Hasson *et al.*, 1999b; Anggraeni and Owens, 2000; van de Braak *et al.*, 2002b; Rodríguez *et al.*, 2003). Even though this technique is cheap and simple, definitive diagnosis of tissue changes cannot be made by applying routine histology (Hasson *et al.*, 1999b), because the same changes may be produced by many factors. Therefore, other diagnostic methods are required to overcome the limitation of this technique in lymphoid organ studies.

Later, the electron microscope became an important tool in the lymphoid organ studies. Transmission electron microscopy (TEM) has been used widely in disclosing the normal structure, the pathological changes and the presence of virions within the lymphoid organ (Martin *et al.*, 1987; Owens *et al.*, 1991; Bonami *et al.*, 1992; Spann *et al.*, 1995; Spann *et al.*, 1997; Wang *et al.*, 1997; Park *et al.*, 1998; van de Braak *et al.*, 2002b; Rodríguez *et al.*, 2003; Shao *et al.*, 2004). More recently, scanning electron microscopy (SEM) was used to visualise the three dimensional structure of the normal lymphoid tubules and the histological changes of lymphoid organ due to spheroid formation (Duangsuwan *et al.*, 2008a;

Duangsuwan*et al.*, 2008b). However, the high cost of these tools limits their use, especially in developing countries.

Immunofluoresence (Nadala *et al.*, 1992), immunohistochemistry (Anggraeni and Owens, 2000; van de Braak *et al.*, 2002b; Soowannayan *et al.*, 2003; Shao *et al.*, 2004; Rodríguez *et al.*, 2012), and *in situ* hybridization (Hasson *et al.*, 1999a; Hasson *et al.*, 1999b; Anggraeni and Owens, 2000; Soowannayan *et al.*, 2003; Spann *et al.*, 2003; Cowley *et al.*, 2005a; Tang *et al.*, 2005; Sritunyalucksana *et al.*, 2006) were also performed to differentiate between the normal tubules and the spheroid cells and to elucidate the localization of antigens in the lymphoid organ. However, ISH was limited by its sensitivity (Speel *et al.*, 1999). False negatives resulted from low viral load.

In addition, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay has been applied to detect the occurrence of apoptosis in the lymphoid organ (Anggraeni and Owens, 2000; Wu and Muroga, 2004). However, this technique was also restricted by its specificity and sensitivity (Labat-Moleur *et al.*, 1998). Computer software (Sigma Scan and Sigma Scan Pro) has also been used to analyse the area of stromal matrix and spheroids (Anggraeni and Owens, 2000). To make this analysis cheap and simple, a quantitative measurement using a transect technique was introduced (Littik, 2003) and later it was modified to overcome the error of double counting in determining spheroids in the LO (Rusaini and Owens, 2007). However, this technique is time consuming in processing and viewing, and therefore limits the number of samples that can be assessed in a given time.

Recently, molecular biology methods such as reverse transcriptase- polymerase chain reaction (RT-PCR), quantitative RT-PCR (qRT-PCR) and gene sequencing have been utilised to identify viral genomes in the lymphoid organ(Soowannayan *et al.*, 2003; Cowley *et al.*, 2005a; Rajendran *et al.*, 2006). Expression of genes encoding antimicrobial peptides (AMPs)(Supungul *et al.*, 2004), transglutaminase (TGase) and clotting protein (CP)(Maningas *et al.*, 2008a)have been determined in the LO using RT-PCR. Differential display RT-PCR was applied to identify gene that encoded Argonaute (Pm Ago1) (Unajak *et al.*, 2006) and real timeRT-PCR was

used to quantified gene expression of lysozyme within the lymphoid organ (Burge *et al.*, 2007). In addition, rapid amplification of cDNA ends (RACE) has been performed to determine genes encoding leucine-rich repeat (*Pm*LRR), apoptosis-linked-gene-2 (ALG-2)-interacting protein X (*Pm-Alix*) in the LO of *P. monodon* and toll-like receptor (FcToll) in *P.chinensis*(Sangsuriya *et al.*, 2007; Sriphaijit and Senapin, 2007; Yang *et al.*, 2008). Furthermore,expressed sequence tag (EST) has been used to analyse the expression of immune-related genes within the lymphoid organ of penaeid prawns(O'Leary *et al.*, 2006; Pongsomboon *et al.*, 2008).

2.2.6. Immune components in the lymphoid organ

Recently, there has been an increasing number of studies on immune-related genes illustrated by molecular cloning, sequencing and expression analysis of these genes in penaeid prawns. It seems that most studies in gene-related immunity of penaeids pay particular attention to the haemocytes (Gross *et al.*, 2001; Rojtinnakorn *et al.*, 2002; Supungul *et al.*, 2002; Dong and Xiang, 2007; Sriphaijit and Senapin, 2007; Chen *et al.*, 2008; Costa *et al.*, 2009). However, several studies on other defence-related tissues including the lymphoid organ (Sriphaijit and Senapin, 2007; Bourchookarn *et al.*, 2008; Pongsomboon *et al.*, 2008) have also been conducted on the localization of these genes. It is crucial to discover immune-related genes that may be actively expressed in the lymphoid organ to better understand the immunological role of this organ in penaeid prawns.

Expression of various immune-related genes or molecules has been reported within the LO (Appendix 1). Compared to other tissues tested, there was a relatively higher expression of genes-related to immunity including FcToll (Yang *et al.*, 2008), serine protease inhibitor (Fc-serpin) (Liu *et al.*, 2009b) and Argonaute (Unajak *et al.*, 2006), and significantly higher expression of anti-lipoplysaccharide factor (*Lv*ALF1) (de la Vega *et al.*, 2008), lysozyme (Burge *et al.*, 2007) and *PmAlix* (Sangsuriya *et al.*, 2007) in the lymphoid organ. Expression of some of these genes underwent alteration following challenge with microbial, microbial components or environmental stress.However, to some extent the data presented is questionable. In some cases, poor experimental design and the absence of statistical analysis or at

least lack of significant differences between treatment and control groups made the claims doubtful.

An example of this problem is given in several articles describing that some immune-related genes such as peroxiredoxin (*mj*Prx), leucine-rich repeat (*Pm*LRR), Argonaute (Pm Ago), Drosha, transglutaminase and clottable protein were down-regulated in the lymphoid organ following peptidoglycan (PG), viral and bacterial treatments (Unajak *et al.*, 2006; Sriphaijit and Senapin, 2007; Maningas *et al.*, 2008b; Chaikeeratisak *et al.*, 2012; Huang *et al.*, 2012). Unfortunately, these conclusions suffered from unstated or small sample size, unclear number of replicates and the absence of any statistical analysis. A similar problem appeared in the published paper that claimed the up-regulation of transglutaminase and the down-regulation of clottable protein upon YHV injection (Bourchookarn *et al.*, 2008). The small sample size (n = 5) also weakened the statistical analysis of this work. Curiously, these authors concluded the involvement of these two proteins in defence mechanism against YHV.

Gene expression of toll-like receptor from *P. chinensis* was reported to be up regulated after bacterial infection, but down-regulated upon viral exposure (Yang *et al.*, 2008). Furthermore, these authors went further with the interpretation that in bacterial treatment, FcToll gene needs at least five hours post-infection (hpi) to predominate in the signal transduction pathway. On the other hand, in viral infection, FcToll gene expression was inhibited in the early stage because viral proteins have already taken over the host-transcription pathway for replication. However, their published figures showed that gene transcription was significantly higher in the control group than in both challenge groups until five hpiand there was no significant differences in gene expression between viral and control group from 14 hpi until the end of the experiment. Again, this conclusion was based on a small sample size (n = 3) which limits statistical analysis.

In another study, lysozyme transcripts in the LO of whiteleg prawn (*P. vannamei*) were claimed to be obviously different between control and injected groups (Burge *et al.*, 2007). However, the only significant difference between control and the

injected group occurred at four hpi in which lysozyme transcripts were significantly decreased after bacterial injection. In addition from this time onward, the number of transcripts of the lysozyme genes returned to the initial level. These authors predicted that the down regulation of lysozyme gene at four hpi resulted from the regulation of individual haemocyte in lysozyme expression or haemocytes trafficking to injection site and leaving the circulation and infiltrating tissues. Paradoxically, these authors also stated that at 12 hpi lysozyme gene increased because the gene was highly up-regulated in the remaining haemocytes or new haemocytes were released from the haematopoietic tissue. However, again there was no significant difference between treatment and control groups.

Another problem of immune-related gene expression in lymphoid organ studies is the temporary nature of the alteration after microbial administration. Most immune-related gene studies in the lymphoid organ were conducted over a short time. For example, the down regulation of FcToll only lasted eight hpi after WSSV injection before it returned to the same level as the control group during the 23 h experiment. However following bacterial treatment, its up regulation occurred after eight hpi until the end of experiment (Yang *et al.*, 2008). Other work on antimicrobial peptides (AMP) and protein-related clotting system confined their results only at 0 – 48 h (Burge *et al.*, 2007; Bourchookarn *et al.*, 2008). In addition, other studies on Fc-serpin and Pm Ago were conducted only between 0 – 60 h (Unajak *et al.*, 2006; Sriphaijit and Senapin, 2007). Slightly longer observation on *mj*Prx has been carried out for seven days post-treatment. On day 0 *mj*Prx was constitutively more expressed than on days 1, 3 and 7 (Maningas *et al.*, 2008b).

A further complication in studies of immune-related genes in the lymphoid organ is that most authors claimed an immunodefence role of these genes due to their alteration upon microbial challenge or their homology (shared sequence identity) with immune-related genes previously found in either vertebrates or invertebrates. However, activity analysis, precise function and the exact regulatory mechanism of these genes in prawn immunological state are still unclear and remain to be

elucidated as has been stated by some authors (Unajak *et al.*, 2006; Sangsuriya *et al.*, 2007; Maningas *et al.*, 2008b; Yang *et al.*, 2008; Liu *et al.*, 2009b).

All the changes listed above not only occurred in immune-related gene expression associated with the lymphoid organ but also appeared in the other defence-related tissues. The same problems can be found in the AMP gene studies such as ALF*Pm*s, *Lv*ALF, crustin*Pm*s and Pen*Pm*s in haemocytes, epipodite, gills, hepatopancreas, and haematopoietic tissue (Supungul *et al.*, 2004; de la Vega *et al.*, 2008; Somboonwiwat *et al.*, 2008; Vatanavicharn *et al.*, 2009). Studies on the gene expressions of proPO, cysteine aspartate protease (caspase-3) and Fc-serpin in haemocytes at different time points also showed similar results (Chang *et al.*, 2008; Liu *et al.*, 2009b; Yeh *et al.*, 2009a). Therefore, in general immune-related gene expression in penaeid prawns still requires further investigation.

Localisation of genes related to immunity within the LOS cells is also poorly investigated. Only a few studies have been conducted describing the localisation of immune components in the lymphoid organ spheroids, but different articles described different results. By *in situ* hybridization, the antisense proPO, crustin, pen-3, TGase, and lysozymes probes hybridised only in the haemocytes within the lumen and endothelial cells surrounding the lumen of the LO, but not in the stromal matrix cells or the spheroids (Wang *et al.*, 2007b). However, cytochemical studies showed that spheroid cells contained prophenoloxidase (proPO) and peroxidase (Anggraeni and Owens, 2000; Shao *et al.*, 2004). In addition, immunohistochemistry analysis by purified goat polyclonal IgG antibody showed localisation of cathepsin L within the LOS cells of prawns infected with WSSV (Pongsomboon *et al.*, 2008). The discovery of immune-related factors within the LOS cells strengthens the hypothesis that LOS cell are spent haemocytes (Anggraeni and Owens, 2000). Therefore, the visualisation of immune-related factors within the spheroid cells is not unexpected because most of the immunocomponents of the prawns are stored in the haemocytes (Soderhall and Cerenius, 1992; Rodriguez and Le Moullac, 2000; Smith *et al.*, 2003).

The last point is that most authors working on the function of the LO fail to take into consideration the role of the haemocytes in the immune-related function of the LO. Most authors examine the lymphoid organ without removing the haemocytes from the tissue. In fact, the LO is composed of highly branched lymphoid tubules (Oka, 1969; Martin *et al.*, 1987; Duangsuwan *et al.*, 2008a) and supplied with haemocytes directly from the heart via the subgastric artery into the lumen which diapedise through stromal matrix cells to the open haemocoel, the haemal sinuses (Anggraeni and Owens, 2000; van de Braak *et al.*, 2002b). This implies that all tissues within the lymphoid organ are bathed by the haemocytes. Therefore, the haemocytes could actually be responsible for the immune related activities within the LO, not the stromal matrix cell themselves. Distribution of some genes related to immunity in the haemocytes as well in the lymphoid organ (Burge *et al.*, 2007; Sangsuriya *et al.*, 2007; Sriphaijit and Senapin, 2007; Wang *et al.*, 2007b; Chang *et al.*, 2008; de la Vega *et al.*, 2008; Maningas *et al.*, 2008a; Maningas *et al.*, 2008b; Yang *et al.*, 2008; Liu *et al.*, 2009b; Vatanavicharn *et al.*, 2009; Yeh *et al.*, 2009a) supports this supposition. A similar phenomenon may also occur in other immune-related tissues such as gill, hepatopancreas and heart.

2.2.7. Conclusion

In crustacean taxa, the lymphoid organ is found exclusively in penaeid prawns. This organ plays an important role in immune defence again invading pathogens. The most striking feature at the cellular level in the lymphoid organ is the formation of spheroid cells as a defence response to invading pathogens. Since the formation of these spheroids is associated with many viral diseases, it is believed that these changes are a nonspecific reactive form to viral infection but not to bacterial antigens. The origin of spheroid cells has been debated, but it is most likely that these cells were produced from spent haemocytes, entering via the tubule lumen, migrating through the stromal matrix and coalescing in the haemal sinuses to form spheroids.

There may be an elimination mechanism leading to the fluctuation in the number of spheroid cells during the life span of the animals. Its likely LOS cells are being

disposed of rather than *in situ* destruction via necrosis or apoptosis. This process is not related to animal size, sex or moulting cycles. Probably this mechanism is associated with lunar rhythms. However, what factors or processes are driving the fluctuation in the number of spheroids with the moon phases is still unclear and remains to be elucidated. The discovery of genes related to immunity within this organ confirms that some cells have an immunological function, even though it might be the haemocytes bathing and within the lymphoid tissue. However, the exact regulatory mechanism of this gene expression against foreign invaders or environmental changes in penaeid prawns still needs further investigation.

2.3. Suppression Subtractive Hybridization

Alteration in gene transcripts is related to a wide spectrum of physiological and pathological changes in cells. Therefore, the identification of differential gene expression can be used to understand biological process at a molecular level (Porkka and Visakorpi, 2001; Ji *et al.*, 2002; Huang *et al.*, 2007; Ghorbel and Murphy, 2011). Various techniques such as expressed sequence tags, differential display, serial analysis of gene expression (SAGE), cDNA microarray, and subtractive hybridization have been developed to analyse differential gene expressions in any given sample (Carulli *et al.*, 1998; Byers *et al.*, 2000). The majority of methods for differentially expressed genes are based on differential display and subtractive hybridization. As it is technically demanding and labour intensive due to the library screening to identify isolated genes, subtractive hybridization is less widely used than differential display (Hedrick *et al.*, 1984; Byers *et al.*, 2000). However, subtractive hybridization produces fewer false positives compared to differential display (Carulli *et al.*, 1998; Byers *et al.*, 2000).

2.3.1. Early development of suppression subtractive hybridization

Initially, subtractive hybridization was used to study different expression of genes in the developmental stage of *Xenopus laevis* (Sargent and Dawid, 1983) and in T-helper hybridoma cell lines (Hedrick *et al.*, 1984). Modifications of this method have been applied to a cDNA library in scrapie-infected brain of the hamster

(Duguid *et al.*, 1988; Duguid and Dinauer, 1990). Commonly, this technique includes cDNA hybridization from one population (tester) to excess cDNA from another population (driver) and separation of unhybridised fragments (target) from hybridised common fractions (Diatchenko *et al.*, 1996). Mostly, the common sequences in the two populations were eliminated by hydroxylapatite chromatography (Sargent and Dawid, 1983; Hedrick *et al.*, 1984), an avidin-biotin system (Duguid *et al.*, 1988; Duguid and Dinauer, 1990) or oligo(dT)30-latex beads (Hara *et al.*, 1991; Hara *et al.*, 1993). Later, integration of PCR into subtractive hybridizationincreased the sensitivity of the subtraction method and led to the possibility of using low abundance mRNA (Gurskaya *et al.*, 1996). This integrated method has been used in identifying differentially expressed genes in thyroid hormone-induced tadpole tail regression (Wang and Brown, 1991), in the human placenta of a newborn female, in Epstein-Barr virus-immortalised lymphoid cell lines (Wieland *et al.*, 1990), human embryonal carcinoma cells (Hara *et al.*, 1991), and human diploid fibroblasts (Hara *et al.*, 1993).

However, to some extent, the subtractive hybridizationis limited by its complexity due to the nature of subtraction kinetics. Low abundance genes may be hybridised slower and obtain a lower level of completion compared to high abundance genes. Thus, it works poorly in indentifying low abundance genes. Unfortunately, many target genes involved in cellular process are in low abundance, so to maximise the identification of rare messages, high concentration of primers were necessary at the expense of insert length. In addition, several rounds of hybridizations may be required to prevent retention of sequences that are not differentially expressed (Sargent and Dawid, 1983; Hedrick *et al.*, 1984; Duguid *et al.*, 1988; Duguid and Dinauer, 1990; Hara *et al.*, 1991; Gurskaya *et al.*, 1996; Carulli *et al.*, 1998; Diatchenko *et al.*, 1999; Byers *et al.*, 2000). However, the number of common cDNA clones still remained high in the two samples (Hara *et al.*, 1993).

To overcome these problems, recently subtractive hybridization has been modified or combined with other differential gene expression methods leading to the establishment of a number of related methods. These techniques include phagemid

subtractive hybridization, subtractive hybridization and shot-gun sequencing, direct random oligonucleotide prime (DROP) subtractive hybridization, chemical cross-linking subtraction (CCLS), and representational difference analysis (RDA). The modification of subtractive hybridization has also resulted in several other methods called combined subtractive and display techniques, subtractive differential display, differential subtractive hybridization, gene profiling using subtractive hybridization, and suppression subtractive hybridization (SSH) (Byers *et al.*, 2000). Indeed, every method has advantages and disadvantages and careful consideration is needed to choose a suitable method for a specific sample, because not all methods are well-matched with all samples.

2.3.2. Principle of suppression subtractive hybridization

The principle of suppression subtractive hybridization is based on cDNA sequences of two samples being compared by hybridization. These two different cDNA populations are referred as "tester" and "driver" (Wieland *et al.*, 1990). Tester contains specific target sequences that are not present in driver, the reference cDNA. The common sequences of both samples are subtracted leaving a cDNA population enriched for sequence preferentially expressed in the tester but absent in the driver (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996; Diatchenko *et al.*, 1999).Thus in the SSH method, target cDNA is selectively amplified, while an undesirable sequence is simultaneously suppressed during PCR amplification. The suppression PCR effect is mediated by long inverted terminal repeats attached to the end of thecDNA fragments. The inverted repeats form stable panhandle-like loop structures following denaturation and annealing procedures. This panhandle-like structure cannot be exponentially amplified in a PCR with primers obtained from long inverted repeats sequences, because intramolecular annealing of the long inverted terminal repeats is highly favoured and is more stable than intermolecular annealing of shorter PCR primers (Diatchenko *et al.*, 1996; Diatchenko *et al.*, 1999).

In general, SSH involves several steps (Figure 2.7). Firstly, isolation of total RNA or poly(A)$^+$ RNA is carried out from tissues or cells being compared. The tester and driver double stranded (ds) cDNA are synthesised separately from mRNAs and

digested with a four-base cutting restriction enzyme (Rsa I) to obtain blunt ended cDNAs. Following this, tester cDNA is divided into two portions and each sample ligated with different adaptors (adapter 1 and adapter 2R) to the 5' end of cDNA, creating two populations of tester. The adaptor's end has no phosphate group. Therefore, only the longer strand of each adaptor can covalently attach to 5' ends of the cDNAs. Next, the excess driver cDNA is mixed with each sample of testers, heat denatured and allowed to anneal, generating type a, b, c and d fractions in each sample (Figure 2.7). During this first hybridization, normalisation of the single stranded (ss) cDNA tester fragment (a) occurs, meaning the concentration of high and low abundancecDNAs become approximately equal. This equalisation happens as a result of the annealing process generating homohybrid (b) and heterohybrid (c) fragments more rapidly for more abundant fractions due to the second-order kinetics of hybridization. Annealing of low abundance cDNAs is slower and these remain single stranded (a). Simultaneously, the ss cDNA tester molecules (a) are enriched significantly for differential gene expression while common non-target fragments form type c fractions with the driver (Diatchenko *et al.*, 1996; Diatchenko *et al.*, 1999).

In the next step, both samples from the first hybridization are mixed and annealed with fresh denatured driver to enrich differentially expressed gene fractions. During this second hybridization, only the remaining normalised and subtracted ss tester cDNAs can reassociate and form a new type of hybrids (e). However, the type e molecules can only be formed if the transcript is over expressed in the tester cDNA. Addition of freshly denatured driver cDNA increases the extent of hybridization, causing further enrichment of type e fractions for differentially expressed sequences. These new hybrids (e) have a different feature from the other fragments in that they have two different adaptor sequences in their 5' ends, one from sample 1 (adaptor 1) and the other from sample 2 (adaptor 2R). Prior to the initiation of PCR amplification, an extension reaction is conducted to fill in the adaptor ends by DNA polymerase. This process provides a primer binding site for PCR amplification. Several types of molecules are formed with diverse combinations of adaptor sequences at their ends. Type e hybrids have distinct annealing sites for nested

primer on their 5' and 3' ends (Diatchenko *et al.*, 1996; Diatchenko *et al.*, 1999; Clontech Laboratories, 2008).

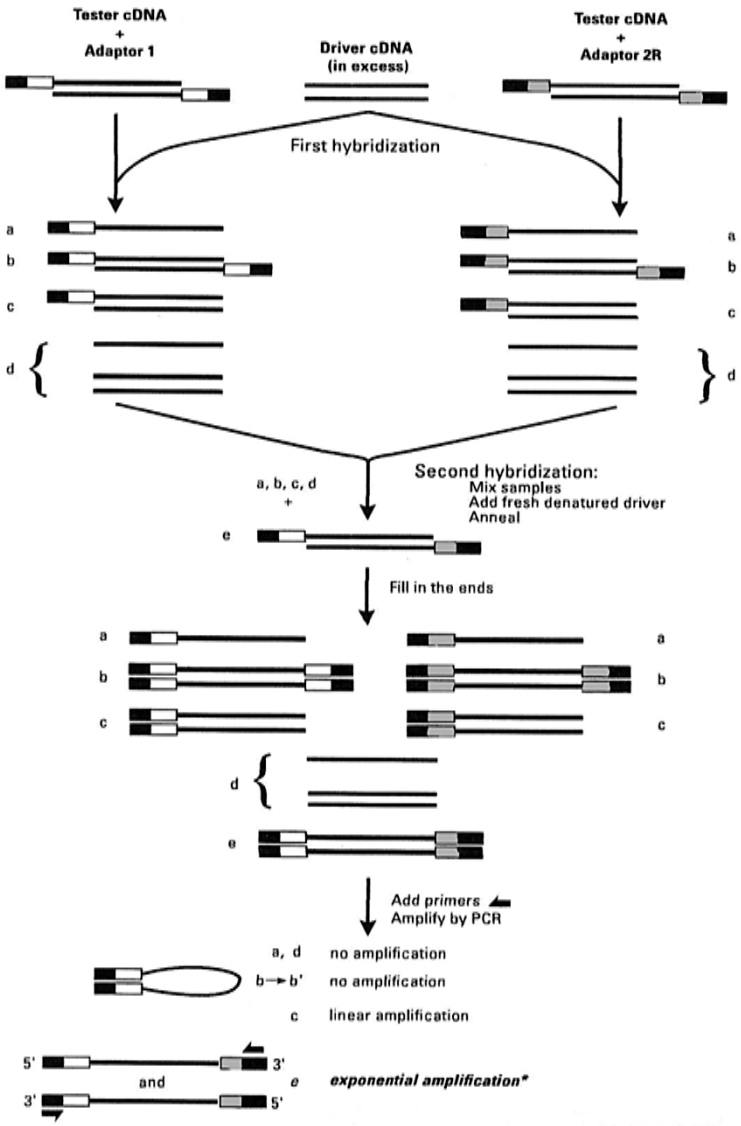


Figure 2.7. Schematic diagram of suppression subtractive hybridization (SSH) technique.The two different cDNA populations are synthesised from mRNA generated from tissues or cells being comparedand refer as “tester” and “driver”. Tester contains specific sequence being targets that are not present in driver, the reference cDNA. Solid lines symbolize digestion of tester and driver cDNAs with a four-base cutting restriction enzyme (Rsa I). Solid boxes characterise the outer part of adaptor 1 and adaptor 2R that associated to PCR primer 1 sequencing. Clear boxes represent the inner part of adaptor 1 that corresponds to nested PCR primer 1 sequencing. Grey boxes symbolize the inner part of adaptor 2R that associate with nested PCR primer 2R sequencing. Type e fractions can only be formed if there is up regulation of transcripts in the tester cDNA. From Clontech Laboratories, I. PCR-Select^{TM} cDNA Subtraction Kit User Manual, 44 pp. Clontech Laboratories, Inc., Mountain View, California(Clontech Laboratories, 2008).

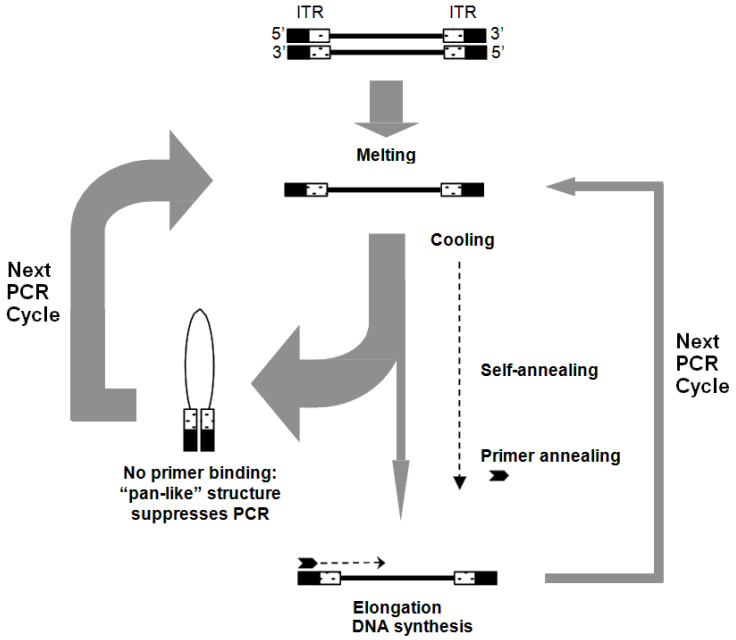


Figure 2.8. The suppression PCR effect is mediated by long inverted terminal repeats attached to the end of cDNA fragment. The inverted repeats form stable panhandle-like loop structure following denaturation and annealing procedures. This panhandle-like structure cannot be exponentially amplified in a PCR with primers obtained from long inverted repeats sequences, because intramolecular annealing of the long inverted terminal repeats is highly favoured and is more stable than intermolecular annealing of shorter PCR primers. From Ghorbel, M.T. and Murphy, D. Suppression Subtractive Hybridization. In: A. Merighi (Ed), Neuropeptides: Methods and Protocols, Vol. 789.pp. 237-259(Ghorbel and Murphy, 2011).

Finally, the mixture is subjected to two rounds of PCR to exponentially amplify the target differentially expressed genes that contain both adaptors. During the primary PCR, type a and d fractions cannot be amplified because they have no primer binding sites. Due to the suppression PCR effect, type b fragments are also unsuitable for amplification because these molecules have long inverted repeats on the ends and form a stable panhandle-like structure following the denaturation and annealing cycles (Figure 2.8). Type c molecules can only be amplified linearly because they only contain one primer annealing sites. Only type e molecules, the differentially expressed sequences, that have different adaptor sequences on the ends can be exponentially amplified by PCR. In the secondary PCR amplification, the differentially expressed sequences are further enriched and any background PCR products are reduced (Diatchenko *et al.*, 1996; Diatchenko *et al.*, 1999; Clontech

Laboratories, 2008). The PCR products can then be inserted to a cloning vector to construct the cDNA libraries.

2.3.3. Advantages and disadvantages of suppression subtractive hybridization

Construction of a cDNA library usingsuppression subtractive hybridization is one of the most powerful approaches in identifying differentially expressed genes in a given sample (Hara *et al.*, 1993; He *et al.*, 2004; He *et al.*, 2005). This technique offers many advantages in the study of the profile of gene transcripts. It enriches the sequence of interest over 1000-fold in a single round of hybridization, allows the representation of very rare messages in the library because high and low abundance sequences are equalised, and prevents undesirable cDNA amplification (Duguid and Dinauer, 1990; Wieland *et al.*, 1990; Diatchenko *et al.*, 1996; Diatchenko *et al.*, 1999; Rebrikov *et al.*, 2000; Porkka and Visakorpi, 2001; Huang *et al.*, 2007; Reyes *et al.*, 2007). This methodmakesit possible to use non-renewable tissues because only small amount of mRNA is needed (Duguid *et al.*, 1988).

Suppression subtractive hybridization also appears to be more discriminating and allowscomparison of differentially expressed genes from two different isolates (Reynaud *et al.*, 2008). This technique can be used to define gene alteration, both up-regulated and down-regulated genes (Wang and Brown, 1991; Munir *et al.*, 2004). The up-regulated transcripts can be detected if the cDNA containing the specific target (differentially expressed) sequences is used as a tester, while the reference cDNA is used as a driver and this is termed forward subtraction. Conversely, the down-regulated genes can be identified if the reference cDNA serves as the tester, while the the specific target cDNA serves as the driver (reverse subtraction) (Clontech Laboratories, 2008). The ability of this technique to identify alteration of genes has been confirmed using independent protocols such as real-time PCR (de Lorgeril *et al.*, 2005; Reyes *et al.*, 2007) and real-time quantitative (q)RT-PCR (de la Vega *et al.*, 2007a; de la Vega *et al.*, 2007b; Leelatanawit *et al.*, 2008)

Another advantage of this PCR-based cDNA subtraction method is that recombinants can be amplified by a single oligonucleotide primer in the PCR. In addition, a wider representation of sequences can be produced because of the efficiency of bacterial transformation can be obtained (Duguid and Dinauer, 1990). This technique makes it possible to simultaneously isolate expression of many genes (Rebrikov *et al.*, 2000). The diversity of genes found in the SSH libraries offers an opportunity to discover functionally important genes (Leelatanawit *et al.*, 2008). Furthermore, subtractive hybridization libraries can be used continuously to carry out different experiments (Duguid *et al.*, 1988).

Despite its benefit, the subtractive method has disadvantages. These drawbacks include that under the standard procedure, commonly 2 – 4 µg of poly(A) RNA are needed as a starting material to construct both forward and reverse libraries. To some extent this quantity of poly(A) RNA maybe difficult to obtain. In order to be expressed in the SSH libraries, the amount of target cDNA needs to be more than fivefold concentrated in the tester than in the driver or should account for at least 0.01% of the total mRNA, because the completion of hybridization in the second hybridization step cannot be achieved if the target cDNA is too low. However, the large amount of mRNA does not accurately reflect the amount of encoded protein and very low abundance genes have the higher probability of being over expressed due to the lack of a site for the restriction enzyme. In addition, genes that may have an important function in a biological process may not be detected if their mRNA abundance is not significantly altered by the event in question. Furthermore, genes with limited poly(A) tail may also not be detected by this method. At least the genes in question should have four or more poly(A) tail in order to be expressed using this PCR-subtraction method because the cDNA synthetic primer of this technique contain four poly(T) at the first 5′ end. Low efficiency and high number of false positive clones may occur due to annealing with nonspecific PCR primers, non-specific annealing of suppression adaptors during subtractive hybridization, or incomplete subtraction of particular genes in the library (Wang and Brown, 1991; Hara *et al.*, 1993; Rebrikov *et al.*, 2000; Ji *et al.*, 2002; de la Vega *et al.*, 2007a; Huang *et al.*, 2007; Reyes *et al.*, 2007; Clontech Laboratories, 2008).

The four-base cutting restriction enzyme (Rsa I) used to produce small fragments (~ 600 bp) in order to increase the efficiency of SSH technique, may be a disadvantage if a full length differentially expressed cDNA is required. However, creating each cDNA into multiple fragments has advantages. A complex network may result from long cDNA fragments preventing the formation of appropriate hybrids which are needed to bind the two adapters (adaptor 1 and adaptor 2R) at the end of the target sequences. In addition, small cDNA fragments produce a better representation of individual genes. Furthermore, the average small size (~ 200 bp) of inserts in the cDNA libararies is another problem of the SSH method. This is due to the fact that short fragments are hybridized, amplified and cloned more efficiently than long fragments (Diatchenko *et al.*, 1996; Diatchenko *et al.*, 1999).

To circumvent the limitations and increase the efficiency in determining differential gene transcripts, suppression subtractive hybridization has also been integrated with the other techniques. Different components of several methods including differential display, SAGE, EST and SSH have been integrated into a linear system for gene identification. This integrated procedure results in reduction of sequence redundancy, a decrease in the requirement of mRNA, an increase the accuracy of subtraction efficiency, and an increase in the likelihood of EST database matches and novel sequence identification (Wang and Rowley, 1998). Combination SSH with subtractive hybridization and differential screening has been performed to ensure the expression of rare messages, eliminate undesirable common transcripts, and reduce the probability of false positives (Nakata and McConn, 2002). SSH has also been combined with cDNA array hybridization(Porkka and Visakorpi, 2001), mirror orientation selection (MOS) (Rebrikov *et al.*, 2000; Rebrikov *et al.*, 2004) and microarray analysis (Munir *et al.*, 2004)to eliminate the proportion of background clones that represent non-differentiated genes in the SSH libraries. In addition, two different approaches of SSH called direct forward subtractive library (Dfsl) and enriched forward mirror orientation selection (efMOSl) have been performed. It was found that Dfsl was less labour intensive, less technically demanding, and had higher diversity in gene hits than efMOSl (Ascenso *et al.*, 2007). Furthermore, SSH has

been modified to permit the identification of differential viral and cellular genes expressions from two cDNA populations (Kiss *et al.*, 2003).

2.3.4. Application of suppression subtractive hybridization in penaeid prawn's gene studies

Suppression subtractive hybridization as a single method for gene expression has been applied in a large number of circumstances. However, this review will focus on aquaculture studies with respect to prawn genes and diseases. The SSH method has been usedto determine genes involving in growth (Tangprasittipap *et al.*, 2010) and reproductive maturation (Leelatanawit *et al.*, 2008; Wonglapsuwan *et al.*, 2009; Preechaphol *et al.*, 2010; Xie *et al.*, 2010) of penaeid prawns. This PCR-based cDNA subtraction method has been performed to elucidate the expression of immune-related genes in response to white spot syndrome virus (WSSV)(Bangrak *et al.*, 2002; Pan *et al.*, 2005; Zhao *et al.*, 2007; James *et al.*, 2010) andyellow head virus (YHV) (Junkunlo *et al.*, 2010; Prapavorarat *et al.*, 2010) infections in penaeid prawns. Some genes that encode proteins involving in the immune response of penaeid to heat-killed microorganism challenges including the fungus *Pichia pastoris*, Gram-positive bacteria *Bacillus subtilise* and Gram-negative bacteria *Escherichia coli*have been identified using the SSH technique (He *et al.*, 2004). Subtracted cDNA libraries have also been constructed to identify genes of penaeids that are expressed differentially upon challenge with *Vibrio penaeicida* (de Lorgeril *et al.*, 2005), *V. harveyi* (Nayak *et al.*, 2010; Nayak *et al.*, 2011) and lipopolysaccharide (LPS), a predominant component of Gram-negative bacterial cell-walls (Lu *et al.*, 2009). In addition, differential gene expression of prawns due to environmental challenge including osmotic, hypoxic and hyperthermic stresses has been studied using the SSH method (de la Vega *et al.*, 2007a; Gonçalves-Soares *et al.*, 2012).

Combination of suppression subtractive hybridization with the other techniques has been applied to determine the differential gene expression profile of penaeid prawns challenged by virus, bacteria and environmental stress. The SSH combined with EST (O'Leary *et al.*, 2006), differential hybridization (He *et al.*, 2005; Reyes *et al.*,

2007), mirror orientation selection (García *et al.*, 2009) or microarray analysis (Arts, 2006; Wang *et al.*, 2006; Robalino *et al.*, 2007a) has been used to elucidate genes involved in the immune response of penaeid prawns during WSSV infection. The SSH coupled with microarray analysis has also been applied to isolate differentially expressed genes in prawns challenged with heat-killed microorganisms and dsRNA injection (Robalino *et al.*, 2007a) and to identify and characterise genetic markers of *V. nigripulchritudo* virulence in prawn (Reynaud *et al.*, 2008). In addition, the integration of these two methods has been used to study the alteration of gene expression of penaeids due to environmental stress such as hypoxic, hypoosmotic and hyperthermic conditions (de la Vega *et al.*, 2007b).

2.3.5. Differential gene expression from SSH library of penaeid prawns

Several species of penaeid prawns including the black tiger prawn *P. monodon* (Bangrak *et al.*, 2002; de la Vega *et al.*, 2007a; de la Vega *et al.*, 2007b; Leelatanawit *et al.*, 2008; Nayak *et al.*, 2010; Prapavorarat *et al.*, 2010; Preechaphol *et al.*, 2010; Tangprasittipap *et al.*, 2010), the whiteleg prawn *P. vannamei* (O'Leary *et al.*, 2006; Reyes *et al.*, 2007; Robalino *et al.*, 2007a; Zhao *et al.*, 2007; García *et al.*, 2009; Junkunlo *et al.*, 2010; Gonçalves-Soares *et al.*, 2012), the kuruma prawn *P. japonicas* (He *et al.*, 2004; He *et al.*, 2005; Pan *et al.*, 2005), the Pacific blue prawn *P. stylirostris* (de Lorgeril *et al.*, 2005), the fleshy prawn *P. chinensis* (Wang *et al.*, 2006; Xie *et al.*, 2010), the banana prawn *P. merguiensis* (Loongyai *et al.*, 2007a; Wonglapsuwan *et al.*, 2009), the Indian white prawn *P. indicus*(James *et al.*, 2010; Nayak *et al.*, 2011) and the giant freshwater prawn *Macrobrachium rosenbergii* (Cao *et al.*, 2006; Lu *et al.*, 2009) have been used to construct the cDNA libraries for differentially expressed genes. Most of these libraries were constructed from the haemocytes (Bangrak *et al.*, 2002; He *et al.*, 2004; de Lorgeril *et al.*, 2005; He *et al.*, 2005; O'Leary *et al.*, 2006; de la Vega *et al.*, 2007a; de la Vega *et al.*, 2007b; Robalino *et al.*, 2007a; García *et al.*, 2009; Lu *et al.*, 2009; Prapavorarat *et al.*, 2010), the hepatopancreas (Pan *et al.*, 2005; O'Leary *et al.*, 2006; Robalino *et al.*, 2007a; Zhao *et al.*, 2007; James *et al.*, 2010) and the gills (O'Leary *et al.*, 2006; Robalino *et al.*, 2007a; Junkunlo *et al.*, 2010; Gonçalves-Soares *et al.*, 2012). The

SSH libraries have also been constructed from cephalothorax (Wang *et al.*, 2006; Reyes *et al.*, 2007), testis (Leelatanawit *et al.*, 2008), ovaries (Wonglapsuwan *et al.*, 2009; Preechaphol *et al.*, 2010; Xie *et al.*, 2010), optic lobes of eyestalks (Tangprasittipap *et al.*, 2010) and whole postlarvae (Nayak *et al.*, 2010; Nayak *et al.*, 2011).

Many geneencoding proteins have been identified in the SSH cDNA libraries of penaeid prawns (Appendix 2). These genes were clustered according to their predicted functionalities. For an instance, in WSSV infected prawns, differentially expressed genes were classified belonging to defence-related proteins, defence-related enzymes, apoptotic-related proteins, antioxidant enzymes, regulation and signal transduction factors (Pan *et al.*, 2005; Zhao *et al.*, 2007; James *et al.*, 2010). In general, these differentially expressed genes were clustered based on the sequences having significant homology in the GenBank database (National Centre for Biotechnology Information). However, the regulatory mechanism and the exact role of these genes in the prawns' immune response are poorly understood and needs further investigation.

Alteration in gene expressions from SSH cDNA libraries of penaeids has been reported in a number of articles (Supplementary Table 1). Among those, genes encoding proteins for haemocyanin, transglutaminase (TGase), lysozyme, penaeidins, thioredoxin reductase, trypsin, and elongation factors were found to be up-regulated in prawns due to viral, bacterial and environmental challenges (de Lorgeril *et al.*, 2005; Pan *et al.*, 2005; de la Vega *et al.*, 2007a; de la Vega *et al.*, 2007b; Robalino *et al.*, 2007a; Zhao *et al.*, 2007; García *et al.*, 2009; Nayak *et al.*, 2010; Prapavorarat *et al.*, 2010; Gonçalves-Soares *et al.*, 2012) suggesting the important role of these genes in the defensive response of penaeid prawns. Over expression of genes that encode viral proteins, has also been identified in the cDNA libraries of penaeids experimentally infected with WSSV and YHV (Reyes *et al.*, 2007; García *et al.*, 2009; Prapavorarat *et al.*, 2010). Conversely, genes coding for C-type lectin and cathepsin were down-regulated in prawns challenged with viruses

and environmental stressors (de la Vega *et al.*, 2007a; Junkunlo *et al.*, 2010; Gonçalves-Soares *et al.*, 2012; Junkunlo *et al.*, 2012)

A problem with the study of altered genes from SSH cDNA libraries is the inconsistency of gene alterations reported by different authors and also within the same papers. Some of these genes were reported to be undergoing both up-regulation and down-regulation following different or the same treatments (Appendix 2). For example, the gene coding for an antioxidant glutathione S-transferase was reported as being overexpressed in the hepatopancreas of *P. japonicus* following WSSV infection (Pan *et al.*, 2005). In contrast, this gene was suppressed in the hepatopancreas of *P. vannamei* upon WSSV infection (Robalino *et al.*, 2007a). An antimicrobial peptide, lysozyme, was up-regulated following WSSV infection at 32°C, but it was down regulated upon dsRNA treatment in the haemocytes of *P. vannamei*(Robalino *et al.*, 2007a) perhaps due to the interfering RNA phenomenon. The most worrying example on the discrepancy of gene alteration in which genes encoding for tachylectin-5A and serine protease inhibitors (serpin) experienced alteration in both directions in the haemocytes of *P. vannamei* following WSSV infection at 32°C (Robalino *et al.*, 2007a) without any explanation on the phenomenon from the authors. Therefore, many more attempts in improving the quality and quantity of immune-related gene studies are needed before the suppression subtractive hybridization data gains its full potential as a technique to provide better knowledge in the immune system of the penaeid prawns.

2.3.6. Conclusion

Suppression subtractive hybridization is a powerful method that offers many advantages in disclosing differentially expressed genes in any given sample. Despite its advantages, this technique also has a drawback that genes with limited poly(A) or with mRNA which is not quantitatively altered by the event in question cannot be detected. Therefore, several methods have been combined with SSH to circumvent this limitation. In respect to prawn gene studies, this technique has opened a great opportunity to identify functionally important genes of penaeid species. Many genes have been identified from SSH cDNA libraries of penaeid prawns affected by viral,

bacterial and environmental challenges. However, many aspects of immune-related genes of penaeid prawns including the regulatory mechanism, the exact role, and the alteration of these genes in prawns' immunity are still awaiting further investigation.

2.4. General Conclusion

The biggest challenge facing the prawn aquaculture industry worldwide is to control the devastating impact of infectious pathogens. Therefore, many efforts have been made to control this problem. To some extent good management practices are effective, but other protection methods are needed to prevent epidemics. Application of biological or chemical substances has been seen as essential to reduce catastrophic diseases. However, most of these methods seem to have no clear benefit to this industry (Smith *et al.*, 2003). To some extent progess has been made. These include but are not restricted to widespread use and standardization of diagnostic tests, wider application and improvement of biosecurity, and more effort on epidemiology and molecular studies of microbial dynamics in ponds and tanks (Flegel *et al.*, 2008). However, some serious problems still remain to be resolved since infectious pathogens continue to emerge in aquaculture facilities including ponds, tanks and hatcheries.

Currently, the immune system of these aquacultured animals has become the prime interest for many researchers to overcome the problem. Studying the immunological role of the lymphoid organ may provide a better understanding of penaeid prawn'simmunology. Therefore, the high throughput molecular method, suppression subtractive hybridisation seems essential in elucidating viral genomes and the differentially expressed genes in particular immune-related genes in the lymphoid organ during viral infection. Knowledge on the pathogens and host interactions at the molecular level may provide a better and more effective management strategy to combat the catastrophic impact of infectious pathogens in order to maintain or increasing prawn production.

CHAPTER3

GENERAL MATERIALS AND METHODS

3.1. ExperimentalAnimals

Wild banana prawns *Penaeus merguiensis* (De Man, 1888) were caught with a cast net in creeks around Townsville and transported to the Aquatic Pathology Laboratory of School of Veterinary and Biomedical Sciences, James Cook University for lymphoid organ extraction. *Penaeus merguiensis* with detectable spheroids in the lymphoid organ were sourced from a hatchery in northern Queensland where the lymphoid organs were extracted *in situ*. Prior to LO extraction, prawns were anesthetised by placing in iced water for a few minutes. Wild banana prawns were used as a driver cDNA, while farmed prawns were used as a tester cDNA for suppression subtractive hybridization.

3.2. Histology

Prior to histological examination, experimental animals were fixed in Davidson's fixative (formaldehyde 220 ml, acetic acid 115 ml, absolute ethanol 313 ml and tap water 352 ml) by injecting 0.5 ml of the fixative into the hepatopancreas and adjacent area of the chephalotorax and then immersed in the fixative at a ratio of the tissue to fixative 1:10. After 48 hours, the cephalothorax was cut in half longitudinally, placed in a histocassette and preserved in 70% ethanol and then processed for routine histological examination using standard paraffin embedded protocol (Bell and Lightner, 1988). Paraffin blocks were cut at 5 µm and stained with haematoxylin and eosin (H & E) and examined under a light microscopy.

3.3. RNA Extraction

3.3.1. Total RNA extraction

Total RNA wasextracted from the lymphoid organ of *P. merguiensis*by SV Total RNA Isolation System (Promega, USA catalogue # Z3100) according to manufacturer's instruction. Briefly, 10 - 15 mg of lymphoid organ was transferred to a 1.5 ml microcentrifuge tube containing 175 µl RNA lysis buffer. Tissue was disrupted with a plastic pestle and homogenised by vortexing. After addition of 350 µl RNA dilution buffer to each tube, the lysates were heated to 70°C in a dry block

heater (Ratek, Australia) for 3 minutes (mins) and then centrifuged at 14,000 *g* for 10 mins. Clear lysate was transferred to a sterile microcentrifuge tube and 200 µl 95% ethanol was added. The mixture was transferred to a spin basket assembly and centrifuged for 1 min. To the spin basket, 600 µl RNA wash solution (RWA) was added and centrifuged for 1 min. Next, 50 µl of DNase mix was applied to the membrane and incubated for 15 mins at room temperature. Following the incubation, 200 µl DNase stop solution was added to the membrane and centrifuged for 1 min. Then the membrane was washed twice with RWA. Finally, the spin basket (membrane) was transferred to an elution tube, 100 µl nuclease-free water was added and centrifuged for 1 min to elute the total RNA. The concentration and purity of total RNA was checked in a spectrophotometer (NanoPhotometer™, Implen, Germany). The total RNA was frozen at -80 °C until further examined.

3.3.2. Poly(A)$^+$ RNA extraction

Polyadenylated (Poly(A)$^+$) RNA was isolated from total RNA using Poly A Tract mRNA Isolation System III (Promega, USA catalogue # Z5300) according to the protocol of the manufacturer. In brief, 500 µl total RNA was transferred to a 1.5 ml sterile microcentrifuge tube and heated at 65°C in a heating block for 10 mins. After addition of 3 µlof biotinylated-oligo(dT) probe and 13µl of 20× SSC (87.7 g NaCl; 44.1 g sodium citrate; 500 ml nuclease free-H_2O), the annealed mixture was incubated at room temperature until completely cooled. Streptavidin magnesphere paramagnetic particles (SA-PMPs) were resuspended and washed three times with 300 µl 0.5× SSC and resuspended again with 100 µl 0.5× SSC. After each washing, the SA-PMPs were captured with a magnetic stand and the supernatant was removed. The entire annealing reaction was added to the tube containing the washed SA-PMPs and incubated at room temperature for 10 mins. Following this, the SA-PMPs were washed four times with 300 µl 0.1× SSC. Finally the pellet was resuspended with 100 µl RNase-free water, the SA-PMPs were captured and the eluted mRNA was transferred to a 1.5 ml sterile tube. Once again, the SA-PMPs pellet was resuspended with 150 µl RNase-free water, and then the mRNA was pooled. Pooled mRNA was stored at -80°C until used. Prior to use, 1000 µl poly(A)

RNA was concentrated by freeze drying (Telstar 23750 - Cryodos -50/230 V 50 Hz, the UK), re-dissolved in 25 µl RNase-free water and kept at -80°C until used. The concentration and purity of nucleic acids were determined using spectrophotometry (NanoPhotometer™, Implen, Germany).

3.4. Suppression Subtractive Hybridization

Suppression subtractive hybridization (Figure 3.1) was performed using the PCR-Select cDNA Subtraction Kit (Clontech, USA catalogue # 637401) as instructed by the manufacturer to generate cDNA forward library between wild animals (driver) and farmed animals (tester).

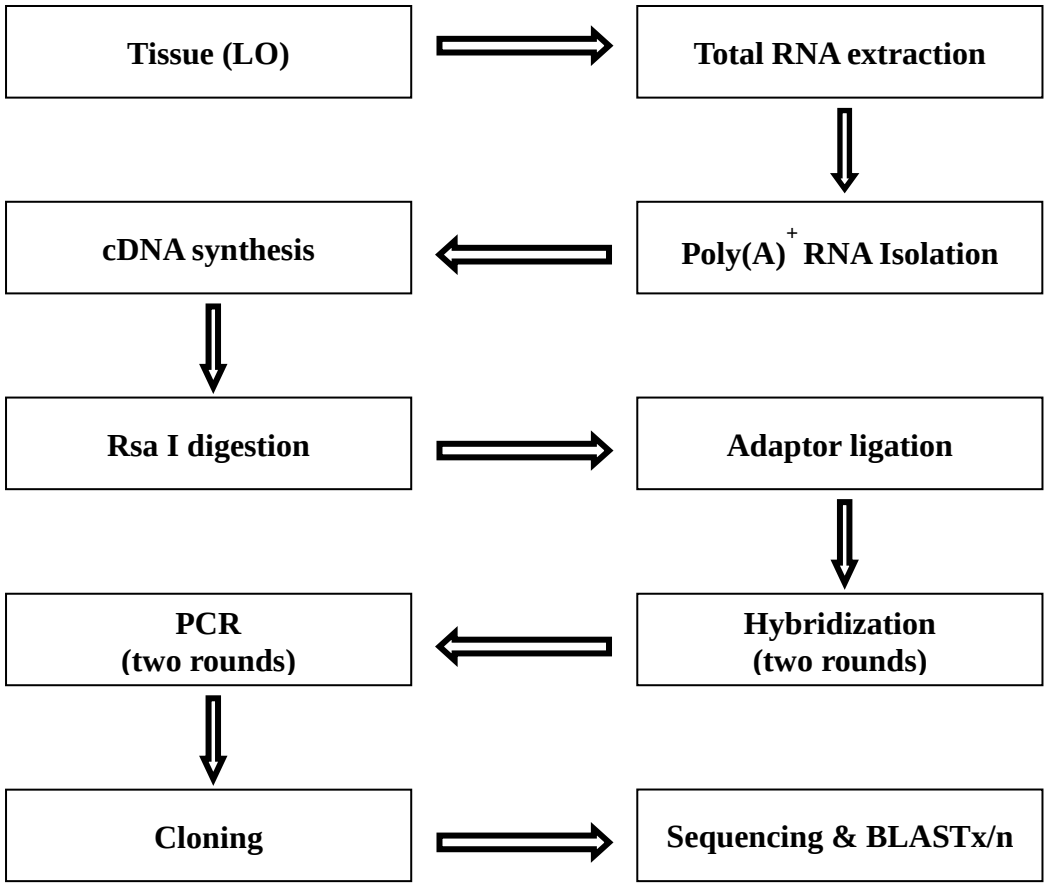


Figure 3.1. Overall diagram of the suppression subtractive hybridization process in the present study.

3.4.1. Complementary DNA (cDNA) synthesis

Synthesis of cDNA was conducted in two steps, first-strand cDNA synthesis and second-strand cDNA synthesis. In the first step, 4 µl of each driver and tester was combined with 1 µlcDNA Synthesis primer (10 µM) in a sterile 0.5 ml tube, while 2 µl of control Poly $(A)^+$ RNA (provided by the company), was combined with 1 µl cDNA synthesis primer and 2 µl sterile H_2O in another 0.5 ml tube. After incubation at 70°C, the mixture was cooled on ice for 2 mins before centrifugation. First-strand buffer, dNTP mix (10 mM), sterile water and AMV reverse transcriptase was added to each mixture and incubated in a dry block heater at 42°C for 90 minutes and then placed on ice to terminate the first strand cDNA synthesis.

In the second-strand cDNA synthesis, the mixture of first strand cDNA synthesis was mixed with sterile H_2O, second strand buffer, dNTP mix and second-strand enzyme cocktail. After incubation in a thermal cycler (Mastercycler gradient 5333, Eppendorf Germany) at 16°C for 2 hours, T4 DNA polymerase was added to the mixture. Following incubation at 16°C for 0.5 hour in a thermal cycler, the reaction was terminated by EDTA/glycogen mix and transferred to a 1.5 ml tube. To extract the cDNA from the mixture, 100 µl phenol:chloroform:isoamyl alcohol (25:24:1) was added to the cDNA mixture, vortexed and centrifuged at 14,000 rpm for 10 minutes to separate phases. The top aqueous phase was transferred to a new tube, mixed with chloroform:isoamyl alcohol (24:1) and centrifuged. The aqueous phase was precipitated with 4 M ammonium acetate (NH_4OAc) and 95% ethanol and centrifuged for 20 mins. Supernatant was discarded and the pellet was overlayed with 80% ethanol and centrifugedonce again. Finally, the pellet was air dried to let the residual ethanol evaporate and it was then dissolved with 50 µl of sterile water.

3.4.2. Rsa I digestion

This step was carried out to obtain shorter blunt-ended molecules. Briefly, each tester and driver ds cDNA was mixed with 10× Rsa I restriction buffer and Rsa I by vortexing. Following incubation at 37°C for 1.5 hours, the reaction mixture was terminated with 20× EDTA/glycogen mix. A solution of

phenol:choloroform:isoamyl alcohol (25:24:1) was added to the digested mixture to separate phases by centrifugation. The aqueous phases were mixed with chloroform:isoamyl alcohol (24:1) and centrifuged again. Following this, the aqueous phase was precipitated with NH_4OAc and 95% ethanol and subjected to centrifugation. The supernatant was removed and the pellet was overlaid with 80% ethanol. After centrifugation, the pellet was air dried, then re-dissolved in 5.5 µl sterile H_2O and kept at -20°C until used.

3.4.3. Adaptor ligation

In the following procedure, only tester cDNA was ligatedwith two different adaptors. In brief, Rsa I-digested tester cDNA was divided into two 0.5 ml microcentrifuge tubes and each tube contained 2 µl cDNA. One portion was ligated with adaptor 1, another one with adaptor 2R and each portion was mixed with ligation master mix (3 µl H_2O, 2µl 5× ligation buffer and 1 µl T_4 DNA ligase). The ligated mixture was incubated in a thermal cycler at 16°C for 20 hours. After it was terminated with 1 µl EDTA/glycogen mix, the reaction was heated at 72°C for 5 mins to inactivate the ligase and then samples were stored at -20°C until used for the next step.

3.4.4. Hybridization

There are two steps in hybridization. In the first step, an excess of driver cDNA was mixed with each tester cDNA, heat denatured and allowed to anneal to generate several fractions of molecules. Briefly, Rsa I-digested driver cDNA and 4× hybridization buffer were added toeach tester cDNA. Then the mixture was incubated in a thermal cycler at 98°C for 90 seconds and at 68°C for 8 hours. In the second hybridization step, the two tester cDNAs from the first step were mixed together in the presence of freshly denatured driver cDNA to further enrich the differentially expressed genes. In brief, a mixture of digested driver cDNA, hybridization buffer and sterile water was incubated at 98°C for 90 secs in a thermal cycler. After denaturing, the mixture was added to tester cDNA and then incubated at 68°C for 20 hours. In the next step, dilution buffer was added to the mixture,

incubated at 68°C for 7 mins and then the reaction was kept at -20°C until used for PCR amplification.

3.4.5. Polymerase chain reaction (PCR) amplification

Finally, the mixture was subjected to two rounds of PCR using specific primersfor both adaptors to exponentially amplify the target differentially expressed genes and suppresses the common sequence of the two cDNA populations. In the first PCR, 24 µl master mix (19.5µl ddH2O, 2.5 µl 10× PCR reaction buffer, 0.5 µl dNTP mix, 1.0 µl PCR primer 1 and 0.5 µl 50× advantage cDNA polymerase mix) was added to 1 µl diluted cDNA from the second hybridization step. Amplification was performed with an initial extension at 75°C for 5 minutes, and followed by 30 cycles at 94°C for 30 secs denaturation, 66°C for 30 secs annealing and 72°C for 90 secs for extension.

The product of the first amplification was used as a template for the secondary PCR with nested primers. In brief, 24 µl second PCR mixture containing 18.5 µl ddH2O, 2.5 µl 10× PCR reaction buffer, 1.0 µl nested PCR primer 1 (10 µM), 1.0 µl nested PCR primer 2R (10 µM), 0.5 µl dNTP mix (10 mM) and 0.5 µl 50× advantage cDNA polymerase mix was mixed with 1 µl of primary PCR product. The second PCR profile consisted of 15 cycles denaturation at 94°C for 30 secs, annealing at 68°C for 30 secs and extension at 72°C for 90 secs. Both PCR products were visualised on a 1.2% agarose/ethidium bromide gel running in 1× TAE buffer.

3.5. Cloning and Sequencing

The subtracted PCR products were cloned into the pGEM®-T Easy Vector System (Promega, USA catalogue # A1380) following the manufacturer's protocol. Briefly,ligation reaction containing 5 µl 2× rapid ligation buffer, 1 µl pGEM®-T easy vector, PCR products and 1 µl T4 DNA ligase were incubated overnight at 4°C. Transformation of recombinant plasmid was performed by mixing 2 µl ligation reactions with 50 µl JM109 High Efficiency Competent Cells. After incubation on ice for 20 min, the mixture was heat-shocked in a heating block at 42°C for 45-50 s and returned to ice for 2 min. Into the tube containing transformed mixture, 950 µl

super optimal broth with catabolite repression (SOC) medium was added and then incubated at 37°C in a shaking incubator at 150 rpm for 90 mins. Following this, 100 µl transformation cultures were inoculated onto duplicate Luria-Bertani (LB) agar plates containing ampicillin, X-gal (5-bromo-4-choloro-3-indodyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated overnight in an incubator at 37°C.

The following day, the transformation culture was selected by blue-white colony screening. Well-isolated white colonies from each plate were inoculated into separate 10 ml LB medium containing the same antibiotic and incubated overnight in a shaking incubator (150 rpm) at 37°C. Next day, 500 µl overnight bacterial cultures from LB medium were mixed with 500 µl 50% glycerol in a sterile tube and immediately frozen at -80°C for storage. The remaining bacterial culture was subjected to plasmid DNA purification using Wizard® *Plus* SV MiniprepsDNA Purification System (Promega, USA catalogue # A1470). The bacterial culture was harvested and pelleted in a tabletop centrifugation at 4,500 *g* for 5 mins. The supernatant was discarded and the pellet was resuspended with 250 µl cell resuspension solution by vortexing and transferred to a sterile 1.5 ml microcentrifuge tube. Next, 250 µl cell lysis solution was added to the resuspended pellet and incubated for 5 mins until the cell suspension cleared. Following this, 10 µl alkaline protease solutions was mixed to the cell suspension and incubated for 5 min at room temperature. Into the tube, 350 µl Wizard®*Plus* SV Neutralisation Solution was added and then centrifuged (14,000 *g*) for 10 min at room temperature.

Clear lysate was transferred to a spin column and centrifuged at maximum speed for 1 min. The spin column was washed with 750 µl column wash solution and centrifuged for 1 min. The spin column was further washed with 250 µl column wash solution and centrifuged for 2 min. Next, the spin column was transferred to a sterile 1.5 ml microcentrifuge tube and the DNA plasmid was eluted with 50 µl nuclease-free water and centrifuged for 1 min. The eluted plasmid was stored at -20°C for further use. The purified plasmid was sent to Macrogen, Korea for sequencing using universal M13F-pUC (5`-GTTTTCCCAGTCACGAC-3`) and

M13R-pUC (5`-CAGGAAACAGCTATGAC-3`) primers (provided by the company). Sequencing analysis and contig assembly were performed using Sequencher® 4.10.1 software (Gene Codes Corporation). Nucleotide sequences were submitted for basic local alignment search tool (BLAST)x and BLASTn for comparison to known amino acid/nucleotide sequences on GenBank databases (National Centre for Biotechnology Information, NCBI). A probability with E-values $<1e^{-05}$ were considered to be significant matches.

CHAPTER 4

INVESTIGATION OF AN IDIOPATHIC NUCLEAR CHANGE IN *Cherax quadricarinatus* USING SUPPRESSION SUBTRACTIVE HYBRIDIZATION: A PILOT STUDY

Partly published. Rusaini, Ariel, E., Burgess, G.W. and Owens, L. (2013) Investigation of an idiopathic lesion in redclaw crayfish *Cherax quadricarinatus* using suppression subtractive hybridization. *J Virol Microbiol***2013, Article ID 569032**: 15 pages (Appendix 4.2).

4.1. Introduction

Presumptive parvovirus infection in farmed Australian freshwater crayfish has been reported on several occasions. The first occurrence of a parvovirus infection in freshwater crayfish was described in a moribund cultured *Cherax destructor* and called *Cherax destructor* systemic parvo-like virus (CdSPV) (Edgerton *et al.*, 1997). Secondly, a case of spawner-isolated mortality virus (SMV) infection in *C. quadricarinatus* was reported by Owens and McElnea (2000), followed by a putative parvo-like virus associated with chronic mortality in this species (Edgerton *et al.*, 2000). The last report was *Cherax quadricarinatus* gill parvo-like virus (CqPV) causing mass mortalities in juvenile and adult farmed freshwater redclaw crayfish (Bowater *et al.*, 2002).

Histopathological features of crayfish putatively infected with parvovirus were commonly hypertrophic nuclei and rarefied chromatin with or without Cowdry type A (CA) intranuclear inclusion bodies in the infected tissues (Edgerton *et al.*, 1997; Edgerton *et al.*, 2000; Bowater *et al.*, 2002). The CAs were phloxophilic and Feulgen-negative (Edgerton *et al.*, 1997). Early and late stage intranuclear inclusion bodies that stain eosinophilic and basophilic respectively with haematoxylin and eosin could also be observed in infected tissues (Bowater *et al.*, 2002). By transmission electron microscopy, these virions measured 18 – 25 nm in diameter (Edgerton *et al.*, 1997; Edgerton *et al.*, 2000; Bowater *et al.*, 2002).

Studies on parvovirus in redclaw crayfish are traditionally conducted using histopathology with the aid of light microscopy and TEM to describe the tissue changes and the viral morphology (Edgerton *et al.*, 1997; Edgerton *et al.*, 2000; Bowater *et al.*, 2002), because successful culture of crustacean cell lines has not been achieved. Therefore, genetic characterisation of the viruses has not been carried out. Hypertrophic nuclei with rarefied chromatin but without Cowdry type A (CA) intranuclear inclusion bodies in the gills were identified in a population of redclaw crayfish (hypertrophied nuclei population), while these changes were not observed in another crayfish population (non-hypertrophied nuclei population) in the aquaculture facilities of the School of Veterinary and Biomedical Sciences, James Cook

University. These nuclear changes resemble those associated with putative parvovirus infection previously reported in this species (Edgerton *et al.*, 2000).

Several studies have been conducted into disclosing the structure, expression and function of genes in *C. quadricarinatus*, including those coding for a cellulose, glycosyl hydrolase family (GHF) 9 gene (Crawford *et al.*, 2004), heat shock proteins (HSPs) (Cimino *et al.*, 2002; Fang *et al.*, 2012), red pigment concentrating hormone (RPCH), β-actin (Martínez-Pérez *et al.*, 2005), farnesoic acid *0*-methytransferase (FaMeT) (Kuballa *et al.*, 2007), the vitellin precursor protein, vitellogenin (Abdu *et al.*, 2002; Serrano-Pinto *et al.*, 2004; Shechter *et al.*, 2005) and moult inhibiting hormone (MIH) (Pamuru *et al.*, 2012). Moreover, functional activities of genes in response to white spot syndrome virus (WSSV) such as those coding for phenoloxidase (PO), peroxidise (POD), superoxidase dismutase (SOD) and lysozyme have been investigated in the redclaw crayfish (Wang *et al.*, 2012).

Few studies have been carried out to identify differential expression of genes in the redclaw. Expression patterns of multi-transcripts related to the moult cycle of redclaw have been identified in the hepatopancreas, gastrolith disk, and hypodermis using cDNA microarray (Shechter *et al.*, 2007) or cDNA microarray hybridization techniques (Yudkovski *et al.*, 2007; Yudkovski *et al.*, 2010). Only one study has been done using suppression subtractive hybridisation to elucidate differentially expressed genes in the redclaw, but this study was carried out *in vitro* using WSSV infected haematopoietic cell culture of crayfish (Liu *et al.*, 2011a). Therefore, this study was performed to uncover the aetiology of these nuclear changes and profiling differentially expressed genes in particular immune-related genes from the gills of the crayfish using suppression subtractive hybridization.

4.2. Materials and Methods

4.2.1. Experimentalanimals

Two separate populations of redclaw crayfish *C. quadricarinatus* were sourced from stock redclaw crayfish at the School of Veterinary and Biomedical Sciences, JCU. Both of these crayfish populations were reared in 1,000 l plastic bins with a

recirculating system under two different conditions. One population which comprised about 200 animals was maintained in an outdoor facility (hypertrophied nuclei population) while the other population which consisted of about 500 animals was in an indoor facility (non-hypertrophied nuclei population). Prior to gill excision and histological examination, animals were anaesthetised by placing them in iced water for a few minutes.

4.2.2. Histology

Gills were fixed in Davidson's fixative for 24 hours, transferred to 70% ethanol and then processed for routine histological examination using a standard wax embedded procedure (Bell and Lightner, 1988). Sections were cut at 5 µm using a rotary microtome and mounted on glass slides. Tissue sections were stained with H & E and examined under a light microscopy.

4.2.3. Transmission electron microscopy (TEM)

For TEM, gills from hypertrophied nuclei and non-hypertrophied nuclei animals were fixed in 3% glutaraldehyde in a single strength phosphate buffer saline (PBS) and sent to the Analytical Electron Microscopy Facility, Queensland University of Technology, Brisbane, Australia. After arrival, samples were transferred into 3% glutaraldehyde in 0.1 M cacodylate buffer, embedded in Spurr's epoxy resin and the ultrathin sections were cut and viewed under an electron microscopy.

4.2.4. Challenge trial

Prior to use in the challenge experiment, all glass aquaria and equipment were disinfected with liquid sodium hypochlorite (100 g/l) at 30 ppm active Cl overnight, rinsed three times with filtered fresh tap water (Aqua-Pure water filter housing, AP11S with 5 µm filter cartridge, AP117R USA) overnight, then refilled with filtered fresh tap water and allowed to stand for 3 days before use. Each aquarium (60 × 60 × 30 cm) was connected with a recirculation pump and an air lift corner filter (coral rubble, filter wool and an air stone). Non-hypertrophied nuclei redclaw crayfish from the indoor facility were transferred and kept individually in the

aquarium and acclimatised for three days prior to commencement of the experiment. During the challenge trial, water temperature was measured daily and ranged between 20-23ºC. The experimental animals were fed with commercial chicken pellets once a day. Uneaten food and debris were siphoned from the aquaria every two days and water was refilled.

Tissue extract from hypertrophied nuclei crayfish was prepared from 2.75 g of pooled gills of five *C. quadricarinatus* (mean body weight of 21.47 ± 7.64 g) from the outdoor facility. The tissues were put into stomacher bags and homogenised in 5 ml single strength PBS. The homogenised tissues were clarified by centrifugation at 224 *g* for 10 minutes and the supernatant was further centrifuged at 1,398 *g* in a bench-top centrifuge (Eppendorf Centrifuge 5415 D, Hamburg, Germany) for 15 minutes. Then 10% foetal bovine serum (FBS, batch number 31301108, kindly provided by Jan Smith, TropBio JCU) was added to the supernatant and filtered through a 0.45 µm filter using a 1 ml syringe to obtain a cell-free extract. The extract was stored at -80ºC prior to use.

The experimental animals were divided into two groups: a challenge group and a control group. The challenge group consisted of six crayfish with a mean body weight of 15.14 ± 4.65 g, which were injected with 100 µl gill extract each from hypertrophied nuclei crayfish into the first and second abdominal segments. For control animals, six crayfish with an average body weight of 12.43 ± 2.99 g received 100 µl single strength PBS in the first and second abdominal segments. All injections were conducted using a sterile 1 ml Livingstone disposable syringe with a Terumo needle (29 G × ½"). After 31 days, each group was boosted, either with 50 µl gill extract for the challenge group or with 50 µl PBS for control animals and the experiment was then continued for another 31 days.

4.2.5. RNA extraction

Total RNA was extracted separately from the gills of hypertrophied nuclei (6 crayfish) and non-hypertrophied nuclei (6 crayfish) *C. quadricarinatus*. Polyadenylated (Poly(A)$^+$) RNA was isolated from pooled total RNA. Before use,

750 µl Poly $(A)^+$ RNA was concentrated by freeze drying and re-dissolved in 25 µl RNase-free water (see Section 3.3).

4.2.6. Suppression subtractive hybridization

The tester cDNA was prepared from 0.31 µg of poly$(A)^+$ RNA and the driver cDNA was synthesised from 0.29 µg of poly$(A)^+$ RNA. Suppression subtractive hybridization protocol can be seen in Section 3.4.

4.2.7. Cloning and sequencing

Subtracted PCR products were cloned into T & A cloning vectors (RBC, Taiwan) or pGEM-T easy vectors (Promega, USA) and transformed into HIT-DH5α or JM109 competent cells. For cloning and sequencing procedure, refer to Section 3.5.

4.3. Results

4.3.1. Histology

Histological examination of sections stained with H & E showed that 10 out of 10 crayfish in the hypertrophied nuclei population had cellular changes typical of putative parvovirus infection, consisting of hypertrophic nuclei with rarefied chromatin but without Cowdry type A inclusion bodies in the gill tissues (Figure 4.1a), while these changes were not found in crayfish (7/7) from the non-hypertrophied nuclei population (Figure 4.1b). Pyknotic and karyorrhectic nuclei were also sometimes observed in gills of the hypertrophied nuclei population. Haemocyte aggregation and the presence of granulomatous reactions (Figure 4.2) were observed in the gill tissues of both populations revealing the possibility of bacterial infections as subclinical infection.

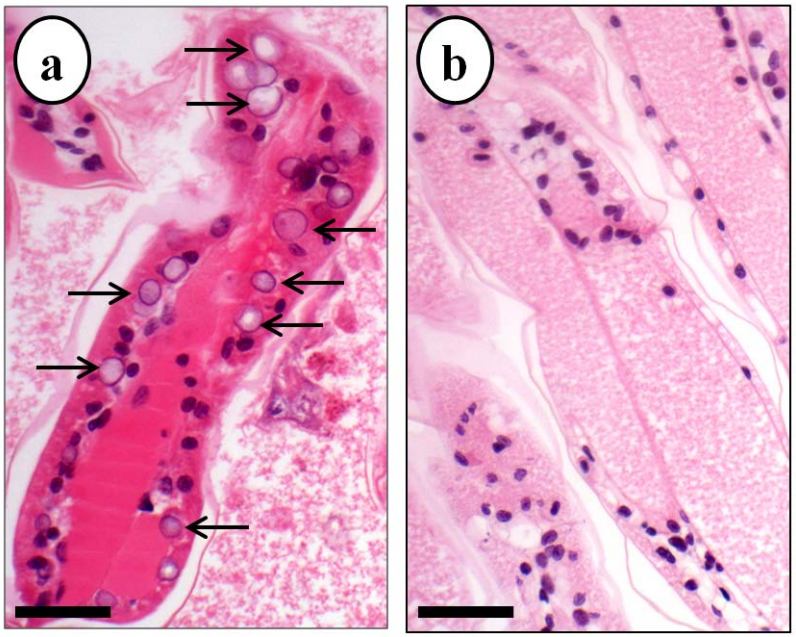


Figure 4.1. Longitudinal section of the gills of *Cherax quadricarinatus*. Hypertrophy of nuclei with marginated chromatin (arrow) in the gill epithelium of infected animal from the hypertrophied nuclei population (a) compared to normal gill epithelium of uninfected animals from the non-hypertrophied nuclei population (b). Haemotoxylin and eosin stain. Scale bar = 50 µm.

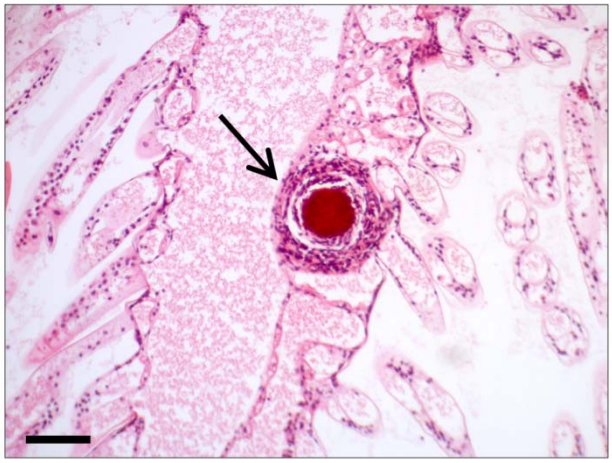
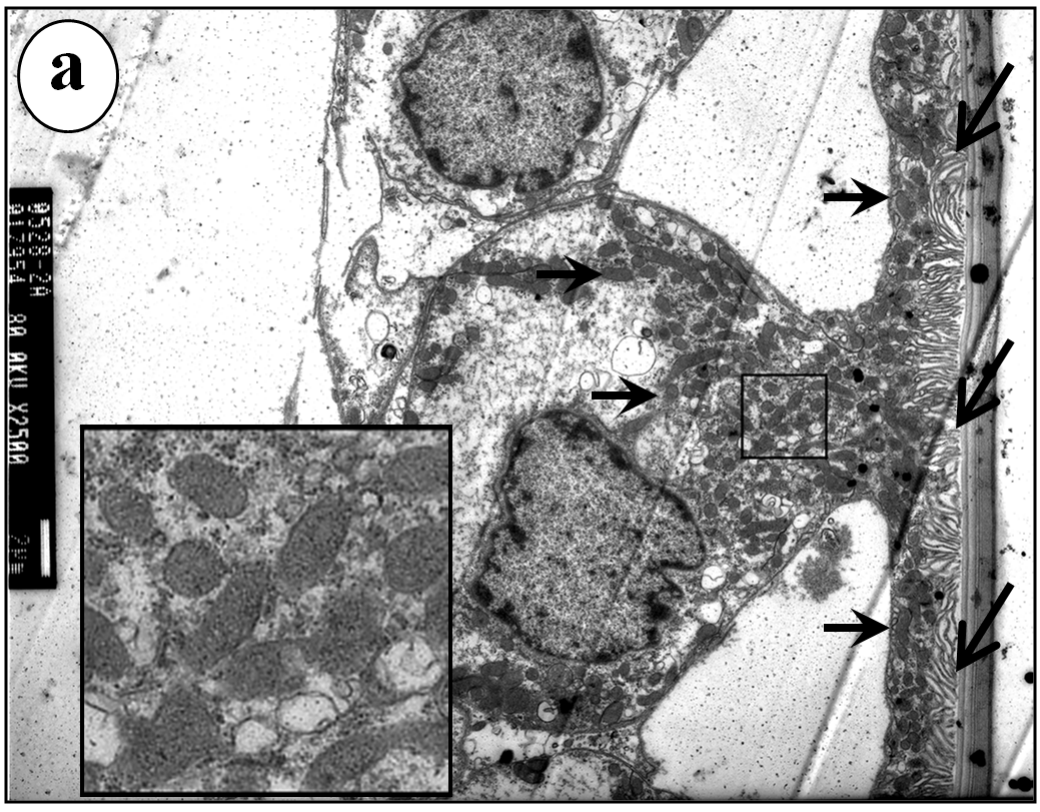
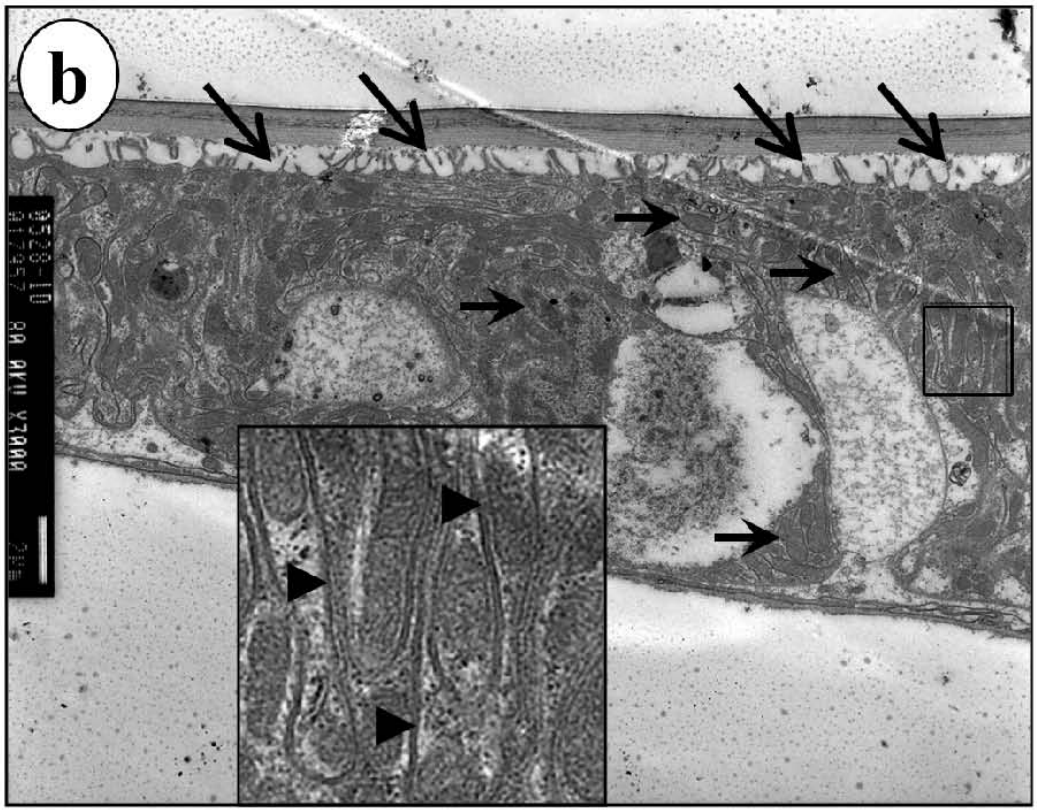


Figure 4.2. A melanised nodule surrounded by multiple layers of haemocytes (arrow) in the gills of *Cherax quadricarinatus* can be observed in both hypertrophied and non-hypertrophied nuclei populations. Longitudinal section. Haemotoxylin and eosin stain. Scale bar = 100 µm.

4.3.2. Transmission electron microscopy (TEM)

Transmission electron microscopy of gill tissues of *C. quadricarinatus* showed that in the non-hypertrophied nuclei population, mitochondria appeared normal and finely distributed in the cytoplasm (Figure 4.3a), but in the hypertrophied nuclei animals, these organelles were surrounded by proliferative endoplasmic membranes (Figure 4.3b). In the non-hypertrophied nuclei animals subcuticular microvilli appeared normal in the cells (Figure 4.3a) while in idiopathic animals, these microscopic cellular membranes were structurally disrupted (Figure 4.3b). Inclusion body-like structures were observed in the gill tissues of the non-hypertrophied nuclei crayfish (Figure 4.3c), while inclusion bodies with rarefied chromatin that may be caused by viral infection were found in the hypertrophied nuclei animals (Figure 4.3d).

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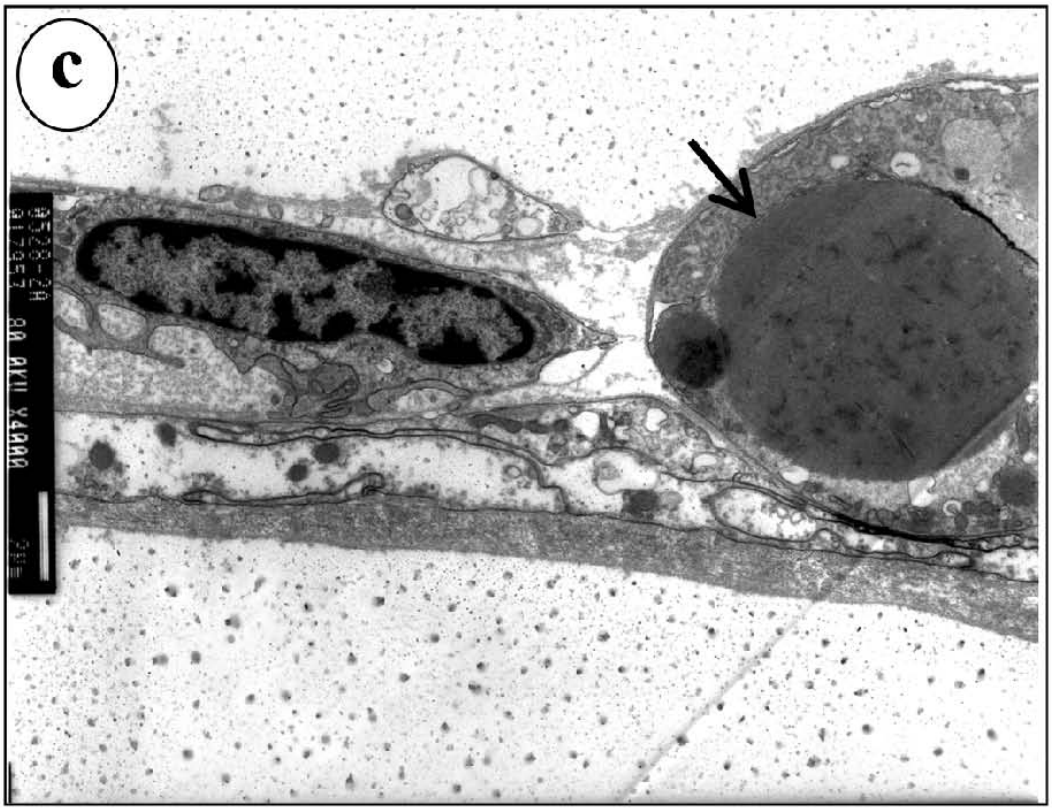
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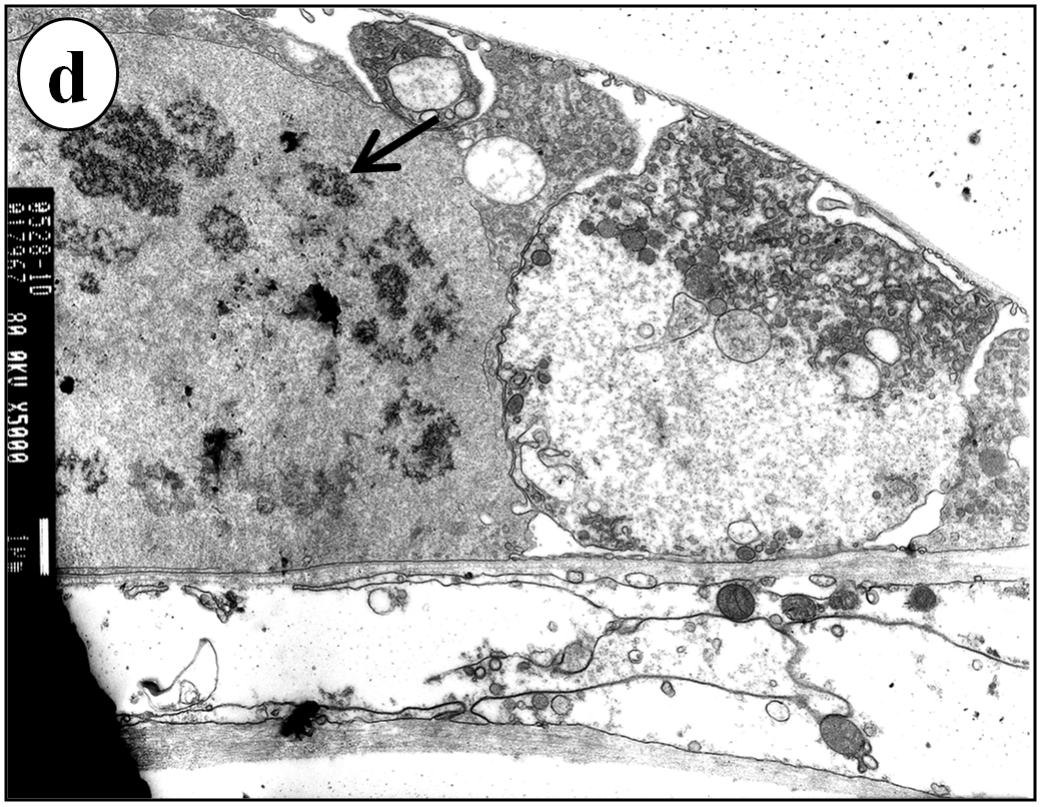

Figure 4.3. Electron micrographs of gills of *Cherax quadricarinatus* from the non-hypertrophied nuclei population (a and c) and the hypertrophied nuclei population (b and d). (a) Intact mitochondria (stealth arrows) distributed in the cytoplasm of non-hypertrophied nuclei population. The enlarged area indicated by the square, shows more clearly normal mitochondria in the cytoplasm. Microvilli (open arrows) are fine and intact below the chitin in non-hypertrophied nuclei animals. (b) Mitochondria (stealth arrows) are surrounded by proliferative endoplasmic reticulum (ER) in the hypertrophied nuclei group. The enlarged area indicated by the square, shows more clearly the proliferative ER (arrow heads) surrounding mitochondria. (c) An inclusion body (arrow) in the non-hypertrophied nuclei animals. (d) An inclusion body with rarefied chromatin (arrow) in hypertrophied nuclei animals. Scale bar = 2 µm (a, b and c) and 1 µm (d).

4.3.3. Transmission trial

Two crayfish from each group died during the experiment. In the challenge group, one crayfish died at 44 days post-injection (dpi) and another one was dead at 56 dpi. In the control group, one animal was dead at 28 dpi due to failure to shed the old cuticle during moulting, while another one was dead at 38 dpi. Moribund animals were observed to be lethargic and ceased feeding. The crayfish that died at 56 dpi

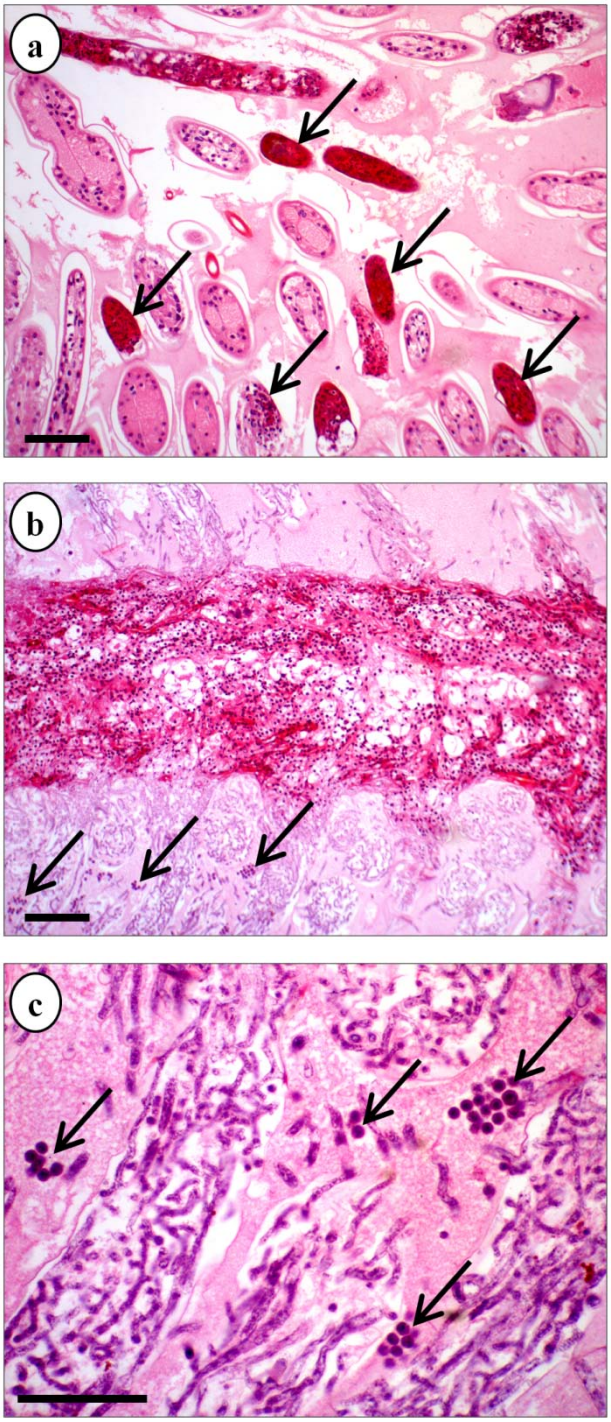

Figure 4. 4. Longitudinal section of gills of *Cherax quadricarinatus* from the challenge groups with haematoxylin and eosin stain. (a) Necrotic eosinophilic filaments (arrows). Scale bar = 100 µm. (b) Haemocytic aggregation in the central axis of the gills with surrounding branching gill filaments colonised by fungal hyphae. Fungal spores (arrows) can also be observed. Scale bar = 100 µm. (c) High magnification of fungal hyphae and fungal spores (arrows) in the infected gill filaments. Scale bar 50 µm.

from the challenge group, also had amputated appendages and melanisation in the abdomen, telson, uropods and the claws.

In histological examination, hypertrophic nuclei with rarefied chromatin and Cowdry type A inclusion bodies could not be observed in the gills in either treatments. However, all animals from both the challenge and the control groups had other pathological changes. Melanisation of the gills was found in all animals in both groups. Formation of melanised nodules can also be observed in the challenge and control groups (challenge group: three animals; control group: four animals). Necrotic eosinophilic gill filaments (Figure 4.4a) were seen in five animals from each group. Haemocytic aggregations were also seen in the central axis of the gills (Figure 4.4b) of two animals from the control group. One animal from each group was also infected by fungus. Fungal hyphae and spores were observed in infected gills (Figure 4.4c).

4.3.4. Suppression subtractive hybridization

Ligation efficiency and PCR amplification analysis of suppression subtractive hybridization can be seen in Figure 4.5 and Figure 4.6, respectively. Suppression subtractive hybridization was conducted three times resulting in several libraries (Table 4.1). A total of 339 clones were sequenced. After removing vector sequences and the poor quality sequences of these three attempts, a total of 323 sequences were grouped into 76 consensus sequences (contigs) with a range of insert sizes between 61 bp and 484 bp. The homology search revealed that around 61.6% of the total clones (199 out of 323 clones) shared significant similarities to known proteins in the GenBank database (Table 4.2).

These transcripts were clustered into 8 categories based on sequence homology from the public database (Figure 4.7). Significantly matching transcripts were clustered to immune-related genes (15.2%), energy and metabolism factor genes (3.1%) and muscle and cytoskeletal-related proteins (0.6%). Transcripts that had significant similarity to amino acids of unknown functionalities in the public database were grouped into ribosomal (2.8%) and hypothetical protein sequences (39.9%).

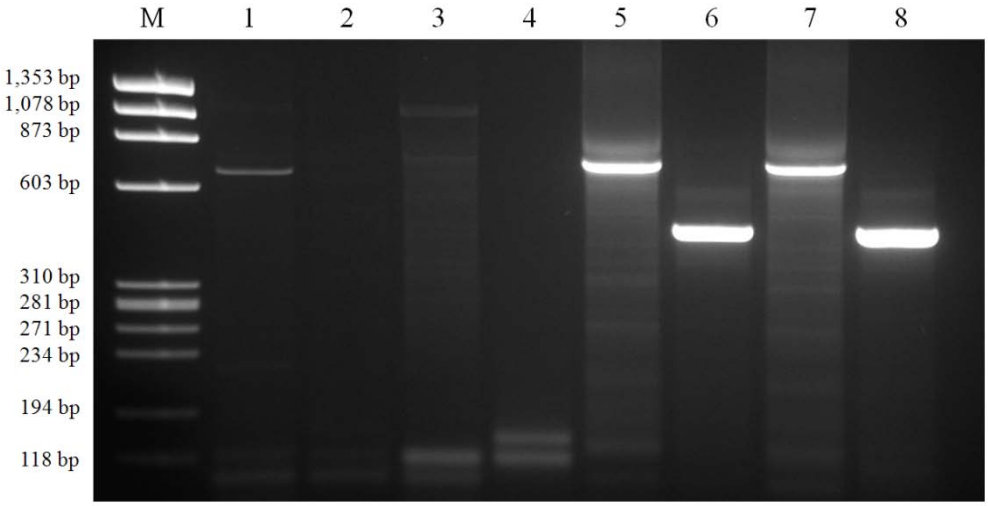

Figure 4.5.PCR product for ligation efficiency analysis of experimental samples (Lane 1 – 4) and control skeletal muscle (Lane 5 – 8) cDNA. Lane M: ΦX174 DNA/Hae III digest size marker (Takara, Japan). Lane 1: Experimental Tester 1 (Adaptor 1-ligated) template cDNA, G3PDH 3' Primer and PCR Primer 1. Lane 2: Experimental Tester 1 (Adapter 1-ligated) template cDNA, G3PDH 3' Primer and G3PDH 5' Primer. Lane 3: Experimental Tester 2 (Adaptor 2R-ligated) template cDNA, G3PDH 3' Primer and PCR Primer 1. Lane 4: Experimental Tester 2 (Adaptor 2R-ligated) template cDNA, G3PDH 3' Primer and G3PDH 5' Primer. Lane 5: Control Tester 1 (Adapter 1-ligated) template cDNA, G3PDH 3' Primer and PCR Primer 1. Lane 6: Control Tester 1 (Adapter 1-ligated) template cDNA, G3PDH 3' Primer and G3PDH 5' Primer. Lane 7: Control Tester 2 (Adapter 2R-ligated) template cDNA, G3PDH 3' Primer and PCR Primer 1. Lane 8: Control Tester 2 (Adapter 2R-ligated) template cDNA, G3PDH 3' Primer and G3PDH 5' Primer. Samples are electrophorosed on a 1.2% agarose/EtBr gel.

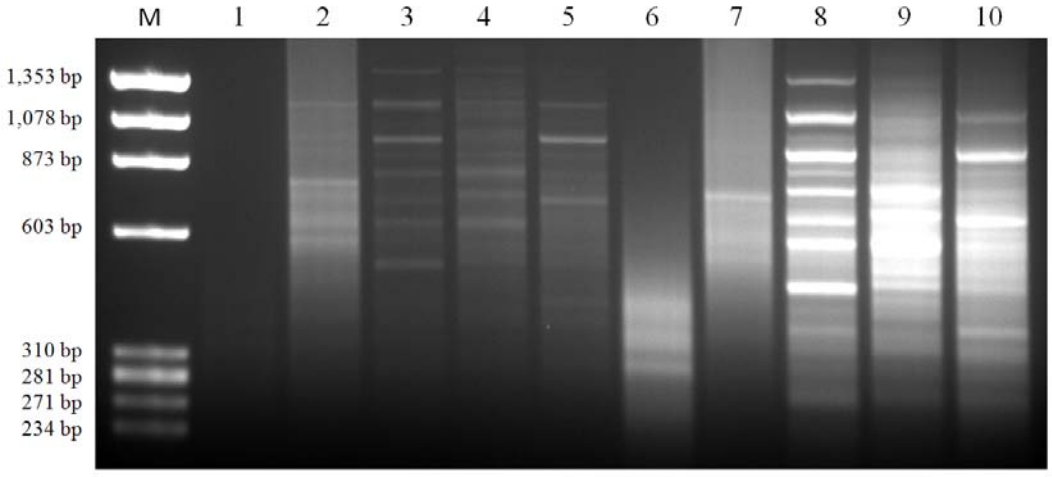

Figure 4.6. First (Lane 1 – 5) and second (Lane 6 – 10) PCR amplification of experimental samples and control skeletal muscle cDNA. Lane M: ΦX174 DNA/Hae III digest size marker (Takara, Japan); Lane 1 & 6: forward-subtracted cDNA tester; Lane 2 & 7: unsubtracted cDNA tester control; Lane 3 & 8: subtracted control skeletal muscle cDNA; Lane 4 & 9: unsubtracted control skeletal muscle cDNA; Lane 5 & 10: PCR control-subtracted cDNA. Samples are electrophorosed on a 1.2% agarose/EtBr gel.

Table 4.1. Gill cDNA libraries obtained from approximate PCR product amplicons of suppression subtractive hybridization trials.

Trial	Libraries	PCR products
1	1a	270 bp
	1b	200 bp
2	2a	200 bp
3	3a	450 bp
	3b	350 bp
	3c	300 bp

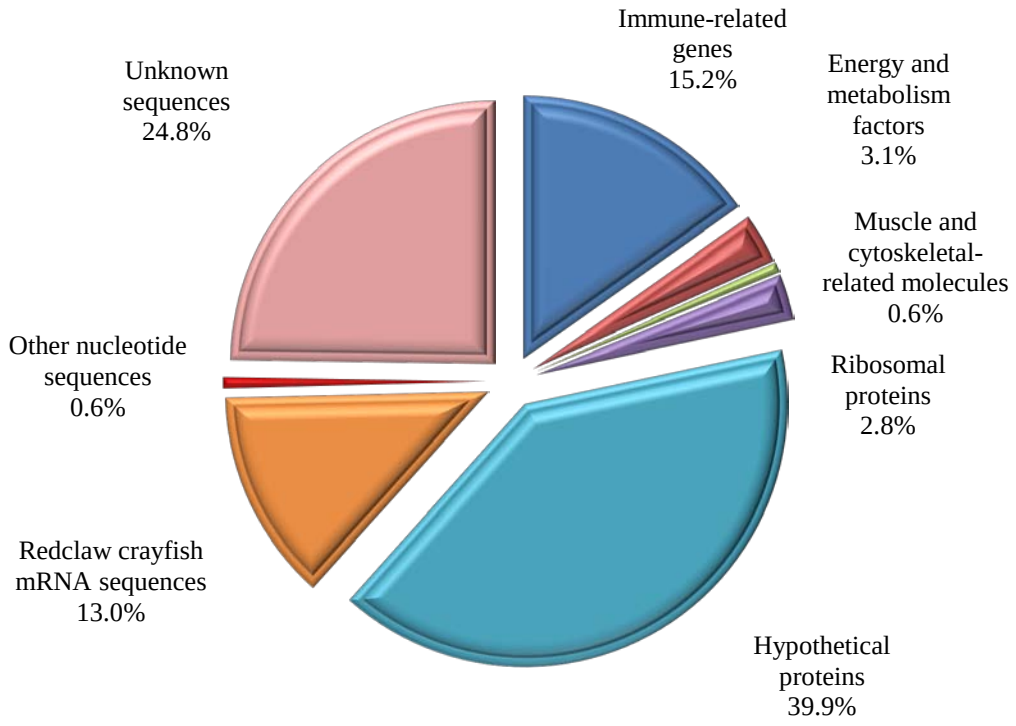

Figure 4.7. Functional categories of differentially expressed genes from the gill cDNA suppression subtractive hybridization libraries of redclaw crayfish *Cherax quadricarinatus*.

These were the most abundant transcripts found in the SHH libraries. Transcripts that did not match any protein sequences but had significant matches with nucleotides in the GenBank were clustered into redclaw crayfish mRNA (13.0%) and other sequences (0.6%). Sequences that had no significant matches either with amino acids or nucleotides in the public database were grouped into unknown sequences (24.8%).

Table 4.2. Differentially expressed genes from suppression subtractive hybridization libraries of the gills of freshwater redclaw crayfish, *Cherax quadricarinatus* with amino acids/sequences similarity to amino acids/sequences in the public database (NCBI).

Contig	Number of clones	Library	Fragment size (bp)	BLAST type	Accession number	Closest homology	Species	E-value	Identity (%) - (q/s)
						Immune-related genes			
CqG003	1	3	302	x	ABC59529.1	Cytosolic manganese superoxide dismutase	*Penaeus vannamei*	2.00E-09	93 (26/28)
CqG008	6	3a	360	x	ACD76641.1	C-type lysozyme	*Penaeus stylirostris*	3.00E-08	63 (24/38)
CqG015	17	3, 3a, 3b, 3c	235	x	P19857.2	Serum amyloid A protein	*Equus cabalus*	9.00E-06	76 (22/29)
CqG018	1	3c	243	x	ACL79888.1	Putative elastin a	*Rimicaris exoculata*	1.00E-22	75 (41/55)
CqG025	1	3c	240	x	ACY66442.1	Eukaryotic initiation factor 4A	*Scylla paramamosain*	7.00E-39	99 (79/80)
CqG026	1	3c	150	x	ACY66461.1	Translationally-controlled tumor protein	*Scylla paramamosain*	1.00E-18	78 (38/49)
CqG027	1	3c	161	x	ACY66388.1	Chaperonin 10	*Scylla paramamosain*	3.00E-14	85 (45/53)
CqG029	1	3c	237	x	ABZ90154.1	Translationally-controlled tumor protein	*Penaeus japonicus*	1.00E-29	86 (49/57)
CqG030	2	3a, 3c	368	x	ACY64752.1	Crustin 2	*Procambarus clarkii*	4.00E-49	74 (64/87)
CqG047	1	3	388	x	AEL23029.1	Insulin-like growth factor binding protein 7-like protein	*Cherax quadricarinatus*	2.00E-37	96 (54/56)
CqG048	14	2a, 3, 3a, 3c	343	x	ADI96221.1	Kazal-type serine proteinase inhibitor I	*Procambarus clarkii*	2.00E-17	76 (34/45)
CqG050	2	3, 3b	269	x	ABH10628.1	Laminin receptor	*Penaeus vannamei*	1.00E-38	92 (79/86)
CqG075	1	3a	388	x	ADM21460.1	Anti-lipopolysaccharide factor (ALF) isoform 6	*Penaeus monodon*	1.00E-43	71 (62/87)
						Energy and metabolism factors			
CqG007	1	3	189	x	YP_022769.1	NADH dehydrogenase subunit 3	*Cherax destructor*	2.00E-05	67 (33/49)
CqG021	1	3c	215	x	AAM11778.1	Cytochrome oxidase subunit I	*Engaeus strictifrons*	1.00E-36	89 (63/71)
CqG024	4	2a, 3, 3c	254	x	YP_022768.1	Cytochrome c oxidase subunit III (COIII)	*Cherax destructor*	2.00E-35	78 (62/79)

Contig	Number of clones	Library	Fragment size (bp)	BLAST type	Accession number	Closest homology	Species	E-value	Identity (%) - (q/s)
CqG035	1	3	350	x	CBW54880.1	Putative DEAD box ATP-dependent RNA helicase	*Cancer pagurus*	1.00E-17	95 (37/39)
CqG041	1	3	225	x	ACR54103.1	ATP synthase subunit g	*Palaemonetes varians*	2.00E-20	79 (37/47)
CqG056	1	1a	81	x	YP_004563978.1	NADH dehydrogenase subunit 4	*Homarus americanus*	1.00E-06	81 (21/26)
CqG076	1	3a	175	x	YP_022765.1	Cytochrome c oxidase subunit II (COII)	*Cherax destructor*	5.00E-15	89 (32/36)
						Muscle/cytoskeletal related-molecules			
CqG037	1	2	76	x	AAS98886.1	Allergen Pen m2	*Penaeus chinensis*	2.00E-10	100 (25/25)
CqG060	1	3a	405	x	BAJ14323.1	Alpha tubulin	*Pinctada fucata*	1.00E-66	96 (96/100)
						Ribosomal proteins			
CqG014	1	3c	240	x	AEB54647.1	Ribosomal protein S18	*Procambarus clarkii*	2.00E-33	100 (76/76)
CqG022	1	3c	265	x	ADY39535.1	Putative 60S ribosomal protein L7-like	*Hottentotta judaicus*	1.00E-41	75 (61/81)
CqG058	2	2, 2a	62	x	ACY66551.1	Ribosomal protein L10	*Scylla paramamosain*	2.00E-05	95 (19/20)
CqG062	2	3b	191	x	XP_002733250.1	PREDICTED: Ribosomal protein L38-like	*Saccoglossus kowalevskii*	6.00E-20	95 (40/42)
CqG064	2	3b, 3c	275	x	ADW95789.1	Ribosomal protein S30-like protein	*Pectinaria gouldii*	3.00E-11	51 (31/61)
CqG070	1	3c	138	x	ACN44179.1	Ribosomal protein S16	*Cavia porcellus*	2.00E-17	89 (50/56)
						Hypothetical proteins			
CqG023	4	3b, 3c	229	x	DAA34691.1	TPA_inf: hypothetical secreted protein 323	*Amblyomma variegatum*	2.00E-11	39 (30/77)
CqG028	121	1, 1a, 1b, 2, 2a, 3, 3a, 3b, 3c	437	x	CAM36311.1	Hypothetical protein	*Thermobia domestica*	8.00E-07	71 (23/32)
CqG065	1	3b	305	x	EFZ23151.1	Hypothetical protein SINV_03072	*Solenopsis invicta*	3.00E-20	62 (61/98)
CqG066	1	3b	155	x	XP_002739723.1	PREDICTED: Protein-like	*Saccoglossus kowalevskii*	1.00E-15	87 (34/39)

Contig	Number of clones	Library	Fragment size (bp)	BLAST type	Accession number	Closest homology	Species	E-value	Identity (%) - (q/s)
CqG068	1	3b	240	x	EFX85348.1	Hypothetical protein DAPPUDRAFT_230545	*Daphnia pulex*	5.00E-22	68 (54/79)
CqG073	1	3c	166	x	XP_780871.2	PREDICTED: Hypothetical protein	*Strongylocentratus purpuratus*	1.00E-06	43 (23/53)
						Redclaw crayfish mRNA sequences			
CqG002	21	1, 1a, 1b, 2, 2a	61	n (h)	EF692627.1	Clone y9_B8 mRNA sequences	*Cherax quadricarinatus*	3.00E-21	98 (60/61)
CqG005	3	1b, 2a, 3c	86	n (h)	GQ286092.1	Clone GB_1A mRNA sequences	*Cherax quadricarinatus*	1.00E-27	93 (82/88)
CqG012	1	3b	233	n (h)	DQ847728.1	Clone cherax_207 mRNA sequences	*Cherax quadricarinatus*	1.00E-67	100 (143/143)
CqG016	1	3c	170	n (h)	DQ847803.1	Clone y1_a2 mRNA sequences	*Cherax quadricarinatus*	7.00E-78	99 (163/164)
CqG020	2	3, 3c	242	n (h)	DQ847728.1	Clone cherax_207 mRNA sequences	*Cherax quadricarinatus*	3.00E-98	99 (203/205)
CqG031	2	3, 3c	221	n (h)	DQ847679.1	Clone cherax_157 mRNA sequences	*Cherax quadricarinatus*	1.00E-106	99 (220/223)
CqG034	3	1, 3	374	n (h)	DQ847684.1	Clone cherax_163 mRNA sequences	*Cherax quadricarinatus*	7.00E-171	99 (333/335)
CqG036	2	1, 1b	73	n (h)	DQ847743.1	Clone cherax_223 mRNA sequences	*Cherax quadricarinatus*	9.00E-28	99 (72/73)
CqG045	2	3	233	n (h)	DQ847664.1	Clone cherax_141 mRNA sequences	*Cherax quadricarinatus*	6.00E-90	96 (200/208)
CqG046	1	3	135	n (h)	DQ847565.1	Clone epi2_G11 mRNA sequences	*Cherax quadricarinatus*	2.00E-38	98 (94/96)
CqG049	1	3	260	n (h)	EF692615.1	Clone y17_B11 mRNA sequences	*Cherax quadricarinatus*	1.00E-132	100 (260/260)
CqG051	1	3	484	n (h)	DQ847548.1	Clone epi1_B3 mRNA sequences	*Cherax quadricarinatus*	0.00E+00	99 (464/467)
CqG053	2	1a, 1b	66	n (h)	GQ286117.1	Clone GI_2D mRNA sequences	*Cherax quadricarinatus*	4.00E-21	97 (63/65)
						Other nucleotides sequences			
CqG040	1	3c	229	n (s)	AM439566.1	Whole genome shotgun sequence contig VV78X26936.8	*Vitis vinifera*	4.00E-05	83 (48/58)
CqG059	1	3a	255	n (s)	HM020387.1	Secretory eggshell protein precursor (SEP18.7) mRNA	*Clonorchis sinensis*	3.00E-20	79 (93/117)

When no homology found with a BLASTx [**x**] against non-redundant sequences in the public database, BLASTn optimised for highly similar sequences (megablast) [**n (h)**] was conducted against sequences in database. If no similarity obtained from n (h), then BLASTn optimised for somewhat similar sequences (blastn) [**n (s)**] was performed. If multiple significant similarities matched with a single cDNA (sequence consensus), only the highest scoring hit was included in the table. Library 1a and 1b were produced from the first SHH trial with amplicon size of 270 bp and 200 bp, respectively. Library 2a was constructed from the second SSH trial with amplicon size of 200 bp. Library 3a, 3b and 3c were constructed from the third SSH trial with amplicon size of 450 bp, 350 bp, and 300 bp respectively. All these bands (amplicons) were cut, purified and cloned to construct the libraries. Library 1, 2 and 3 were constructed from the first, second and third SSH trial respectively, directly purified and inserted to the cloning vector without cutting the bands. q/s: number of identical amino acids (nucleotides) between query and subjects sequences/number of amino acids (nucleotides) for alignment. A similarity was considered significant at E-value < 10^{-5}.

4.4. Discussion

In the present study gills were selected as a target tissue because this tissue had different histopathological features between the hypertrophied nuclei and non-hypertrophied nuclei populations. In addition, as a multifunctional organ, gills not only play an important role in respiration, osmotic and ionic regulation, and detoxification (Clavero-Salas *et al.*, 2007; Freire *et al.*, 2008), but are also considered to be involved in the immune response to invading pathogens (Clavero-Salas *et al.*, 2007; Yeh *et al.*, 2007; Somboonwiwat *et al.*, 2008) and were therefore ideal for detecting up regulation of immune-related genes using suppression subtractive hybridization.

Using TEM, inclusion body-like structures that may be caused by viral infectionwere observed both in hypertrophied and non-hypertrophied nuclei populations. Whilst very suggestive, virions were not observed in these inclusion bodies nor in the adjacent areas, therefore the viral aetiology for the pathological changes was not confirmed. Moreover, in the challenge trial, the changes with hypertrophic nuclei and marginated chromatin were not successfully transmitted from the hypertrophied nuclei population to the non-hypertrophied nuclei animals via tissue extract and the challenge did therefore not fulfil River's postulate. The only pathological changes that could be observed in the gills of both treatments were melanisation of the gill filaments, formation of melanised nodules, necrotic eosinophilic filaments, aggregation of haemocytes and fungal hyphae and spores that may cause the death of a few of the experimental animals.

Histopathological observations consistently revealed similar changes indicative of putative parvovirus infection in *C. quadricarinatus* as previously reported (Edgerton *et al.*, 2000), but the transmission electron microscopy and the challenge trial failed to confirm the viral aetiology. Perhaps the cellular changes in hypertrophied nuclei population may be caused by infection and recovery may have taken place during the experiment in which viruses have been eradicated, therefore there would be no transmission, but the changes still could be observed in the previously infected tissues. How fast the cells can return to normal is unknown. In addition, possibly

both populations have reached “a viral accommodation equilibrium” (Flegel and Pasharawipas, 1998; Flegel, 2007) in which additional infection with new virus will not produce any adverse effect.

In the suppression subtractive hybridization trial, many differentially expressed genes were identified in the libraries, but for the purpose of this study, only immune-related genes will be discussed. Among a variety of transcripts related to immune response, three antimicrobial peptides belonging to lysozyme, crustin and the ALF family were detected. Lysozymes have the ability to lyse bacteria by splitting the glycosidic linkage between N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan in the bacterial cell wall (Jolles and Jolles, 1984; Bachali *et al.*, 2002). Bacteriolytical activity of these enzymes in crustacea has been reported against both Gram-positive and negative bacteria including pathogenic *Vibrio* species (Fenouil and Roch, 1991; Hikima *et al.*, 2003; Burge *et al.*, 2007; Yao *et al.*, 2008). Lysozymes are also thought to play a role in an antiviral response in crustacea (He *et al.*, 2005; Pan *et al.*, 2005; Mai and Wang, 2010). Senapin and Phongdara (2006) found that lysozymes could bind to viral capsid proteins (VP1 and VP2) of Taura syndrome virus (TSV). Similarly, crustin, a cysteine-rich peptide that contains a whey acidic protein (WAP) domain also has antibacterial activity against Gram-positive bacteria, but some type II and III crustins have the ability to respond to both Gram-positive and negative bacteria (Donpudsa *et al.*, 2010b). Type III crustins may also have proteinase inhibitory activities (Amparyup *et al.*, 2008).

Like lysozymes, anti-lipopolysaccharide factor can be found across a variety of organisms and has multiple biological activities. This molecule inhibits both Gram-positive and negative bacteria and fungus (de la Vega *et al.*, 2008; Yedery and Reddy, 2009; Sun *et al.*, 2011). This antimicrobial peptide is predicted to have an opsonising function for haemocytes in phagocytosing bacteria (Sun *et al.*, 2011). The ALF may also have a defensive role in viral infected crustacea (Liu *et al.*, 2006; de la Vega *et al.*, 2008; Antony *et al.*, 2011; Liu *et al.*, 2011a). It was found that the ALF was up regulated in the WSSV-experimentally infected crayfishand silencing this protein enhanced viral propagation (Liu *et al.*, 2006; Liu *et al.*, 2011a).

Furthermore, this peptide is considered a potential therapeutic agent for prophylactic treatment of viral and bacterial infectious diseases and septic shock (Somboonwiwat *et al.*, 2008)

The transcripts that dominated the immune-related genes from the SHH libraries had highest similarity to Kazal-type serine proteinase inhibitor of *P. clarkii* and serum amyloid A of *Equus caballus*. The Kazal-type serine proteinase inhibitors are believed to have a role in regulation of immune reactions of crustacea, inhibition of proteinase from microorganisms, bacteriostatic activities against both Gram-positive and negative bacteria (Donpudsa *et al.*, 2009; Li *et al.*, 2009b) and are probably involved in an antiviral response as well (Donpudsa *et al.*, 2010a; Liu *et al.*, 2011a). Serum amyloid A (SAA) is an acute phase protein (APP) that has a role in inflammatory processes in vertebrates. This acute phase protein increased in viral and bacterial pathogens infected animals (Cray *et al.*, 2009). Its role in invertebrates, in particular crustaceans, has hardly been investigated. However, in the sea cucumber *Holothuria glaberrima*, the serum amyloid A was predicted to be involved in intestinal morphogenesis (Santiago-Cardona *et al.*, 2003).

Translationally controlled tumour proteins (TCPT) or fortilins have been implicated in cell cycle progression, malignant transformation, anti-apoptotic activity and cell stress (Bommer and Thiele, 2004). In the banana prawn, *P. merguiensis*, fortilin was suggested to be involved in early oocyte maturation and may be related to cell proliferation and differentiation (Loongyai *et al.*, 2007b). This protein also has binding ability to calcium, tubulin, myeloid cell leukaemia (MCL)-1 protein, elongation factor (EF)-1α (Bangrak *et al.*, 2004; Bommer and Thiele, 2004; Loongyai *et al.*, 2007b) and some transcription factors (Chen *et al.*, 2009). In *P. monodon*, TCTP was suggested to protect virally infected cells from dying, thus keeping the prawns healthy (Bangrak *et al.*, 2004; Graidist *et al.*, 2006). The TCTP could also inhibit viral replication, thus decreasing the amount of viral infection (Tonganunt *et al.*, 2008).

Additional transcripts related to immune factors found in the gill cDNA SSH libraries were chaperonin 10 and eukaryotic initiation factor (eIF) 4A. Chaperonin is

a protein that plays an essential role in mediating folding of unfolded polypeptides such as newly translated, imported and stress-denatured proteins. The type of chaperonin determines the process of protein-folding activity. The protein-folding activity of chaperonin I is related to the interaction of chaperonin 60 and chaperonin 10 activities. The type I chaperonin can be found in the chloroplast, eubacteria and mitochondria. Type II chaperonin has only chaperonin 60 and can be found in *Archaebacteria* and eukaryotic cytosol (Levy-Rimler *et al.*, 2002; Valpuesta *et al.*, 2002). Chaperonin also plays an important role in cellular functions. For example, deletion of mitochondrial yeast and bacterial chaperonins can be lethal to both organisms. It is suggested that in humans hereditary spastic paraplegia spg 13 occurs due to mutation of mitochondrial chaperonin 60 (Levy-Rimler *et al.*, 2002). Accumulation of toxic protein aggregating in systemic and neurological diseases of humans such as Parkinson's and Huntington's may be related to protein misfolding (Spiess *et al.*, 2004). Chaperonin 10 was also implicated as growth and differentiation factors and may have immunosuppressive activity such as an anti-inflammatory activity (Dobocan *et al.*, 2009).

The eukaryotic initiation factor 4 families including eIF4A, eIF4B, eIF4E and eIF4F have a role in catalysing the initiation reaction of protein synthesis (Hernández and Vazquez-Pianzola, 2005). Like other DEAD box proteins, eIF4A is involved in the formation of a translation initiation complex (Surakasi and Kim, 2010). The eIF4A complex consists of eIF4A-I, eIF4A-II and eIF4A-III and presents in various taxonomic groups of organisms. The eIF4A-I and eIF4A-II have an essential role in initiation of translation, while the eIF4A-III may provide a link between splicing, mRNA localization, mRNA decay and differentiation of cells (Hernández and Vazquez-Pianzola, 2005; Low *et al.*, 2007). In *Xenopus*, the eIF4A-II was predicted to play a role in neuroectodermus development. In humans, the eIF4A may also be involved in the growth status of the cell. For example eIF4A-I expression was induced in melanomas (Hernández and Vazquez-Pianzola, 2005). The eIF4A of fruit fly, *Drosophila melanogaster* was up-regulated in wing imaginal discs in the tumour suppressor mutant where cells proliferated excessively (Hernández *et al.*, 2004).

A transcript similar to a matrix cellular protein, putative elastin A of *Rimicaris exoculata* was also identified in the gill cDNA library. In vertebrates, the presence of elastic fibres in the extracellular space of the connective tissue determines their resilience and maintains pressure related to liquid and air flow. This protein can be found abundantly in the skin, ligament, cartilage, lungs and vascular tissue (Duca *et al.*, 2004; Foster, 2004; Muiznieks *et al.*, 2010). Accordingly, the extracellular matrix content of various organs also determines the susceptibility of the organs to tumour progression. In an experimental tumour model, elastin has been implicated as a factor involved in inhibition of the metastatic processes (Lapis and Tímár, 2002). This protein also has the ability to induce motility signals in cancer cells (Lapis and Tímár, 2002). Despite their biological activities related to cancer, elastin peptides are also suggested to be involved in vasorelaxation, stimulation of leukocytes' oxidative burst, release of lysosomal enzyme, synthesis of endogenous cholesterol, modification of ion flexus and inducing apoptosis (Duca *et al.*, 2004).

A gene representing laminin receptor was also expressed in the SSH libabries. Laminin receptor is a protein with a molecular mass about 67kDa that has high affinity and specificity for laminin (Nelson *et al.*, 2008). The binding of the laminin protein to the laminin receptor have been implicated in many biological activities such as cell adhesion, proliferation, differentiation and migration. This receptor has also binding ability to elastin and its degradation products (Fülöp and Larbi, 2002). Elastin-laminin receptor plays an important role in extracellular matrix remodelling in aging, atherosclerosis, extravasations, tumor invasion and metastasis (Kunecki and Nawrocka, 2001; Fülöp and Larbi, 2002). In addition, laminin receptor may contribute in bacterial and viral infection (Fülöp and Larbi, 2002; Senapin and Phongdara, 2006). In penaeid prawns, laminin receptor was observed to bind to viral protein (VP) of Taura syndrome virus (TSV), yellow head virus (YHV) and infectious myonecrosis virus (IMNV) (Senapin and Phongdara, 2006; Busayarat *et al.*, 2011). Up-regulation of the laminin receptor was found in WSSV-infected redclaw crayfish (Liu *et al.*, 2011a), suggesting this receptor has protective function against viral infections in decapod crustacea through binding to viral envelop

proteins and this prevents viruses binding to target host cells (Busayarat *et al.*, 2011; Liu *et al.*, 2011a).

An antioxidant enzyme of cytosolic manganese superoxide dismutase (cMnSOD) has been implicated in the immune response of crustacea. The principal function of SOD is to protect host cells against the cytotoxic effect of reactive oxygen species (ROS) produced during the activation of host NADPH-oxidase in the phagocytosis process (Li *et al.*, 2010b; Lin *et al.*, 2010). Marchand *et al.*(2009) found that cMnSOD mRNA expression in hydrothermal crab species, *Bythograea thermydron* and *Segonzacia mesantlantica* was significantly higher than in coastal crab species, *Necora puber* and *Cancer pagurus*. These authors suggested that the environmental conditions of the hydrothermal vent might induce the cMnSOD expression in the crabs as an adaptive response to the higher exposure to oxidative stress compared to less exposure of littoral crabs.

Finally, within the group of genes related to immune factors, transcript encoding insulin growth factor binding protein (IGFBP) 7 was detected in the SSH library. The IGFBP is a family of secreted proteins that bind to insulin-like growth factor (IGF)-I and –II with high affinity and determines their biological activities (Clemmons, 1997). This protein is involved in IGFs transport, protects them from degradation, limits their binding to receptors and maintains a reservoir of biologically inactive IGFs (Castellanos *et al.*, 2008). Insulin-like growth factor plays an important role in growth and differentiation of normal and malignant cells (Hwa *et al.*, 1999; Navarro *et al.*, 1999). The up-regulation of IGFBP 7 in WSSV-infected crayfish suggests its involvement in anti-viral defence mechanism (Liu *et al.*, 2011a). Enzymes related to energy and metabolism including NADH dehydrogenase, cytochrome c oxidase, ATP synthase, ATP-dependent RNA helicase were also identified as up-regulated in the gill cDNA SSH libraries. Clearly, an animal will up-regulated its metabolism when it needs to mobilise its immune response to react to an invading pathogen.

In view of the fact that the transmission electron microscopy and the challenge trial failed to provide unequivocal evidence of viral causation suggesting a conclusion of

an idiopathic aetiology, one should keep in mind that the health status of these two populations of redclaw crayfish was different, resulting in differentially expressed immune-related genes in the two populations, with some genes being up regulated in the hypertrophied nuclei animals. These genes represented antimicrobial peptides, proteinase inhibitor, acute phase protein, insulin growth factor binding protein, protein folding, eukaryotic initiation factor and matrix cellular protein, which are all known to be involved in immune reactions. All in all, further study is necessary to uncover the cause of the hypertrophied nuclei with marginated chromatin in the gills of *C. quadricarinatus* (see Chapter 5).

CHAPTER 5

ENDOGENOUS BREVIDENSOVIRUS-LIKE ELEMENTS IN *Cherax quadricarinatus*: FRIEND OR FOE?

Partly published. Rusaini, La Fauce, K.A., Elliman, J., Bowater, R.O. and Owens, L. (2013) Endogenous Brevidensovirus-like elements in *Cherax quadricarinatus*: Friend or foe? *Aquaculture* **396-399**: 136-145 (Appendix 4.3).

5.1. Introduction

Viral fragments integrated into host genomes are called endogenous viral elements, EVEs (Katzourakis and Gifford, 2010), and have been recognised for many years (Benveniste and Todaro, 1974; Zhdanov, 1975; Jaenisch, 1976). Endogenisation of viral elements occurs when a double stranded DNA copy of the viral genome is inserted into the germ line of the host and can potentially be transmitted vertically to the offspring and become fixed in the population of the host (Kapoor *et al.*, 2010; Holmes, 2011; Feschotte and Gilbert, 2012). Retroviruses are probably the only known group of viruses that involve the integration of viral genomes into the host chromosomal DNA as an obligate step of their proliferation process. Therefore, they are predisposed to integrate into the germ line of the host and become inherited as host alleles. Interestingly, about eight percent of human genomes are composed of these elements in the form of endogenous proviruses. Most likely similar proportions of these endogenous elements exist in other mammal genomes (Jaenisch, 1976; Consortium, 2001; Griffiths, 2001; Gifford and Tristem, 2003; Belyi *et al.*, 2010b; Emerman and Malik, 2010; Feschotte, 2010; Horie *et al.*, 2010; Katzourakis and Gifford, 2010; Feschotte and Gilbert, 2012). Some non-retroviruses also establish other genomic replication strategies but are never found in the germ line of the host cells (Horie *et al.*, 2010; Katzourakis and Gifford, 2010).

Integration of viral genomes into the host cells involves a broad range of viral families both RNA and DNA viruses from various organisms (Belyi *et al*., 2010b; Belyi *et al.*, 2010a; Horie *et al.*, 2010; Kapoor *et al.*, 2010; Katzourakis and Gifford, 2010; Holmes, 2011; Liu *et al.*, 2011b; Feschotte and Gilbert, 2012). This phenomenonhas been detected in bacteria (Simpson *et al.*, 2000; Salanoubat *et al.*, 2002), fungi (Frank and Wolfe, 2009; Taylor and Bruenn, 2009; Liu *et al.*, 2010a), algae (Delaroque *et al.*, 1999; Cock *et al.*, 2010), plants (Bejarano *et al.*, 1996; Harper *et al.*, 2002; Tanne and Sela, 2005; Liu *et al.*, 2010a; Chiba *et al.*, 2011) and protozoa (Liu *et al.*, 2010a). In animals, these insertions are commonly found in vertebrates such as mammals, birds and fish (Benveniste and Todaro, 1974; Zhdanov, 1975; Belyi *et al.*, 2010b; Belyi *et al.*, 2010a; Horie *et al.*, 2010; Kapoor *et*

al., 2010; Katzourakis and Gifford, 2010; Liu *et al.*, 2011b; Fort *et al.*, 2012). In invertebrates, virus-related sequences have been reported in trematodes (Liu *et al.*, 2011b), nematodes (Malik *et al.*, 2000; Liu *et al.*, 2010a; Fort *et al.*, 2012), gastropods (Liu *et al.*, 2010a), tunicates (Liu *et al.*, 2011b), but primarily in arthropods such as insects, arachnids and copepods (Lin *et al.*, 1999; Malik *et al.*, 2000; Crochu *et al.*, 2004; Maori *et al.*, 2007; Roiz *et al.*, 2009; Kapoor *et al.*, 2010; Katzourakis and Gifford, 2010; Liu *et al.*, 2010a; Liu *et al.*, 2011b; Cui and Holmes, 2012; Fort *et al.*, 2012). The endogenous viral sequences that have been identified in decapod crustacea include endogenous *Penaeus stylirostris* densovirus-like elements (EPstDNVE) in *Penaeus monodon*(Tang and Lightner, 2006; Saksmerprome *et al.*, 2011) and endogenous white spot syndrome virus-like elements (EWSSVE) in *P. monodon*(Huang *et al.*, 2011) and *P. japonicus*(Dang *et al.*, 2010; Koyama *et al.*, 2010).

The insertion of endogenous viral elements in the host cells may provide an antiviral protection against infection by closely-related, present day exogenous viruses (Griffiths, 2001; Flegel, 2009; Belyi *et al.*, 2010b). This may be achieved by synthesising dominant negative fragments or antisense RNAs that inhibit viral replication cycles (Flegel, 2009; Fort *et al.*, 2012), synthesising a new phenotype of immune response that could recognise and prevent similar infection (Liu *et al.*, 2010a), activating the innate immunity of the host or via the protein expression that act as immunogens (Holmes, 2011). This may be true if some components of the elements are still active and are able to produce proteins that have protective immunity to exogenous viral infection (Feschotte and Gilbert, 2012).

This chapter describes the presence of endogenous Brevidensovirus-like elements (EBreVE) in *Cherax quadricarinatus* when the viral aetiology of “signet ring” hyperthrophied nuclei with marginated chromatin but without Cowdry type A (CA) intranuclear inclusion bodies within the gills of redclaw crayfish was investigated (Chapter 4). Previously (Chapter 4), transmission electron microscopy, challenge trial and suppression subtractive hybridization failed to provide evidence of viral aetiology. Following the presentation of this work at the 1st Fisheries Research and

Development Corporation (FRDC) Australasian Aquatic Animal Health Scientific Conference, 5-8 July 2011 in Cairns, a senior researcher, Dr. Jeff A. Cowley of Commonwealth Scientific and Industrial Research Organisation (CSIRO) Food Futures National Research Flagship, CSIRO Livestock Industries, Queensland Biosciences Precinct, Australia suggested conducting a nested PCR using Mourilyan virus (MoV) primers since proliferative endoplasmic reticulum (ER) also appeared in the tissues of penaeid prawns infected with MoV and this virus has no poly(A) tail. Therefore, several primers were designed and used to amplify suspected viral genomes in the present study.

5.2. Materials and Methods

5.2.1. Experimental Animals

Redclaw crayfish, *Cherax quadricarinatus*, were sourced from two stocks at the School of Veterinary and Biomedical Sciences (SVBMS), James Cook University (JCU). Redclaw crayfish from the Marine and Aquaculture Research Facilities Unit (MARFU) at School of Marine and Tropical Biology, JCU that had experienced chronic mortality were sampled. Redclaw crayfish were also taken from two geographically separated crayfish farms (Farm A and Farm B) in northern Queensland. The crayfish were transported to the Aquatic Pathology Laboratory of SVBMS, JCU for extraction of material from the gills (20 animals from each population). In addition, extracted DNA from the gills of crayfish that had developed characteristics of hypertrophied nuclei with Cowdry type A intranuclear inclusion bodies (*Cherax quadricarinatus* parvo-like virus, CqPV) in the gills (Bowater *et al.*, 2002) from Tropical and Aquatic Animal Health Laboratory, Biosecurity Queensland (North), Department of Agriculture, Fisheries and Forestry (DAFF) was examined. Crayfish were anaesthetised by submerging in iced water. The cephalothorax was cut midsagitally, one half was preserved in 95% ethanol for nucleic acid isolation and the remaining half was fixed in Davidson's fixative for histological examination.

5.2.2. Histology

Histological examination was described in Section 4.2.2.

5.2.3. Nucleic acids extraction

Deoxyribonucleic acid (DNA) was extracted from the gills of crayfish using a High Pure PCR Template Preparation Kit (Roche Diagnostics, USA catalogue # 11796828001) or Wizard® SV Genomic DNA Purification System (Promega, USA catalogue # A2361) following the manufacturer's protocols. To obtain a positive control for the parvovirus primers, DNA was extracted from the pleopods of banana prawn, *Penaeus merguiensis* known to be positive for Australian *Penaeus merguiensis* densovirus (PmergDNV). Total RNA was also extracted from the gills of hypertrophied and non-hypertrophied crayfish (20 crayfish from each population) using SV Total RNA Isolation System (Promega) according to the manufacturer's instructions and kept at -80°C until used.

5.2.4. Polymerase chain reaction (PCR) amplification

5.2.4.1. PCR amplification with parvovirus primers and reverse transcriptase-PCR (RT-PCR) with bunyavirus primers

Several parvovirus primers (Table 5.1) were designed from various penaeid densoviruses, insect densoviruses and a brevidensovirus (IHHNV) of penaeids to amplify any relevant viral sequences from the nuclear changes in the hypertrophied nuclei population. To investigate the possibility of the aetiological agent of the nuclear changes caused by virus with no poly(A) tail, several bunyavirus primers (Table 5.1) were designed from members of the related-genus Phlebovirus within the Family Bunyaviridae (van Regenmortel *et al.*, 2000), including Mourilyan virus (AY927991), Uukuniemi virus (M17417) and Toscana virus (EU003175). Sequences were aligned and primers were designed in areas of genetic similarity using Vector NTI software or Oligo 7 software. Primers were synthesised by Sigma-Aldrich Pty Ltd, Australia or Macrogen, Korea. Some published primers were also used in this study (Table 5.1).

Complementary DNA (cDNA) was synthesised from total RNA using random primers of ImProm-II™ Reverse Transcription System (Promega) according to manufacturer's instruction. Reverse transcriptase (RT)-nested PCR was performed using primers MoV24F/MoV25R in the first PCR. Templates with amplicons that appeared in the gel electrophoresis produced from the primary PCR were subjected to secondary amplification (nested PCR) using primers MoV148F/MoV149R.

Polymerase chain reaction mixture (Table 5.2) contained 12.5 µl of GoTaq®Green Master Mix (Promega, USA catalogue # M7123), 1µl of DNA templates (PCR amplification) or 1 - 2 µl of cDNA templates (RT-PCR amplification), and 0.75 µl (10 µM) of each primer. The PCR reaction volume was adjusted with nuclease free water (Promega) to a final volume of 25 µl. The PCR amplification (Table 5.1) was performed in a Mastercycler gradient 5333 (Eppendorf, Germany). Amplified products (10 µl) were visualised in 1.2% agarose-TAE gels containing GelRed (10,000x in water) at a concentration of 0.5:10,000. Gels were visualised and photographed using an InGenius LHR, gel documentation and analysis system (Syngene, UK).

5.2.4.2. PCR amplification of endogenous virus-like elements in *Cherax quadricarinatus*

Primers QPF1 and QPR1 (sequences kindly provided by Dr. Jeff A. Cowley) were used to amplify the putative endogenous virus-like elements in *C. quadricarinatus*. The QPF1/QPR1 primers are located in the non-structural protein region of IHHNV. The QPF1 is at nucleotide (nt) 1514 – 1533 of IHHNV isolate Au2005 (EU675312) or at nt 1519 – 1538 of Hawaiian isolate (AF218266). The QPR1 primer is at nt 1596 – 1617 of IHHNV isolate Au2005 (EU675312) or 1601 – 1622 of Hawaiian isolate (AF218266). Thus these primers are expected to yield a 104 bp IHHNV amplicon. Two sets of primers were also designed. The 101F21 and 314R23 primers were intended to yield a 236 bp nucleotide portion of Fragment 1 of endogenous virus-like element. Primers 832F21 and 2585R21 were expected to amplify up and down stream of Fragment 7 (Table 5.1).

Table 5.1. Primer sequences and PCR amplification profile used for presumptive viral genomes in the gills of *Cherax quadricarinatus*.

Primer name	Sequences (5' to 3')	PCR amplification profile						Expected amplicon (bp)	References
		Initial denaturation (ºC) [1]	Denaturation (ºC) [2]	Annealing (ºC) [3]	Extension (ºC) [4]	Number of cycles [2-4]	Final extension (ºC) [5]		
Parvovirus Primers									
Primers used to amplified the presumptive parvovirus genomes									
Templates: Hypertrophied nuclei population (n = 6) and non-hypertrophied nuclei population (n = 6)									
Cparvo-R161-F	CAGTCGTGTCGGCAGT**M**G	94 (7 mins)	94 (45 secs)	45 (45 secs)	72 (1 min)	35	72 (5 mins)	161	Present study
Cparvo-R161-R	ATCGTTCTTCGCAGC**R**CTAT								
Cparvo-161-F	CAGTCGTGTCGGCAGTAG	94 (7 mins)	94 (45 secs)	40 (45 secs)	72 (1 min)	35	72 (5 mins)	161	Present study
Cparvo-161-R	ATCGTTCTTCGCAGCACTAT								
Iparvo-192-F	ACCTACTTCTGTGACCTTCT	94 (7 mins)	94 (45 secs)	43 (45 secs)	72 (1 min)	35	72 (5 mins)	192	Present study
Iparvo-192-R	CTCATATTGCTATCTCGCTCTAA								
BreviD-140-F	ACGACGAAGAATGGACAGAA	94 (7 mins)	94 (45 secs)	50 (45 secs)	72 (1 min)	35	72 (5 mins)	140	Present study
BreviD-140-R	GGTGAGAAGGCTTGGAGAA								
HPV140F	CTACTCCAATGGAAACTTCTGAGC	94 (7 mins)	94 (45 secs)	55 (45 secs)	72 (1 min)	35	72 (5 mins)	140	(La Fauce *et al.*, 2007b)
HPV140R	GTGGCGTTGGAAGGCACTTC								
SMV200F	TAGCTATTTTTTGGTCGTCTG	94 (7 mins)	94 (45 secs)	58 (50 secs)	72 (1 min)	40	72 (5 mins)	207	(Owens and Cullen, 2004)
SMV200R	GCCGCAATTTACCAGTGTTTGAAG								
Primers used to amplified endogenous virus-like elements									
Templates: Hypertrophied nuclei population, non-hypertrophied nuclei population, challenge trial, MARFU (JCU), Farm A,Farm B and DAFF DNA sample.									
QPF1	AGCCGAAGCTGAAGCGACTA	94 (1 min)	94 (25 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)	104	(Cowley, Unpublished)
QPR1	GGCCAAGACCAAAATACGAAAG								

Primer name	Sequences (5' to 3')	PCR amplification profile						Expected amplicon (bp)	References
		[1]	[2]	[3]	[4]	[2-4]	[5]		
101F21	TTCATCATCAGAACAAGACCA	94 (1 min)	94 (25 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)	236	Present study
314R23	TATCTGATAAGGTAGAGAATCCA								
Primers used to amplified presumptive parvovirus (IHHNV) genomes									
Template: DAFF DNA sample									
832F21	TTTCAGACGAGGAAGACAACC	94 (4 mins)	94 (30 secs)	55 (30 secs)	72 (1 min)	40	72 (7 mins)	1,774	Present study
2585R21	CTTGTTGAATCGGCGCACATA								
832F21	TTTCAGACGAGGAAGACAACC	94 (4 mins)	94 (30 secs)	55 (30 secs)	72 (1 min)	40	72 (7 mins)	780	Present study
QPR1	GGCCAAGACCAAAATACGAAAG								(Cowley, Unpublished)
QPF1	AGCCGAAGCTGAAGCGACTA	94 (4 mins)	94 (30 secs)	55 (30 secs)	72 (1 min)	40	72 (7 mins)	1,072	(Cowley, Unpublished)
2585R21	CTTGTTGAATCGGCGCACATA								Present study
ihhnA_F158	ATGGAAGATACGAACAACCAC	94 (5 mins)	94 (30 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)	1,350	(Saksmerprome *et al.*, 2010)
ihhnA_R1507	GATATTTCTAACAAGTACCGTAGTCGC								
ihhnA_F1451	GTTACCTTTGCTGCCAGAGC	94 (5 mins)	94 (30 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)	1,119	(Saksmerprome *et al.*, 2010)
ihhnA_R2569	GGGCTTGCTCTTGTTGAATCGG								
ihhnA_F2544	TGCGCCGATTCAACAAGAGC	94 (5 mins)	94 (30 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)	1,082	(Saksmerprome *et al.*, 2010)
ihhnA_R3625	GTATGTATATAGGGTAGGTATAG								
ihhnA_F702	CAAGCCTTCTCACCCCAGG	94 (5 mins)	94 (30 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)	559	(Saksmerprome *et al.*, 2010)
ihhnA_R1260	TCACTCTCTTCCAGTCGCCT								
ihhnA_F3168	GCCTATACAATCCATGGTGAC	94 (5 mins)	94 (30 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)	292	(Saksmerprome *et al.*, 2010)
IHHNV_R3459	CCAAACTTGCGACACATCC								(Saksmerprome *et al.*, 2011)
IHHNV309F	TCCAACACTTAGTCAAAACCAA	94 (5 mins)	94 (30 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)	309	(Tang *et al.*, 2007)
IHHNV309R	TGTCTGCTACGATGATTATCCA								
HPV140F	CTACTCCAATGGAAACTTCTGAGC	94 (7 mins)	94 (45 secs)	65 (45 secs)	72 (1 min)	40	72 (5 mins)	140	(La Fauce *et al.*, 2007b)
HPV140R	GTGGCGTTGGAAGGCACTTC								

Primer name	Sequences (5' to 3')	PCR amplification profile						Expected amplicon (bp)	References
		[1]	[2]	[3]	[4]	[2-4]	[5]		
Bunyavirus Primers									
Primers used to amplify presumptive viral genomes which have no poly(A) tail									
Templates: Hypertrophied nuclei population (n = 20) and non-hypertrophied nuclei population (n = 20)									
MoV24F	GGGATGGTGTTGCCATACAAAGG	95 (2 mins)	95 (30 secs)	60 (30 secs)	72 (40 secs)	40	72 (7 mins)	610	(Cowley *et al*., 2005a)
MoV25R	GTCATTAGCTGGTCTTAGTTTTCAC								
MoV148F	ACAGTTTGTCAAGCTCACAGGATG	95 (2 mins)	95 (30 secs)	58 (30 secs)	72 (30 secs)	40	72 (7 mins)	322	(Cowley *et al*., 2005a)
MoV149R	AGAAGCGCCATTCTGATGAACATC								
MoV210F	GGCCACCCTTACTATCCTTG	95 (2 mins)	95 (30 secs)	58 (30 secs)	72 (40 secs)	40	72 (7 mins)	249	Present study
MoV439R	ATTGTCCTTGTCTCGGGGTC								
UUKV2558F	TTCCAATAAGTGTAGCCCAAG	95 (10 mins)	95 (20 secs)	58 (20 secs)	72 (20 secs)	45	72 (7 mins)	668	Present study
UUKV3205R	AAAGACACGGCTACATGGAAC								
TosV2667F	AGCGAAAAGCAATTTATCTCA	94 (2 mins)	94 (30 secs)	45 (1 min)	68 (1 min)	40	68 (5 mins)	416	Present study
TosV3064R	CTCATAGCCATCAGAACCA								
Primer Walking									
Primers used to amplify the flanking regions of Fragments 1, 3, 4, 8 and 9 (Contig 1) of endogenous virus-like elements									
Templates: Specimens Cq02B, Cq17B, Cq20B (non-hypertrophied nuclei population, n = 20)									
314F23	TGGATTCTCTACCTTATCAGATA	94 (4 mins)	94 (30 secs)	40 (30 secs)	72 (1 min)	40	72 (7 mins)		Present study
314R23	TATCTGATAAGGTAGAGAATCCA	94 (4 mins)	94 (30 secs)	40 (30 secs)	72 (1 min)	40	72 (7 mins)		Present study
101F21	TTCATCATCAGAACAAGACCA	94 (4 mins)	94 (30 secs)	40 (30 secs)	72 (1 min)	40	72 (7 mins)		Present study
101R21	TGGTCTTGTTCTGATGATGAA	94 (4 mins)	94 (30 secs)	40 (30 secs)	72 (1 min)	40	72 (7 mins)		Present study
Templates: Specimens Cq17B (non-hypertrophied nuclei population, n = 20)], CqAq25 (MARFU, JCU), CqTL04 (Farm A)									
755F40	TTCATCATCAGAACAAGACCAAAACCA AGAACAGGAGGAA	94 (5 mins)	94 (45 secs)	50 (1 min)	72 (1 min)	40	72 (7 mins)		Present study
150R40	GTAAGTTCACGAAGTCTTGGCGAGTTT TCTTGCTGCTGAC	94 (5 mins)	94 (45 secs)	50 (1 min)	72 (1 min)	40	72 (7 mins)		Present study

Primer name	Sequences (5' to 3')	PCR amplification profile						Expected amplicon (bp)	References
		[1]	[2]	[3]	[4]	[2-4]	[5]		
Primers used to amplify the flanking region of Fragments 2 and 5 (Contig 2) of endogenous virus-like elements									
Templates: Specimens Cq01A (hypertrophied nuclei population, n = 20), Cq07B, Cq19B (non-hypertrophied nuclei population, n = 20)]									
88R23	GATAAGGTAGAGAATCCAGCATC	94 (4 mins)	94 (30 secs)	40 (30 secs)	72 (1 min)	40	72 (7 mins)		Present study
88F23	GATGCTGGATTCTCTACCTTATC	94 (4 mins)	94 (30 secs)	40 (30 secs)	72 (1 min)	40	72 (7 mins)		Present study
Templates: Specimens Cq01A (hypertrophied nuclei population, n = 20), CqDP03 (hypertrophied nuclei population, n = 5b), Cq19B (non-hypertrophied nuclei population, n = 20)									
160F40	AGAGCCGAAGCTGAAGCGACTACTACT GTTAAACCATACC	94 (5 mins)	94 (45 secs)	50 (1 min)	72 (1 min)	40	72 (7 mins)		Present study
293R40	TGGCCAAGACCAAAATACGAAAGTTTT TCCACTCCGTATC	94 (5 mins)	94 (45 secs)	50 (1 min)	72 (1 min)	40	72 (7 mins)		Present study
Primers used to amplify the flanking region of fragment 6 (Contig 3) of endogenous virus-like elements									
Templates: Specimens Cq2.1(from n = 6) and Cq1.09.606 (from n = 5a) of hypertrophied nuclei population									
145F40	AACGTGATGTGCCAGTGGTCATTGTGG TCTGTGAGCGTGA	94 (5 mins)	94 (45 secs)	50 (1 min)	72 (1 min)	40	72 (7 mins)		Present study
194R40	TTCATCATCAGAACAAGACCAGTCCCA AGAGGAGGAAAAC	94 (5 mins)	94 (45 secs)	50 (1 min)	72 (1 min)	40	72 (7 mins)		Present study
Primers used to amplify the flanking region of fragment 7 (Contig 4) of endogenous virus-like elements									
Templates: DAFF DNA sample (n = 1)									
QPF1	AGCCGAAGCTGAAGCGACTA	94 (1 min)	94 (25 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)		(Cowley, unpublished)
QPR1	GGCCAAGACCAAAATACGAAAG	94 (1 min)	94 (25 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)		(Cowley, unpublished)
CqIHHNVF40	AAGCTGAAGCGACTACGGTACTTATTA AAGATATCAAGAG	94 (5 mins)	94 (30 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)		Present study
CqIHHNVR29	AAAGCCGTTCAATACCGTATCTGATAA GA	94 (5 mins)	94 (30 secs)	55 (30 secs)	72 (30 secs)	40	72 (7 mins)		Present study

Symbols:**M** = A + C, **R** = A + G

Table 5.2. The PCR reaction mixture. The reaction contained 12.5 µl of GoTaq®Green Master Mix (Promega) and the volume was adjusted with nuclease free water (Promega) to a final volume of 25 µl.

Primer name	PCR mixture (µl)			
	Primer F/R (10 µM)	$MgCl_2$ (25 mM)	$DMSO_2$	Template
Parvovirus/Bunyavirus Primers				
Cparvo-R161-F/ Cparvo-R161-R	0.75	-	-	1
Cparvo-161-F/ Cparvo-161-R	0.75	-	-	1
Iparvo-192-F/ Iparvo-192-R	0.75	-	-	1
BreviD-140-F/ BreviD-140-R	0.75	-	-	1
HPV140F/ HPV140R	0.75	-	-	1
SMV200F/ SMV200R	0.75	-	-	1
QPF1/ QPR1	0.75	-	-	1
101F21/314R23	0.75	-	-	1
832F21/2585R21	1.00	-	-	1
832F21/ QPR1	1.00	-	-	1
QPF1/2585R21	1.00	-	-	1
ihhnA_F158/ ihhnA_R1507	0.75	-	-	1
ihhnA_F1451/ ihhnA_R2569	0.75	-	-	1
ihhnA_F2544/ ihhnA_R3625	0.75	-	-	1
ihhnA_F702/ ihhnA_R1260	1.00	-	-	2
ihhnA_F3168/IHHNV_R3459	1.00	-	-	2
IHHNV309F/ IHHNV309R	1.00	-	-	2
MoV24F/ MoV25R	0.75	-	-	1
MoV148F/ MoV149R	0.75	-	-	1
MoV210F/ MoV439R	0.75	-	-	1
UUKV2558F/ UUKV3205R	0.75	-	-	2
TosV2667F/ TosV3064R	0.75	-	-	2
Primer Walking				
314F23	1.00	-	-	2
314R23	1.00	-	-	2
101F21	1.00	-	-	2
101R21	1.00	-	-	2
88R23	1.00	-	-	2
88F23	1.00	-	-	2
755F40	1.00	2	-	2
150R40	1.00	2	-	2
160F40	1.00	2	-	2
293R40	1.00	2	-	2
145F40	1.00	2	-	2
194R40	1.00	2	-	2
QPF1	1.50	2	1.25	2
QPR1	1.50	2	1.25	2
CqIHHNVF40	1.00	-	-	1
CqIHHNVR29	1.00	-	-	1

The PCR mixture contained (Table 5.2) 12.5 µl of GoTaq®Green Master Mix, 1µl of DNA template, 0.75 µl (10 µM) of primers QPF1/QPR1 and 101F21/314R23 or 1.0 µl (10 µM) of primers 832F21/2585R21, 832F21/QPR1 and QPF1/2585R21. This mixture was adjusted with nuclease free water to a final volume of 25 µl. The PCR amplification profile of primers QPF1/QPR1 and 101F21/314R23 consisted of initial denaturation at 94°C for 1 minute (min), 40 cycles denaturation at 94°C for 25 secs, annealing at 55°C for 30 secs, extension at 72°C for 30 secs and followed by final extension at 72°C for 7 mins. The amplification profile was similar for primers 832F21/2585R21, 832F21/QPR1 and QPF1/2585R21 and differed only in denaturation (30 secs) and extension (1 min).

Primers for gene walking were designed for unidirectional extension (i.e. only one primer was used in each reaction) to identify the flanking regions of endogenous viral fragments (Table 5.1). The PCR mixture contained 12.5 µl of GoTaq®Green Master Mix, 1 – 2 µl of DNA template, 1 – 1.5 µl (10 µM) of each primer and was adjusted with nuclease free water to a final volume of 25 µl. The PCR amplification profile is provided in Table 5.1.

5.2.5. Cloning and sequencing

The resolved PCR products were extracted and purified from the agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega) according to manufacturer's protocol. The recovery products were ligated into pGEM-T easy vectors (Promega) and transformed into JM109 competent cells which were plated onto agar containing ampicillin, X-gal and IPTG following the instructions of the manufacturer. Plasmid DNA was extracted from randomly selected white colonies using Wizard® Plus SV Minipreps DNA Purification System (Promega) following manufacturer's protocol and sent to Macrogen, Korea for sequencing. Nucleotide sequences were analysed with BLASTn and BLASTx against known nucleotide and amino acid sequences on GenBank databases (NCBI) and only the highest scoring entry was listed unless otherwise stated.

5.3. Results

5.3.1. Histology

Melanised or necrotic eosinophilic nodules or filaments were observed in the gills of some crayfish from MARFU and Farms A and B. Haemocyte aggregations could be seen in the gills of animals from MARFU and Farm B. Granular haemocytes could be observed in the efferent vessel of gill filaments of Farm B samples. Some of these haemocytes appeared to be undergoing degranulation (lysis).

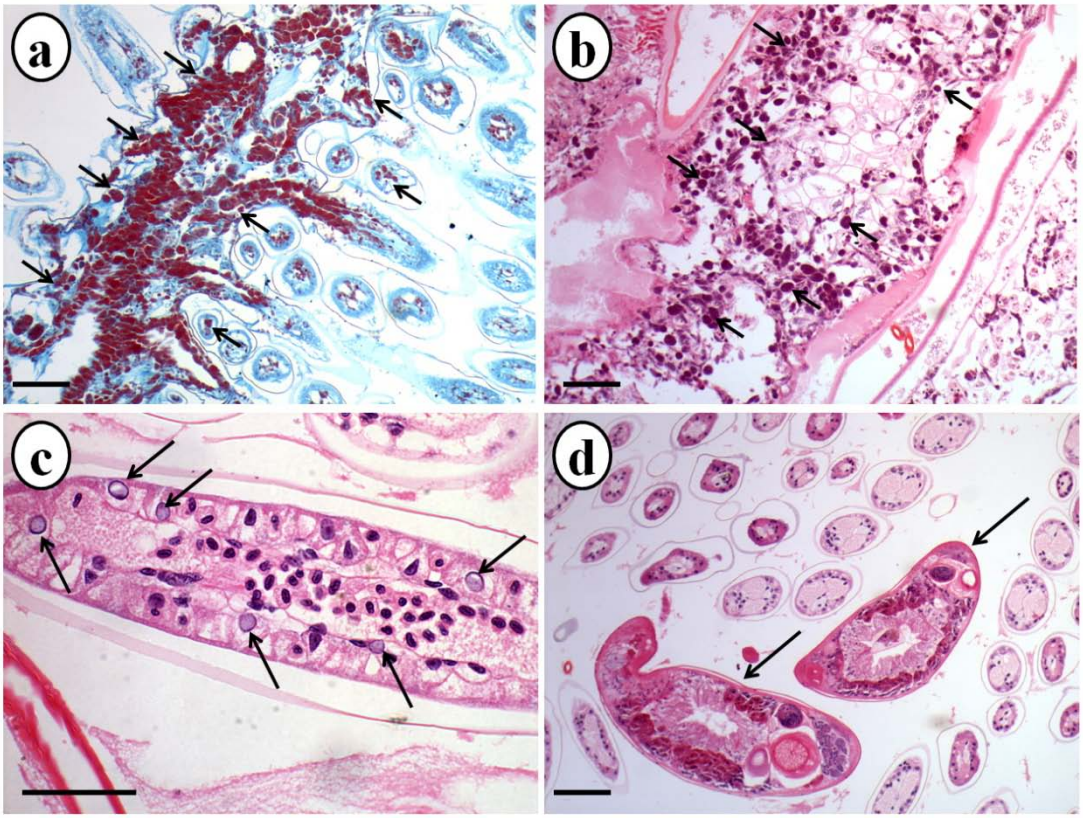

Figure 5.1. Light micrograph of longitudinal section of the gills of *Cherax quadricarinatus*. Numerous inclusion body-like structures of rickettsia-like organism (RLOs) that appeared magenta in the filaments and central axis (a) or basophilic in the connective tissue (b) of the gills of redclaw crayfish from MARFU population. Hypertrophy nuclei with marginated chromatin forming signet ring-like structures but without Cowdry type A (CA) intranuclear inclusion bodies in the gills of redclaw crayfish from Farm B population (c). Temnocephalids in the gills of redclaw crayfish from Farm A (d). Gram Twort stain: a. H & E stain: b, c and d. Scale bars: 100 µm (a, b and d) and 50 µm (c). All described structures are identified with arrows.

Furthermore, some crayfish from MARFU had numerous basophilic inclusion body-like structures of rickettsia-like organisms (RLOs) in the central axis, filaments (Figure 5.1a) and connective tissue (Figure 5.1b) of the gills. Several crayfish from Farm B had a few hypertrophied nuclei with marginated chromatin without CA intranuclear inclusion bodies in the gills (Figure 5.1c) similar to nuclear changes that have been observed in the hypertrophied nuclei population of crayfish at SVBMS, JCU (Chapter 4). Temnocephalids and their eggs were commonly found in the gills of redclaw crayfish from Farms A and B (Figure 5.1d).

5.3.2. Polymerase chain reaction (PCR) amplification

5.3.2.1. PCR amplification with parvovirus primers and reverse transcription-PCR (RT-PCR) with bunyavirus primers

Most of the primers used, produced identical amplicons in both populations, but the HPV140F/R primers did not yield an amplicon. Cparvo-161-F/R was the only primer set that produced the expected amplicon size (161 bp) from the templates of both hypertrophied nuclei and non-hypertrophied nuclei populations (Table 5.3). A 161 bp amplicon was yielded using either Cparvo-161-F/R or Cparvo-R161-F/R primers and 140 bp amplicon was produced using HPV140F/ R primers from templatepositive control of *P. merguiensis*as expected. Sequencing results of the PCR products failed to show any similarity to parvovirus sequences or other viral sequences and only the positive control sequences (with an insert size of 161 bp) had 99% similarity to Australian *Pmerg*DNV (DQ458781) as expected.

Table 5.3. PCR products of primers used to amplify presumptive parvovirus in the gills of hypertrophied nuclei (n = 6) and non-hypertrophied nuclei (n = 6) populations of *Cherax quadricarinatus*.

Primer name	Approximate PCR amplicon size
Cparvo-R161-F/Cparvo-R161-R	300 bp
Cparvo-161-F/Cparvo-161-R	161bp, 300 bp, and 500bp
Iparvo-192-F/Iparvo-192-R	300 bp and 1,000 bp
BreviD-140-F/BreviD-140-R	500 bp
HPV140F/HPV140R	-
SMV200F/SMV200R	500 bp

Table 5.4. PCR products of primers used to amplify possible bunyavirus in the gills of hypertrophied nuclei (n = 20) and non-hypertrophied nuclei (n = 20) populations of *Cherax quadricarinatus*.

Primer name	Approximate PCR amplicon size
MoV24F/MoV25R	100 bp, 250 bp
MoV148F/MoV149R	100 bp, 250 bp
MoV210F/MoV439R	100 bp
UUKV2558F/UUKV3205R	-
TosV2667F/TosV3064R	100 bp, 200 bp, 350 bp

Amplicons were produced in some samples using bunyavirus primers, but were not the expected amplicon size (Table 5.4) in both hypertrophied nuclei and non-hypertrophied nuclei populations. The RT-nested PCR amplification using MoV148F/ MoV149R primers produced the same sized amplicon as the first RT-PCR amplification using MoV24F/MoV25R primers.

5.3.2.2. PCR amplification of endogenous virus-like elements in *Cherax quadricarinatus*

Several amplicons, but not the expected 104 bp amplicon, were visualised in the electrophoretic agarose gel from both hypertrophied nuclei (n = 20) and non-hypertrophied nuclei (n = 20) DNA templates using the QPF1/QPR1 primers (Figure 5.2). The 350 bp and 170 bp amplicons from the hypertrophied nuclei population and the 1025 bp (appeared in approximate size of 1,200 bp amplicon on the agarose gel) and the 170 bp amplicons from non-hypertrophied nuclei population were extracted from the gel, purified and subjected to cloning and sequencing. Two fragments had similarities with IHHNV. The first fragment (Fragment 1)of 983 bp (excluding primers) obtained from a 1025 bp amplicon had 70% ($9e^{-29}$) nucleotide similarity with IHHNV isolate Au2005 (EU675312). This fragment also had 34% ($6e^{-25}$) homology to non-structural protein 2 (NS2) of the Australian IHHNV isolate (ACF32341). The second fragment of 128 bp (Fragment 2) obtained from 170 bp amplicon had 82% ($5e^{-08}$) nucleotide similarities with *Cherax quadricarinatus* clone epi15_C6 mRNA sequences (DQ847767) and it also shared 72% (1.2) amino acids identity to NS1 of IHHNV (AAM93906).

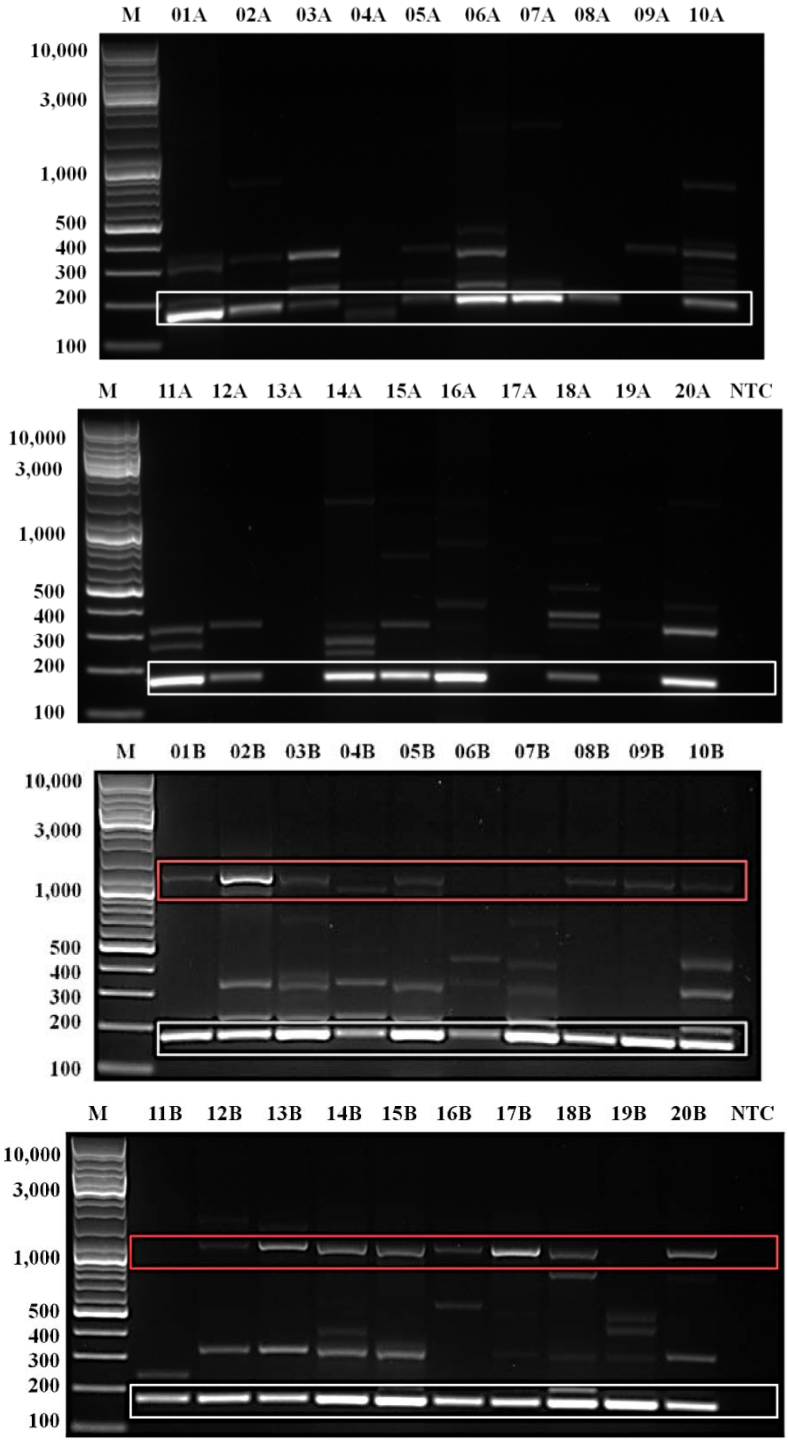

Figure 5.2. PCR amplification with QPF1/QPR1 primers showing 1025 bp amplicon (approximate size of 1,200 bp amplicon in red boxes) from the non-hypertrophied nuclei (01B-20B) population and the absence of these amplicons from the hypertrophied nuclei population (01A-20A), while 170 bp amplicon (white boxes) can be seen in both templates. PCR products are electrophorosed on a 1.2% agarose/GelRed. M: 10 kbp DNA marker (SM0333 Fermentas), NTC: non-template control.

As the QPF1/QPR1 primers amplified the endogenous viral elements rather than expected amplicon, these primers were used to screen the presence of endogenous viral elements in crayfish obtained from other sources. Every sample from all populations produced a 170 bp amplicon and most samples yielded 1025 bp amplicon. Similar results (as described above) were obtained when these two amplicons (from different sources of samples) were subjected to cloning and sequencing (Table 5.5).

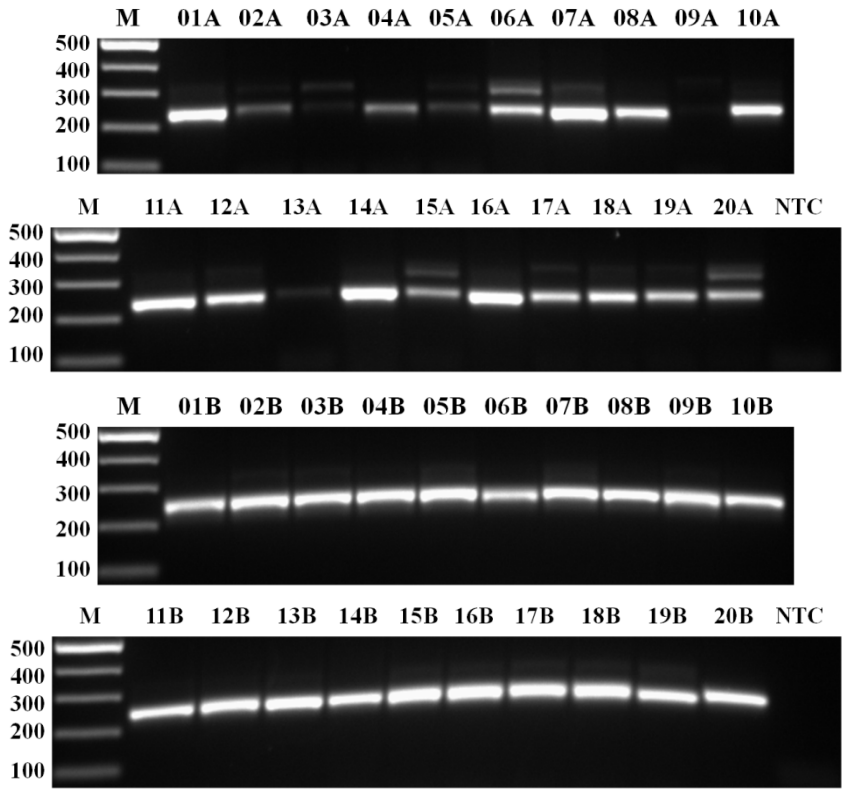

Figure 5.3.PCR amplification with 101F21/314R23 primers showing 236 bp and 296 bp amplicons on hypertrophied nuclei (01A-20A) and non-hypertrophied nuclei (01B-20B) populations. PCR products are electrophorosed on a 1.2% agarose/GelRed. M: 10 kbp DNA marker (SM0333 Fermentas), NTC: non-template control.

The primer set of 101F21 and 314R23 not only yielded a 236 bp amplicons but also a 296 bp amplicons (Figure 5.3). Most of the tested samples produced these two amplicons (Table 5.5). Sequencing results of the clones revealed that a 192 bp fragment (Fragment 3) obtained from 236 bp amplicon that shared 89% ($1e^{-05}$)

Table 5.5.PCR amplification of putative endogenous virus-like elements (EBreVE) in redclaw crayfish (*Cherax quadricarinatus*).

Primers	**QPF1/QPR1**	**101F21/314R23**		**832F21/QPR1**		**QPF1/QPR1**	**101F21/314R23**		**QPF1/QPR1**	**Lineage/ Group**
Amplicon length (including primers)	1025 bp	236 bp		245 bp	190 bp	170 bp	296 bp		104 bp	
Fragment length (excluding primers)	983 bp	192 bp	183 bp	202 bp	147 bp	128 bp	252 bp	189 bp	62 bp	
Fragment number	**1**	**3**	**4**	**8**	**9**	**2**	**5**	**6**	**7**	
Contigs	**Contig 1 (983 bp)**					**Contig 2 (288 bp)**		**Contig 3 (189 bp)**	**Contig 4 (62 bp)**	
Samples (Populations)										
DAFF Oonoomba Townsville										
DNA (n=1, 11/04/2012)	-	√ (2)	√ (1)	√ (6)	√ (1)	√ (3)	-	-	√ (3)	**1**
Hypertrophied nuclei (Paddock, JCU)										
1.09.606 – 5.09.606 (n = 5, 17/11/2009)	-	NA	√ (3)	ND	ND	√	√ (2)	√ (1)	-	**2**
Cq2.1 - Cq2.6 (n = 6, 29/01/2010)	-	NA	√ (3)	ND	ND	√	√ (2)	√ (1)	-	
CqDP01 – CqDP05 (death, n = 5, 12/12/2011)	-	√		ND	ND	√	√ (3)	?		
Cq01A – Cq20A (n = 20, 06/09/2011)	-	√ (1)	√ (2)	ND	ND	√ (9)	√		-	
Challenge trial										
Group A: CqTA01 – CqTA06 (n = 6, 22/06/2011)	-	√ (5)	√ (1)	ND	ND	√	√		-	**3**
Group B: CqTB01 – CqTB06 (n = 6, 22/06/2011)	√	√ (6)	NA	ND	ND	√	√		-	**3**
Non-hypertrophied nuclei (Fish Pathology Laboratory, JCU)										
Cq1.5 – Cq1.10 (n = 6, 28/01/2010)	√ (6)	√ (2)	√ (1)	ND	ND	√	√		-	**3**
Cq01B – Cq20B (n = 20, 07/09/2011)	√ (6)	NA	√ (3)	ND	ND	√ (9)	√		-	
Farm A										
CqTL01 – CqTL20 (n = 20, 11/03/2012)	√ (6)	√ (3)	√ (3)	ND	ND	√	√		-	**3**
Aquaculture (MARFU, JCU)										
CqAq01 – CqAq20 (death, n = 20, 06/03/2012)	√	√		ND	ND	√	√		-	**3**
CqAq21 – CqAq40 (n = 20, 06/04/2012)	√ (6)	√ (3)	NA	ND	ND	√	√		-	
Farm B										
CqBF01-CqBF20 (n = 20, 02/06/2012)	√ (9)	√ (6)	NA	ND	ND	√ (6)	√		-	**3**

Note: Tick with number in bracket [√( )]: Positive for PCR with number of randomly selected clones that are subjected to sequencing and had significant nucleotides/amino acids similarity with IHHNV. Sequencing was conducted on both forward and reverse strands. Tick (√): Positive for PCR as the amplicon appeared on the agarose gel electrophoresis, but cloning was not performed. (-): Negative for PCR as no amplicon visualised on the agarose gel electrophoresis. NA: No data available. ND: Not done, Group A: *C. quadricarinatus* injected with gill extract, Group B: *C. quadricarinatus* injected with phosphate buffer saline. n: Number of animals were sampled on the date indicated.

nucleotide identity to IHHNV (GQ475529) and 53% ($2e^{-06}$) amino acid similarity to NS1 of IHHNV (AAY59892). The 236 bp amplicon also yielded a 183 bp fragment (Fragment 4) with 9 nucleotides missing from the 192 bp and 983 bp fragments. This 183 bp fragment had 87% ($1e^{-04}$) nucleotide identity to IHHNV (GQ475529) and 47% ($1e^{-05}$) amino acid identity to NS1 of IHHNV (AAY59892.1). It also shared 94% (0.006) similarity to *C. quadricarinatus* clone CQ042 microsatellite (HQ895781).

The 296 bp amplicon also yielded two fragments. One fragment consisted of 252 bp (Fragment 5) and had 91% ($7e^{-10}$) similarity with IHHNV strain KLV-2010-01 (JN377975) and 82% ($1e^{-07}$) identity to *C. quadricarinatus* clone epi15_C6 mRNA (DQ847767). This fragment also shared 54% ($3e^{-10}$) amino acid identity to NS1 of IHHNV (AAM94165). The second fragment of 189 bp (Fragment 6) had 84% (0.006) nucleotide identity to IHHNV (GQ475529) and 51% ($1e^{-05}$) amino acids similarity with NS1 of IHHNV (AAY59892).

5.3.2.3. Endogenous virus-like elements in sample from Department of Agriculture, Fisheries and Forestry (DAFF)

QPF1/QPR1 primers produced a 170 bp and 104 bp amplicons (Figure 5.4). The 170 bp amplicon yielded a 128 bp fragment (Fragment 2). A 62 bp fragment (Fragment 7) obtained from the 104 bp amplicon shared 100% ($2e^{-23}$) nucleotide identity to IHHNV isolate Au2005 (EU675312) and had 100% ($3e^{-05}$) amino acid

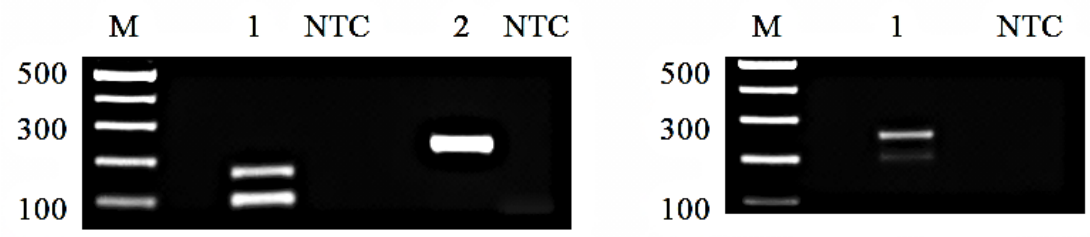

Figure 5.4. PCR amplification of DAFF DNA template with QPF1/QPR1 primers (Lane 1) produced a 170 bp and 104 bp amplicons, while 101F21/314R23 primers (Lane 2) yielded a 236 bp amplicon (left). The 832F21/QPR1 primers yielded 245 bp and 190 bp amplicons (right). PCR products were electrophorosed on a 1.2% agarose/GelRed. M: 10 kbp DNA marker (SM0333 Fermentas), NTC: non-template control.

similarity with NS1 of IHHNV (AAM93906). 101F21/314R23 primers yielded a 236 bp amplicon from which 192 bp (Fragment 3) and 183 bp (Fragment 4) fragments were obtained.

Three sets of overlapping primers that cover most of the IHHNV genome and three sets of nested primers that amplify segments within each region of IHHNV genome (Tang *et al.*, 2007; Saksmerprome *et al.*, 2010; Saksmerprome *et al.*, 2011) were used (Table 5.1) to investigate if the 104 bp amplicons (62 bp fragment) was an exogenous IHHN viral genome. However, no expected amplicons were produced from the PCR amplification. A further set of primers (832F21/2585R21) were designed, used and crossed with QPF1 and QPR1 primers to amplify both sides (up and down stream) of the 62 bp fragment. While other combined primer sets did not produce any amplicon, 832F21/QPR1 primers yielded 245 bp and 190 bp amplicons. A 202 bp fragment (Fragment 8) obtained from 245 bp PCR amplicon had 71% ($9e^{-14}$) similarity with IHHNV isolate Au2005 (EU675312) and 61% ($8e^{-13}$) amino acids identity to NS1 of IHHNV (AAY59892). A 147 bp fragment (Fragment 9) obtained from the 190 bp PCR amplicon shared 79% ($1e^{-09}$) nucleotide similarity to the IHHNV isolate Fujian, China (EF633688) and 64% ($9e^{-04}$) amino acids identity to NS1 of this IHHNV isolate (ABR23509).

5.3.2.4. Primer walking

Different sizes of amplicons were produced from most of the primers used, but not from primers QPR1, CqIHHNVF40 and CqIHHNVR29. Most of the sequence fragments could not be assembled with any pre-existing contigs of the endogenous viral elements. Some fragments could be assembled with several contigs but only overlapped in the primer regions. However, majority of the clones in the libraries have significant similarity to microsatellites of various organisms including *C. quadricarinatus* (Table 5.6).

5.3.3. Sequence contigs of the endogenous virus-like elements

Fragments 2, 3 and 4 were inserted in all of the redclaw populations. It was most likely that Fragments 3 and 4 were not only nucleotide portions of Fragment 1, but

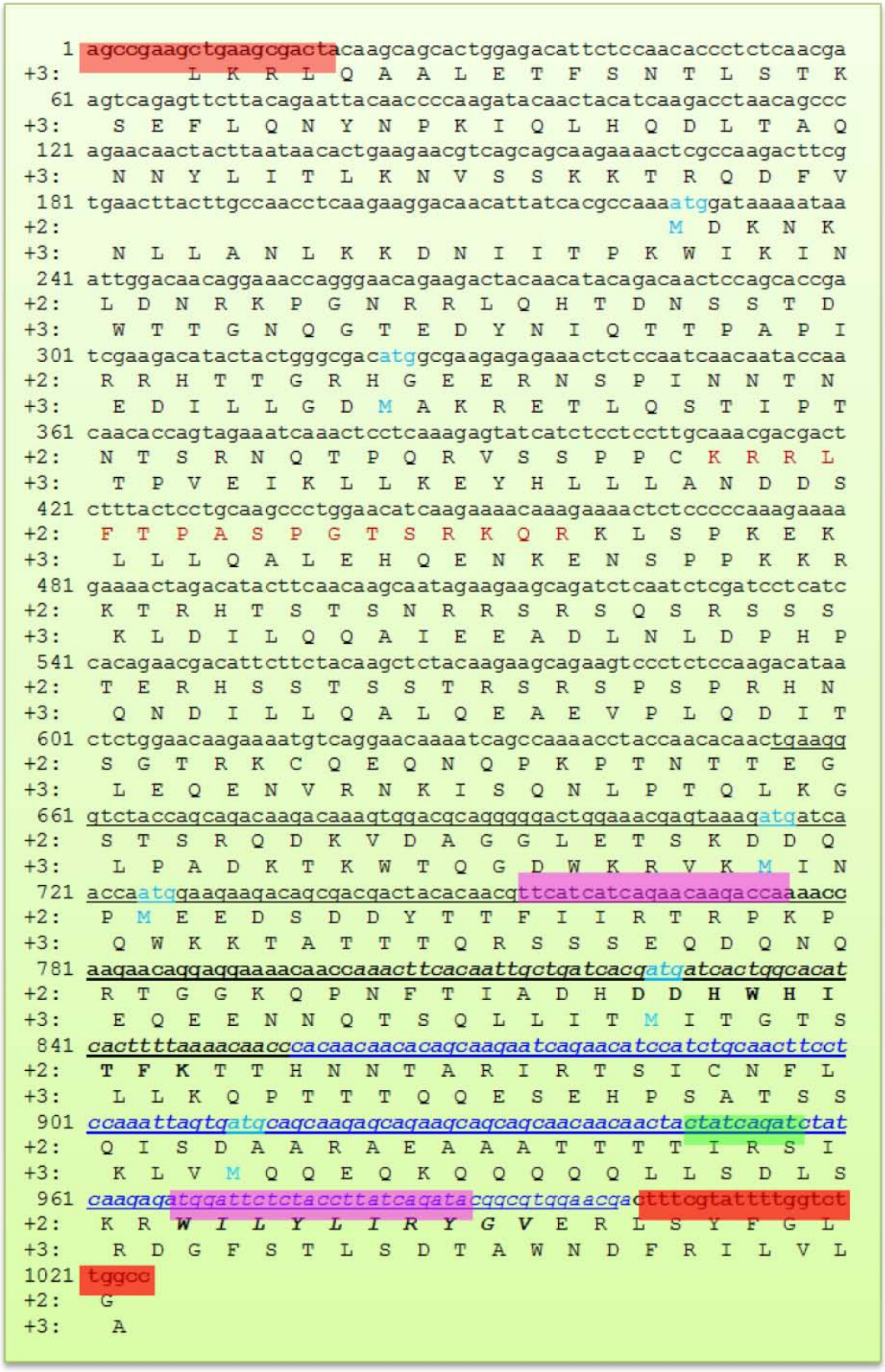
1 agccgaagctgaagcgactacaagcagcactggagacattctccaacaccctctcaacga
+3: L K R L Q A A L E T F S N T L S T K
61 agtcagagttcttacagaattacaaccccaagatacaactacatcaagacctaacagccc
+3: S E F L Q N Y N P K I Q L H Q D L T A Q
121 agaacaactacttaataacactgaagaacgtcagcagcaagaaaactcgccaagacttcg
+3: N N Y L I T L K N V S S K K T R Q D F V
181 tgaacttacttgccaacctcaagaaggacaacattatcacgccaaaatggataaaaataa
+2: M D K N K
+3: N L L A N L K K D N I I T P K W I K I N
241 attggacaacaggaaaccagggaacagaagactacaacatacagacaactccagcaccga
+2: L D N R K P G N R R L Q H T D N S S T D
+3: W T T G N Q G T E D Y N I Q T T P A P I
301 tcgaagacatactactgggcgacatggcgaagagagaaactctccaatcaacaataccaa
+2: R R H T T G R H G E E R N S P I N N T N
+3: E D I L L G D M A K R E T L Q S T I P T
361 caacaccagtagaaatcaaactcctcaaagagtatcatctcctccttgcaaacgacgact
+2: N T S R N Q T P Q R V S S P P C K R R L
+3: T P V E I K L L K E Y H L L L A N D D S
421 ctttactcctgcaagccctggaacatcaagaaaacaaagaaaactctcccccaaagaaaa
+2: F T P A S P G T S R K Q R K L S P K E K
+3: L L L Q A L E H Q E N K E N S P P K K R
481 gaaaactagacatacttcaacaagcaatagaagaagcagatctcaatctcgatcctcatc
+2: K T R H T S T S N R R S R S Q S R S S S
+3: K L D I L Q Q A I E E A D L N L D P H P
541 cacagaacgacattcttctacaagctctacaagaagcagaagtccctctccaagacataa
+2: T E R H S S T S S T R S R S P S P R H N
+3: Q N D I L L Q A L Q E A E V P L Q D I T
601 ctctggaacaagaaaatgtcaggaacaaaatcagccaaaacctaccaacacaactgaagg
+2: S G T R K C Q E Q N Q P K P T N T T E G
+3: L E Q E N V R N K I S Q N L P T Q L K G
661 gtctaccagcagacaagacaaagtggacgcaggggactggaaacgagtaaagatgatca
+2: S T S R Q D K V D A G G L E T S K D D Q
+3: L P A D K T K W T Q G D W K R V K M I N
721 accaatggaagaagacagcgacgactacacaacgttcatcatcagaacaagaccaaaacc
+2: P M E E D S D D Y T T F I I R T R P K P
+3: Q W K K T A T T T Q R S S S E Q D Q N Q
781 aagaacaggaggaaaacaaccaaacttcacaattgctgatcacgatgatcactggcacat
+2: R T G G K Q P N F T I A D H D D H W H I
+3: E Q E E N N Q T S Q L L I T M I T G T S
841 cacttttaaaacaaccacacaacaacacagcaagaatcagaacatccatctgcaacttcct
+2: T F K T T H N N T A R I R T S I C N F L
+3: L L K Q P T T T Q Q E S E H P S A T S S
901 ccaaattagtgatgcagcaagagcagaagcagcagcaacaacaactactatcagatctat
+2: Q I S D A A R A E A A A T T T T I R S I
+3: K L V M Q Q E Q K Q Q Q Q Q L L S D L S
961 caagagatggattctctaccttatcagatacggcgtggaacgactttcgtattttggtct
+2: K R W I L Y L I R Y G V E R L S Y F G L
+3: R D G F S T L S D T A W N D F R I L V L
1021 tggcc
+2: G
+3: A

Figure 5.5. Nucleotide sequence (5′ - 3′) of endogenous virus-like elements (Contig 1). The QPF1/QPR1 primers are in red highlight. The 101F21/314R23 primers are highlighted in pink. Nucleotides that have 70% ($9e^{-29}$) similarity with Australian isolate Au2005 of IHHNV (EU675312) are underlined. Non-underlined nucleotide sequences have no significant similarity with known nucleotides in the public database (NCBI) with BLASTn. Fragment 3 (192 bp) and Fragment 4 (183 bp) are shown in bold font. Bright green outline show the missing nucleotides (9 nts) in Fragment 4 relative to Fragment 3. Fragment 8 (202 bp) is in italic font, while fragment 9 (147 bp) is in blue font. The amino acid sequences under the nucleotide sequence corresponds to two overlapping putative open reading frames of non-structural proteins of IHHNV (ORF finder, NCBI). The first ORF (265 aa) on frame +2 shared 54% (1e-33) similarity with NS1 of IHHNV (AAM94165). The second ORF (338 aa) on frame +3 had 37% (2e-39) amino acid identity to NS2 of IHHNV Au2005 (ACF32342). Initiation codons (atg) in both frames are in light blue font. Putative replication initiator motif I and II of parvovirus (Shike *et al.*, 2000) are indicated in bold type and italic boldface type, respectively. Possible nuclear localisation signal (NLS) similar to mouse α2 RB protein (Efthymiadis *et al.*, 1997; Jans *et al.*, 2000) is in red type.

```
  1 ttcatcatcagaacaagaccaagaacaacaggaagaacaccaaacttcaccattgcagat
                                                N  F  T  I  A  D
 61 cacacagaccactggcacatcacttttaaaacctgccacaacaacacagccagaatcaga
    H  T  D  H  W  H  I  T  F  K  T  C  H  N  N  T  A  R  I  R
121 acatcaatctgcaattacctacaacttgaagatggtgcaagagccgaagctgaagcgact
    T  S  I  C  N  Y  L  Q  L  E  D  G  A  R  A  E  A  E  A  T
181 actactgttaaaccatacccccggccgggattgaacccgcggtcatagagtctcaaaact
    T  T  V  K  P  Y  P  R  P  G  L  N  P  R  S  -  S  L  K  T
241 ccagcccgtcgcgatatcaagtctatcagatgctggattctctaccttatcagatacgga
    P  A  R  R  D  I  K  S  I  R  C  W  I  L  Y  L  I  R  Y  G
301 gtggaaaaactttcgtattttggtcttggcc
    V  E  K  L  S  Y  F  G  L  G
```

Figure 5.6. Nucleotide sequences (5′ - 3′) of endogenous virus-like elements (Contig 2). Fragment 2 (128 bp) is located between QPF1 and QPR1 primers (red type), while Fragment 5 (252 bp) is in the region between 101F21 and 314F23 primers (blue type). Nucleotides that have 72% ($2e^{-12}$) similarity with IHHNV (GQ475529) are in boldface type. Underline type shows the nucleotide that shares 82% (1e-07) identity to *Cherax quadricarinatus* clone epi15_C6 mRNA sequence (DQ847767) on plus/minus strand. Amino acid sequence (frame +1) under the nucleotide sequence has 48% ($7e^{-18}$) identity to NS1 of IHHNV (AAM94165). Putative replication initiator motif I and II of parvovirus (Shike *et al.*, 2000) are indicated in bold type and italic boldface type, respectively.

Figure 5.7. Nucleotide sequences (5′ - 3′) of endogenous virus-like elements (Contig 3). The 101R21/314F23 primers are in blue type. The nucleotides that have 84% (0.006) similarity with IHHNV (GQ475529) are underlined. Amino acid sequences under the nucleotide sequence correspond to open reading frame (+1) of NS1 of IHHNV (AAM94165) with 53% ($1e^{-17}$) similarity. Putative replication initiator motif I and II (incomplete) of parvovirus (Shike *et al.*, 2000) are indicated in bold type and italic boldface type, respectively.

Figure 5.8. Nucleotide sequences (5′ - 3′) of endogenous virus-like element (Contig 4) that have 100% ($2e^{-23}$) similarity with IHHNV isolate Au2005 (EU675312). The QPF1/QPR1 primers are in red type. Amino acid sequences under the nucleotide sequences correspond to open reading frame (+2) of NS1 of IHHNV (AAM93906) with 100% ($1e^{-14}$) similarity. Putative replication initiator II of parvovirus (Shike *et al.*, 2000) is indicated in bold type.

these two fragments also inserted independently into the host genomes as they were present in the populations where the Fragment 1 was absent. Based on the pattern of the presence of these elements, the redclaw can be clustered into three groups (lineages). The DAAF specimen had no insertion of fragments 1, 5 and 6 and was allocated Group 1. The hypertrophied nuclei population of JCU did not have fragments 1 and 7 (Group 2), while all other populations which had no fragment 7 inserted in their genomes clustered in Group 3 (Table 5.5).

Table 5.6. Sequencing results of PCR amplification of DNA templates from gills of *Cherax quadricarinatus* using primer walking.

Contig	Number of clones	Fragment size (bp)	BLAST type	Accession number	Closest homology	Species	E-value	Identity (%) - (q/s)
					101F21			
Cq101F21_01	6	1158	n(s)	NG_012206.1	Protein kinase C alpha (PRKCA) RefSeqGene on chromosome 7	*Homo sapiens*	2e-43	69% (336/486)
Cq101F21_02	1	1742	n(s)	AC154749.2	BAC clone RP24-399C21 from chromosome 17	*Mus musculus*	2e-97	70% (815/1160)
					101R21			
Cq101R21_03	1	1572	n(s)	HM035035.1	Microsatellite CQ010 sequence	*Cherax quadricarinatus*	1e-55	79% (201/254)
Cq101R21_04	3	1185	n(s)	XM 635468.1	AX4 hypothetical protein (DDB_G0281653) mRNA sequence	*Dictyostelium discoideum*	2e-05	88% (42/48)
					88F23			
Cq88F23_05	5	2893	n(s)	DQ847611.1	Clone h1_D3 mRNA sequence	*Cherax quadricarinatus*	2e-22	76% (138/182)
Cq88F23_06	1	829	n(s)	HM035049.1	Microsatellite CQ024 sequence	*Cherax quadricarinatus*	4e-26	78% (124/159)
					88R23			
Cq88R23_07	1	1601	n(s)	AY703870.1	Unidentified sequence clone 1 amplified using OIE WSSV primers	*Cherax quadricarinatus*	2e-38	84% (130/154)
Cq88R23_08	6	1006	n(s)	DQ008073.2	CD45 gene	*Petromyzon marinus*	5e-13	83% (72/87)
					314F23			
Cq314F23_09	3	386	n(s)	CU993818.4	DNA sequence from clone CH1073-468021 in linkage group 23	*Danio rerio*	2e-19	96% (65/68)
Cq314F23_10	3	239	n(h)	HQ895787.1	Clone CQ048 microsatellite sequence	*Cherax quadricarinatus*	3e-88	96% (196/204)
					314R23			
Cq314R23_11	2	792	x(nr)	AAA17752.1	Reverse transcriptase	*Bombyx mori*	9e-11	30% (55/181)
Cq314R23_12	1	714	n(h)	DQ847704.1	Clone cherax_183 mRNA sequence	*Cherax quadricarinatus*	2e-35	80% (140/176)
					755F40			

Contig	Number of clones	Fragment size (bp)	BLAST type	Accession number	Closest homology	Species	E-value	Identity (%) - (q/s)
Cq755F40_13	2	756	n(h)	HM035037.1	Microsatellite CQ012 sequence	*Cherax quadricarinatus*	1e-122	85% (390/459)
Cq755F40_14	1	788	n(h)	HM035051.1	Microsatellite CQ026 sequence	*Cherax quadricarinatus*	1e-94	85% (273/322)
					150R40			
Cq150R40_15	1	1,641	x(nr)	XP_001626638.1	Predicted protein	*Nematostella vectensis*	2e-05	24% (23/96)
Cq150R40_16	1	1,395	n(s)	AC245335.1	Clone sle-34e20 map 10, chromosome 10	*Solanum lycopersicum*	3e-50	81% (51/63)
					160F40			
Cq160F40_17	1	857	n(h)	HM035034.1	Microsatellite CQ009 sequence	*Cherax quadricarinatus*	2e-41	90% (125/139)
Cq160F40_18	1	1,484	n(h)	HM035034.1	Microsatellite CQ024 sequence	*Cherax quadricarinatus*	1e-85	95% (199/210)
					293R40			
Cq293R40_19	2	934	n(s)	GU338226.1	Clone Cabe81microsatellite sequence	*Callinectus bellicosus*	6e-05	80% (52/65)
					145F40			
Cq145F40_20	1	951	n(s)	AC058790.14	Clone b518b9 map 22q11, chromosome 22	*Homo sapiens*	2e-99	71% (543/763)
					194R40			
Cq194R40_21	1	888	x(nr)	EFX63842.1	Hypothetical protein DAPPU DRAFT_118792	*Daphnia pulex*	2e-05	32% (25/77)
Cq194R40_22	1	1,050	x(nr)	XP_002159010.1	PREDICTED: similar to pol-like protein	*Hydra magnipapillata*	1e-05	29% (28/98)
					QPF1			
CqQPF1_23	1	590	n(h)	HM035048.1	Microsatellite CQ023 sequence	*Cherax quadricarinatus*	6e-35	89% (115/129)
CqQPF1_24	1	170	n(s)	HM035050.1	Microsatellite CQ025 sequence	*Cherax quadricarinatus*	1e-41	84% (139/166)

When no homology found with BLASTn optimised for highly similar sequences (megablast) [**n (h**)], then BLASTn optimised for somewhat similar sequences (blastn) [**n (s)**] was performed against sequences in database. A BLASTx [**x (nr)**] against non-redundant sequences in the public database was conducted if no similarity obtained from n (h). If multiple significant similarities matched with a single DNA (sequence consensus), only the highest scoring hit was included in the table. q/s: number of identical nucleotides (amino acids) between query and subjects sequences/number of nucleotides (amino acids) for alignment. A similarity was considered significant at E-value < 10^{-5}.

Fragments 1 (983 bp), 3 (192 bp), 4 (183 bp), 8 (202 bp) and 9 (147 bp) can be assembled into a 983 bp sequence read (excluding primers) and assigned as Contig 1 (Figure 5.5). Fragments 2 (128 bp) and 5 (252 bp) can be assembled (Contig 2) into a 288 bp sequence (Figure 5.6), while Fragment 6 (189 bp) and Fragment 7 (62 bp) were independent from the other fragments and assigned as Contig 3 (Figure 5.7) and Contig 4 respectively (Figure 5.8). All these contigs still retain their open reading frames (ORFs) corresponding to non-structural proteins of IHHNV. Putatively functional motifs such as replication initiator motif I and II of parvoviruses (Shike *et al.*, 2000) could be determined in all contigs, while nuclear localisation sequence (NLS) similar to mouse α2 RB protein (Efthymiadis *et al.*, 1997; Jans *et al.*, 2000) could be seen in Contig 1. This longest element (Contig 1) also maintained its initiation codons but not the stop codons. Interestingly, when examining the IHHNV sequences with respect to the endogenous elements, these integrated fragments are located in the non-structural proteins (NS1 and NS2) of both IHHNV Australian isolate Au2005 (EU675321) and Hawaiian isolate (AF218266) as reference sequences (Figure 5.9), but they do not form one consensus sequences (contig).

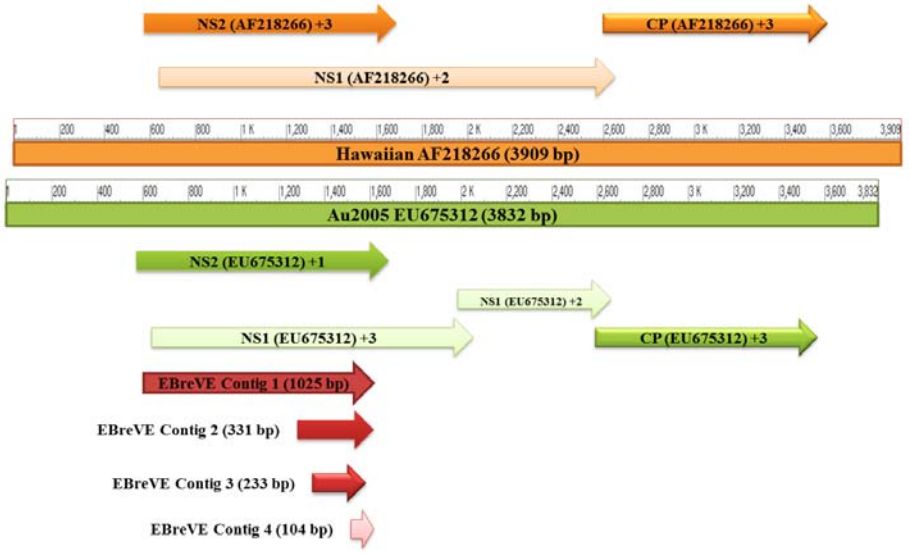

Figure 5.9. Schematic illustration of the location of endogenous virus-like elements in *Cherax quadricarinatus* relative to Australian isolate Au2005 (EU675321) and Hawaiian isolate (AF218266) of IHHNVs. NS, non-structural protein.CP, capsid protein.

5.4. Discussion

Histological examination revealed the presence of a number of commensal temnocephalids in the gills of redclaw from Farms A and B. Melanisation and necrotic eosinophilic nodules and filaments indicative of opportunistic bacterial infection were observed in some crayfish from different sources. These pathological changes may be associated with a number of granular haemocytes observed in the efferent vessel of the gills of redclaw from Farm B population. The mortality that occurred in the MARFU population was most likely due to infection with the rickettsia-like organisms (RLOs). Rickettsia kills their host by destruction of the host cells rather than producing exotoxins (Winkler, 1990; Romero *et al.*, 2000). Surprisingly, some redclaw from Farm B also developed hypertrophic nuclei with rarefied chromatin without CA intranuclear inclusion bodies indicative of parvovirus infection (Edgerton *et al.*, 2000) and similar to the nuclear changes that have been observed in the hypertrophied nuclei population at SVBMS, JCU.

To understand if the hypertrophic nuclei with marginated chromatin in the gills of *C. quadricarinatus* were caused by a parvovirus, several sets of parvovirus primers were designed to amplify possible viral sequences. However, no viral sequence was identified even when amplicons were present in the gel for most of the primer sets. Since the suppression subtractive hybridization also yielded similar results with no viral sequences found in the libraries (Chapter 4), it was hypothesised that these cellular changes may be caused by virus with no poly(A) tail. Thus, RT-PCRs using bunyavirus primers were carried out. Bunyaviral genome comprises negative-sense tripartite single-stranded RNA (ssRNA) and the viral messenger RNAs (mRNAs) do not have a poly(A) tail (van Regenmortel *et al.*, 2000; Elliott, 2008). In addition, Mourilyan virus (MoV) naturally infected wild and farmed *P. monodon* and *P. japonicus*(Cowley *et al.*, 2005c) and may have a quite broad host range (Cowley, pers. commun.). However, in concert with the previous methods, bunyaviruses had no association with these idiopathic nuclear changes.

Nine putative endogenous fragments were found in the gill tissue of crayfish from the different sources, suggesting a widespread occurrence of these elements. These

fragments may have been inserted into the germlines of the host and may be vertically transmitted to the offspring (Belyi *et al.*, 2010b). Most of these elements have undergone various degrees of mutation (Griffiths, 2001; Liu *et al.*, 2011b) that distinguished them from their exogenous counterparts. In addition, at least one of these fragments, the shortest fragment (Fragment 7) had 100% nucleotide and amino acid similarity to IHHNV, and likely represents a recent integration. Other fragments shared between 70 – 91% nucleotide identities and 34 – 72% amino acid similarities with IHHNV and also had various degrees of similarities with insect densoviruses. This suggests that these elements may not have originated from IHHNV (PstDNV) genomes but could be derived from another uncharacterised member of the genus Brevidensovirus within the subfamily Densovirinae, family Parvoviridae (Tattersall *et al.*, 2008) that share nucleotide similarities with IHHNV. Furthermore, while primer walking to confirm the insertion site failed to identify the host ends to each element, one PCR sequence did identify a region of viral-host sequence indicative an insert (Figure 5.6). Thus, these elements are called endogenous Brevidensovirus-like elements (EBreVE) of *C. quadricarinatus*.

The EBreVEs may be integrated into the redclaw crayfish genome following chronic or persistent infection by a corresponding virus that may have occurred as multiple independent integration events years ago leading to the accumulation of several integrated elements in their genomes. These EBreVEs took place randomly throughout the crayfish genomes(Crochu *et al.*, 2004; Roiz *et al.*, 2009; Hawkins *et al.*, 2011; Fort *et al.*, 2012). Even though it cannot accurately be determined where these integrants are inserted within the host genome as there is no whole genome sequence of *C. quadricarinatus* available in the public database, it is most probable these elements were integrated into the microsatellites or transposable elements in various sites along the host genome (Table 5.6). Accordingly, insertions of non-retroviral elements into the host genomes could be mediated either by non-homologous recombination with chromosomal DNA or by interaction with mobile cellular elements (Horie *et al.*, 2010; Katzourakis and Gifford, 2010) such as long interspersed elements (LINEs) via target-primed reverse transcription from ancestral viral mRNAs (Belyi *et al.*, 2010b). Flegel (2009) hypothesised that reverse

transcriptase (RT) of the host may recognise mRNA of viruses and use integrases (IN) to randomly insert cDNA elements into their genome.

These integrated viral sequences still retained their open reading frames (ORF) with several functional protein coding sequences and initiation codons but no stop codons. Belyi *et al.*(2010b) believed that selective pressure is strong in order to retain the full length of the ORF. The most prominent feature of EBreVEs in *C. quadricarinatus* is that in each case, the segment (portion) of viral sequences inserted into the host genomes is identical, but they cannot be assembled into one consensus sequence. This indicates that they have undergone indel (insertion and deletion) over time (Hawkins *et al.*, 2011). These elements are derived from non-structural protein regions of ancestral virus. Non-structural proteins may play crucial role in DNA virus replication and are more likely to be more conserved during the evolution. Therefore, endogenous sequences that have similarity with NS protein are more frequently found than the sequences coding the VP proteins (Belyi *et al.*, 2010a; Kapoor *et al.*, 2010)

It is unknown whether the existence of these EBreVEs is of a benefit or detrimental for redclaw crayfish. However, the results were striking; all the animals which had pathological changes of hypertrophied nuclei with marginated chromatin but without CA intranuclear inclusion bodies (hypertrophied nuclei population) or with basophilic CA intranuclear inclusion bodies (DAFF specimen) in the gills did not possess the 1025 bp fragment, while most of the animals without these nuclear changes had this insert within their genomes. Five animals from Farm B that had this insertion also had the nuclear changes; however they were few or difficult to identify (poorly developed) in the gills of the redclaw. Perhaps this element may have protective function for the host against invasion of their exogenous viral counterparts. Crayfish without this fragment developed these nuclear changes. Previous studiesalso found that animals having endogenous virus-related fragments in their genomes are more resistant to the infection of their more closely related exogenous viruses. The exogenous counterparts may infect the host cells, but replicate poorly with no or little effect in the host (Maori *et al.*, 2007; Belyi *et al.*,

2010b; Liu *et al.*, 2011b). This resistance (tolerance) may be from an RNAi pathway that is able to suppress viral propagation (Maori *et al.*, 2007; Flegel, 2009).

These findings suggest that the nuclear changes developed in the hypertrophied nuclei population and DAFF specimen have originated from two different species of exogenous viruses that are closely related to the EBreVEs. These two exogenous viruses are also closely related, but cause distinct pathological changes within the host cells. Nevertheless, further studies are still needed to uncover the viral aetiology of the nuclear changes in the gills of *C. quadricarinatus*.

To summarise, endogenisation of brevidensovirus-like elements is shown for the first time to be widespread in the freshwater redclaw crayfish populations of northern Queensland. This integrant may contribute to the genomic variation of the redclaw during their evolution history (Crochu *et al.*, 2004; Roiz *et al.*, 2009; Feschotte and Gilbert, 2012) and may play a significant role in the defence response against infection of their present day exogenous counterpart. Identification of integrated viral sequences can be used to determine reservoirs of viruses and uncover the evolution of exogenous viruses (Feschotte and Gilbert, 2012). The viral inserts can also be used as a population marker for tracing history of crayfish movement. The endogenisation of viral fragments also provides fossil records of the past viral infection and also fills the gap on the interaction between host and virus at a molecular level.

Furthermore, the existence of endogenous viral elements may practically interfere with screening and diagnosis of exogenous viral infection in aquaculture species. False positive results may derive from samples having the insertions when the methods used cannot distinguish between the endogenous and exogenous viruses (Tang and Lightner, 2006). Therefore, the development of diagnostic methods that can identify the real viruses, but do not detect endogenous fragments or can differentiate between inserts and exogenous elements, is worthy. This will prevent economic losses from sacrificing cultured animals which are mistakenly diagnosed as being infected with exogenous viral counterparts (Saksmerprome et al., 2011; Saksmerprome et al., 2010; Tang et al., 2007). Discarding false positive specimens

may have negative consequence in breeding programs since the integrants may have protective function to their closely related exogenous viral infections (Saksmerprome et al., 2011).

CHAPTER 6

INVESTIGATION OF SPHEROID CELLS IN THE LYMPHPOID ORGAN OF *Penaeus merguiensis* USING SUPPRESSION SUBTRACTIVE HYBRIDIZATION

6.1. Introduction

Several viruses have been reported to cause diseases in the wild and cultured banana prawn *Penaeus merguiensis*. Hepatopancreatic parvo-like virus (HPV) was the first viral disease in cultured *P. merguiensis* that was recorded to cause growth retardation and 50 – 100% mortality in juveniles (Chong and Loh, 1984; Lightner and Redman, 1985). HPV has also been reported in the wild banana prawn in northern Queensland, Australia (Roubal *et al.*, 1989) and reached 95% prevalence in New Caledonia (Tang *et al.*, 2008). Currently, the Australian strain of HPV (*P. merguiensis* densovirus, *Pmerg*DNV) has been recorded in cultured and wild *P. merguiensis* (La Fauce *et al.*, 2007a; La Fauce *et al.*, 2007b). In addition, *P. mergiensis* was observed to be susceptible to lymphoidal parvo like-virus (LPV) (Owens *et al.*, 1991), white spot syndrome virus (WSSV) (Wang *et al.*, 2002) and spawner-isolated mortality virus (SMV) (Owens *et al.*, 2003). Moreover, even though there is no report on naturally infected banana prawn with gill-associated virus (GAV), experimental infection revealed that this species may be susceptible to GAV (Spann *et al.*, 2000).

Histopathologically, most of these systemic viruses cause spheroid development within the lymphoid organ (LO) of banana prawns. It is believed that the formation of spheroid cells in the lymphoid organ is a major defence mechanism to viral infection in penaeid prawns (Anggraeni and Owens, 2000). Currently, in northern Queensland approximately 75 – 100% cultured banana prawns from different family lines have lymphoid organ spheroid (LOS) cells that occupied more than 40% of the lymphoid organ area (Owens, unpublished data). However, no candidate virus has been identified in causing these spheroid formations. Furthermore, in banana prawns, differential gene transcripts in particular immune-related genes are poorly investigated.

Only few studies have been conducted into disclosing the immunological factors of the banana prawn *P. merguiensis*. Immune-related genes that have been investigated in banana prawns were an antimicrobial peptide (AMP), lysozyme (Mai and Hu, 2009a), agglutinating protein, lectin (Rittidach *et al.*, 2007; Rattanaporn and

Utarabhand, 2011) and protein involved in programmed cell death (apoptosis), cysteine aspartate protease (caspase) (Phongdara *et al.*, 2006). Several other genes that may have immune function have been identified in *P. merguiensis* including translationally controlled tumour protein (TCTP), heat shock protein 70 (HSP 70), shrimp ovarian peritropin (SOP), and thrombospondin (TSP) (Loongyai *et al.*, 2007a; Loongyai *et al.*, 2007b; Wonglapsuwan *et al.*, 2009).

Most of published work on the immune-related factors of *P. merguiensis* pay particular attention to the haemolymph or haemocytes (Phongdara *et al.*, 2006; Rittidach *et al.*, 2007; Mai and Hu, 2009a) and ovaries (Loongyai *et al.*, 2007a; Loongyai *et al.*, 2007b; Wonglapsuwan *et al.*, 2009; Wonglapsuwan *et al.*, 2010). Only a few studies have been conducted on the tissue distribution of the genes including muscle, gonad, gills (Mai and Hu, 2009a), brain, heart, intestine (Wonglapsuwan *et al.*, 2009), hepatopancreas and lymphoid organ (Mai and Hu, 2009a; Wonglapsuwan *et al.*, 2009). Furthermore, application of suppression subtractive hybridization (SSH) in banana prawns has only been performed to identify genes related to the ovarian development (Loongyai *et al.*, 2007a; Wonglapsuwan *et al.*, 2009; Wonglapsuwan *et al.*, 2010). Therefore, this study was carried out to determine the aetiology of the formation of spheroid cells and differentially expressed genes in the lymphoid organ of *P. merguiensis* using suppression subtractive hybridization.

6.2. Materials and Methods

6.2.1. Experimental animals

Refer to Section 3.1 for experimental animals.

6.2.2. Histology

For histological examination, see Section 3.2.

6.2.3. Suppression subtractive hybridization

Twenty *P. merguiensis* with an average body weight of 38.8 ± 6.8 g (SD) from a hatchery population and 20 prawns with a mean body weight of 12.0 ± 5.2 g from the wild caught population were used for the suppression subtractive hybridization. The tester cDNA was prepared from 1.19 µg of poly(A)$^+$ RNA of hatchery population and the driver cDNA was synthesised from 0.02 µg of poly(A)$^+$ RNA of wild caught animals. Refer to Section 3.4 for suppression subtractive hybridization protocol.

6.2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification with bunyavirus primers and PCR amplification with parvovirus primers

Several bunyavirus primers (Table 5.1) were used to investigate the possibility of the aetiological agent of the histopathological changes of spheroid cells in the lymphoid organ of *P. mergueinsis* was caused by virus with no poly(A) tail (see Section 5.2.4). Total RNA was extracted from the lymphoid organ of hatchery *P. merguiensis* (20 prawns) using SV Total RNA Isolation System (Promega) according to the manufacturer's instructions (Section 3.3.1). Complementary DNA (cDNA) was synthesised from total RNA using random hexamer primers (SuperScriptTM III first-strand synthesis system for RT-PCR, Invitrogen, USA catalogue # 18080-051) according to manufacturer's protocol. RT-nested PCR was performed using primers MoV24F and MoV25R in the primary PCR, and primers MoV148F and MoV149R in the nested PCR.

In the primary amplification, PCR mixture contained 12.5 µl of GoTaq®Green Master Mix (Promega), 1 µl of DNA template, and 0.75 µl (10 µM) of each primer. This PCR reaction volume was adjusted with nuclease free water (Promega) to a final volume of 25 µl. The PCR amplification was performed in a Mastercycler gradient 5333 (Eppendorf, Germany) with an initial denaturation 95°C for 2 mins, 35 cycles for 30 secs denaturation at 95°C, 30 secs annealing at 60ºC, 40 secs extension at 72°C, and then followed by final extension for 7 mins at 72°C. In the nested PCR

amplification, 2 µl of primary PCR products was amplified using primers MoV148F and MoV149R in 25 µl reaction mixture. Amplification profile was different from the primary PCR with annealing temperature at 58ºC and a shorter extension time for 30 secs (Cowley *et al.*, 2005a). For other primers including primers TosV2667F/TosV3064R, the reaction mixture was the same as described for the primary PCR of primers MoV24F/MoV25R but it was slightly different in amplification profile. Amplification profile of primers MoV210F/MoV439R and primers UUKV2558F/UUKV3205R consisted of initial denaturation at 94°C for 7 mins, 40 cycles for 45 secs denaturation at 94°C, 45 secs annealing at 58ºC, 1 min extension at 72°C, and then followed by final extension for 5 mins at 72°C.

Deoxyribonucleic acid (DNA) was isolated from the LO of hatchery animals (20 prawns) using a High Pure PCR Template Preparation Kit (Roche Diagnostics), while DNA from the LO of wild population (20 prawns) was extracted using Wizard® SV Genomic DNA Purification System (Promega) following the manufacturers' protocols. Using HPV140F/HPV140R primers, the PCR product was amplified at 94°C for 7 mins for initial denaturation, 40 cycles for denaturation at 94°C for 45 secs, annealing at 65ºC for 45 secs, extension at 72°C for 1 min, and then finally subjected to extension at 72°C for 5 mins (La Fauce *et al.*, 2007b). Amplified products (10 µl) were visualised on a 1.2% agarose-TAE gels containing GelRed (10,000X in water) at a concentration of 0.5:10,000. Gels were visualised and photographed using InGenius LHR, gel documentation and analysis system (Syngene, UK).

6.3. Results

6.3.1. Histology

Routine histological examination with H & E stain (Figure 6.1) showed that 12 out of 12 of hatchery animals had lymphoid organ spheroid cells, while these pathological changes were never observed in the lymphoid organ of wild caught animals (12/12). These abnormal cells had more basophilic cytoplasm and had no central lumen compared to the normal stromal matrix cells of the lymphoid organ.

Some spheroids were bounded by elongated flattened cells or fibrous connective tissue and had cytoplasmic vacuoles.

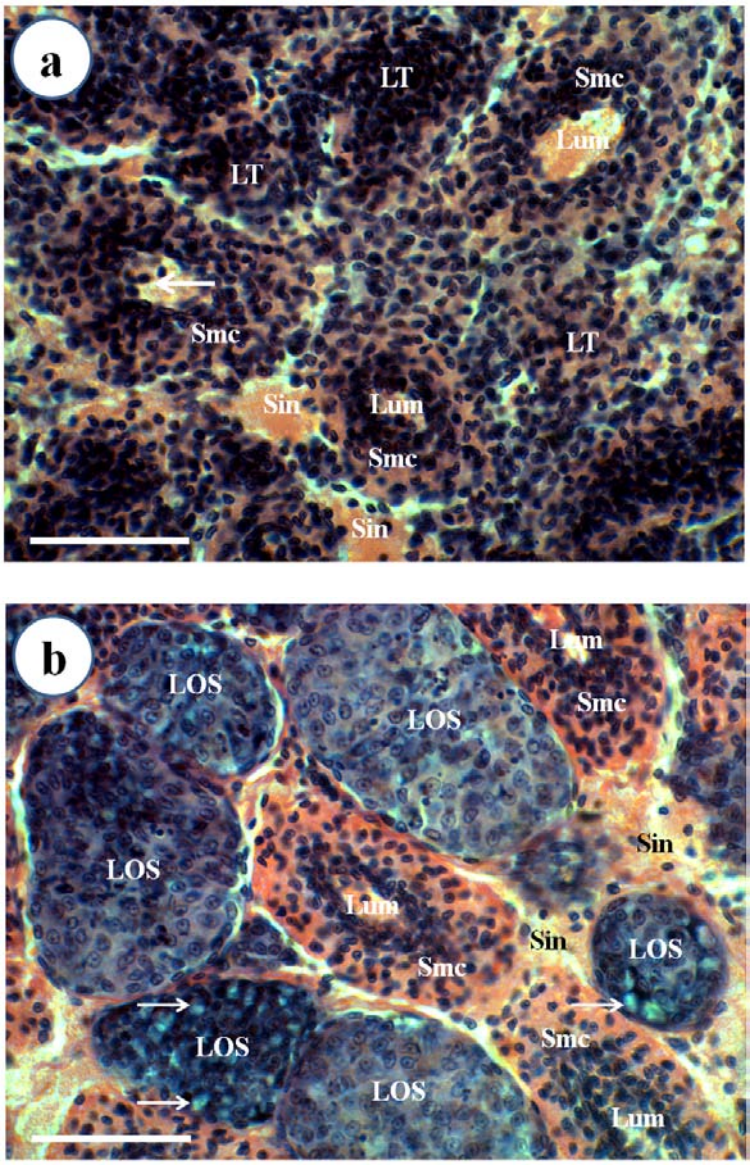

Figure 6.1. Light micrograph of longitudinal section of the lymphoid organ (LO) of *Penaeus merguiensis*. (a) Normal lymphoid tubule (LT) without lymphoid organ spheroid (LOS) cells of wild caught *P. merguiensis*. Lymphoid tubules consist of lumen (Lum) surrounded by stromal matrix cells (Smc). Haemocytes (arrow) sometimes can be observed within the tubular lumen. (b) Spheroid development within the LO of hatchery *P. mergueinsis*. Spheroid cells are formed in the haemal sinuses (Sin) and have a more basophilic cytoplasm and lack a central lumen compared to the normal lymphoid tubule. Some spheroids demonstrated cytoplasmic vacuoles (arrow). H & E stain. Scale bar: 50 µm.

6.3.2. Suppression subtractive hybridization

Penaeus merguiensis that presented spheroid cells (hatchery animals) were used as a tester, whilst the ones without these changes (wild animals) were used as driver to construct forward suppression subtractive hybridization libraries. After the second PCR amplification, two bands with approximate 250 bp and 200 bp were visualised following gel electrophoresis and these PCR product was used as SSH libraries (SSH libraries A and B, respectively). Each band was cut, purified and inserted into a cloning vector. Following hand picking of selected white colonies, 328 clones were sequenced. After removing the vector sequences and the poor quality sequences, a total of 316 sequences were clustered into 141 contigs (consensus sequences) with a range of fragment sizes between 47 bp and 427 bp. Homology search revealed that around 51.6% of the total clones (163 out of 316 clones) shared significant similarities to known amino acids or nucleotides in the GenBank database (Table 6.1).

Transcripts were assigned functions as predicted from sequence homology from the public database and grouped into 8 categories (Figure 6.2). Among transcripts that had a significant matches, structural and cytoskeletal proteins (10.1%) predominated the lymphoid organ cDNA SSH libraries. Sequenced clones that had homology to actin family were identified, with actin 2 (30 clones) being the most represented transcript within this group. Sequences that encoded energy and metabolism factors (7.3%), proteases and inhibitors (7.0%) and ribosomal proteins (6.3%) were the next greatest proportion identified in the SHH libraries. Transcripts that have similarity to mitochondria of penaeids were the most abundant genes representing energy and metabolism factors. Sequences signalling cathepsin B were the most common genes found within the group of proteases and inhibitors. Some transcripts representing both small subunit and large subunit ribosomal proteins from various organisms have also been identified.

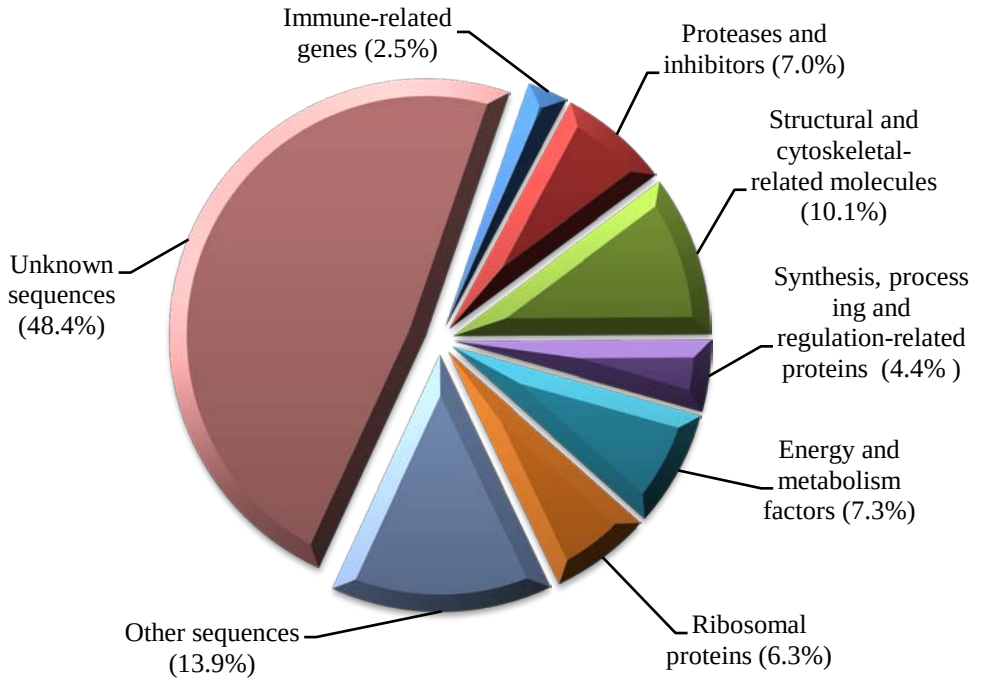

Figure 6.2. Functional categories of differentially expressed genes from the lymphoid organ cDNA suppression subtractive hybridisation (SSH) libraries of banana prawn, *Penaeus merguiensis*.

Transcripts encoding synthesis, processing and regulation-related molecules (4.4%) and the ones that were directly involved in immunity (2.5%) were found in relatively low abundance in the lymphoid organ cDNA SSH libraries. Within the group of synthesis, processing and regulation-related genes, several transcripts encoding regulatory protein of ubiquitins, calreticulin precursor and genes that play a key role in translation process such as eukaryotic translation initiation factor isoform 6 and eukaryotic translation elongation factor 1 delta were identified. Sequenced clones that signal heat shock protein were the most frequently found within the group of molecules related to immunity. Other sequences from various organisms (13.9%) were also found in the SSH libraries. However, many sequenced clones (48.4%) from the SSH libraries had no significant similarity to amino acids/nucleotides in the public database. This indicates the ability of SSH method in revealing new differentially expressed genes in the lymphoid organ of penaeid species.

Table 6.1. Differentially expressed genes from suppression subtractive hybridization (SSH) libraries of the lymphoid organ of Australian banana prawn (*Penaeus merguiensis*) with amino acids/sequences similarity to amino acids/sequences in the public database (NCBI).A, B and AB in the contig indicate that the clones were derived from SSH library A, library B, or both libraries A and B.

Contig	Number of Clones	Fragment Size (bp)	BLAST Type	Accession Number	Closest Homology	Species	E-value	Identity (%) - (q/s)
					Immune-related genes			
PmergAB004	6	110	x	BAJ78982.1	Heat shock protein 70	*Penaeus japonicus*	9.00E-50	100 (36/36)
PmergA056	1	93	x	ADR31352.1	Gamma-interferon-inducible lysosomal thiol reductase	*Penaeus vannamei*	5.00E-09	97 (29/30)
PmergB124	1	62	n (h)	AY859500.1	Antimicrobial peptide (ALF)	*Penaeus chinensis*	5.00E-14	96 (49/51)
					Proteases and inhibitors			
PmergA015	1	105	n (s)	EF213113.1	Cathepsin B mRNA	*Penaeus monodon*	3.00E-27	88 (90/102)
PmergB106	14	64	n (s)	GU571199.1	Cathepsin B mRNA	*Penaeus vannamei*	4.00E-09	93 (42/45)
PmergB131	1	71	x	ABQ10737.1	Cathepsin B	*Penaeus monodon*	2.00E-05	100 (23/23)
PmergB138	1	70	n (s)	EF213113.1	Cathepsin B mRNA	*Penaeus monodon*	1.00E-16	87 (39/45)
PmergAB043	5	92	n (s)	HQ259084.1	Metallothionein mRNA	*Penaeus monodon*	1.00E-06	76 (65/85)
					Structural and cytoskeletal related molecules			
PmergAB010	30	75	x	AEB54623.1	Actin 2	*Procambarus clarkii*	3.00E-05	100 (24/24)
PmergA041	1	94	x	DQ205426	Beta actin	*Penaeus chinensis*	2.00E-12	98 (43/44)
PmergB103	1	93	x	AEJ07990.1	Actin	*Bostrychus sinensis*	3.00E-08	100 (30/30)
					Synthesis, processing, regulation and apoptotic-related proteins			
PmergAB007	2	89	x	EU679002.1	Eukaryotic translation initiation factor 6 mRNA	*Penaeus chinensis*	2.00E-16	83 (74/89)
PmergA013	3	104	n (h)	AY695937.1	Ubiquitin/ribosomal 27a mRNA	*Penaeus japonicus*	5.00E-42	98 (100/102)

Contig	Number of Clones	Fragment Size (bp)	BLAST Type	Accession Number	Closest Homology	Species	E-value	Identity (%) - (q/s)
PmergAB023	2	128	n (h)	DQ323054.1	Calreticulin precursor (CRT) mRNA	*Penaeus chinensis*	2.00E-27	97 (74/76)
PmergA040	2	118	n (h)	XM _963426.1	PREDICTED: similar to ribosomal protein Ubq/L40e (LOC656930), mRNA	*Tribolium castaneum*	7.00E-05	82 (51/62)
PmergA054	1	95	n (h)	HQ630062.1	Protein disulfide isomerase 2 mRNA	*Penaeus chinensis*	2.00E-41	100 (95/95)
PmergA058	1	127	n (h)	DQ323054.1	Calreticulin precursor (CRT) mRNA	*Penaeus chinensis*	3.00E-55	98 (125/127)
PmergA064	1	105	x	AEB54653	Ubiquitin	*Procambarus clarkii*	2.00E-12	100 (34/34)
PmergA067	1	120	x	XP_002089050	eukaryotic translation elongation factor 1 delta	*Drosophila yakuba*	1.00E-09	75 (30/40)
PmergB109	1	94	x	ADD38619.1	Signal peptidase complex catalytic subunit SEC11A	*Lepeophtheirus salmonis*	1.00E-07	87 (26/30)
					Energy and metabolism factors			
PmergA073	2	120	x	AAM96647.1	Cytochrome oxidase subunit I (COI)	*Alpheus angulosus*	2.00E-13	95 (38/40)
PmergA083	2	121	x	ABG65672.1	NADH dehydrogenase subunit 5	*Penaeus chinensis*	1.00E-08	74 (29/39)
PmergB108	15	158	n (h)	DQ656600.1	Mitochondrion	*Penaeus chinensis*	4.00E-55	92 (148/161)
PmergB110	2	69	n (h)	EU517503.1	Mitochondrion	*Penaeus stylirostris*	1.00E-16	94 (59/63)
PmergB125	1	66	n (h)	AY595740.1	Voucher KACa0348 cytochrome oxidase subunit II (COII)	*Aegla spinipalma*	4.00E-23	91 (59/65)
PmergB126	1	47	n (h)	AY261445.1	Isolate VG1 cytochrome oxidase subunit II (COII)	*Afrochlus harrisoni A*	1.00E-08	100 (33/33)
					Ribosomal proteins			
PmergA002	3	98	x	AEB54645.1	Ribosomal protein L9	*Procambarus clarkii*	5.00E-10	94 (30/32)
PmergA005	2	123	x	AEB54637.1	Ribosomal protein S24	*Procambarus clarkii*	5.00E-13	88 (35/40)
PmergA006	8	109	x	ACY66538.1	60S ribosomal protein RPL34 (putative)	*Scylla paramamosain*	4.00E-09	88 (30/34)
PmergA032	1	105	x	ACY66537.1	60S ribosomal protein L27	*Scylla paramamosain*	3.00E-05	68 (23/34)
PmergA060	1	98	x	ACQ91223.1	Ribosomal protein	*Penaeus monodon*	3.00E-10	100 (32/32)

Contig	Number of Clones	Fragment Size (bp)	BLAST Type	Accession Number	Closest Homology	Species	E-value	Identity (%) - (q/s)
PmergA072	1	129	x	ADM64579.1	Ribosomal protein S24	*Hypophthalmichthys nobilis*	3.00E-12	86 (36/42)
PmergB091	1	84	x	ABW23211.1	Ribosomal protein rps21	*Eurythoe complanata*	3.00E-05	96 (25/26)
PmergB104	2	107	x	ACY66547.1	Ribosomal protein L5	*Scylla paramamosain*	4.00E-07	76 (25/33)
PmergB134	1	72	n (s)	NM_001160683.1	40S ribosomal protein S21 (rs21) mRNA	*Oncorhynchus mykiss*	3.00E-05	87 (39/45)
					Other sequences			
PmergA017	1	121	x	EF206694.1	PL10A mRNA	*Penaeus chinensis*	3.00E-25	99 (68/69)
PmergA022	1	114	x	XP_310188.3	AGAP009508-PA	*Anopheles gambiae str. PEST*	7.00E-05	62 (21/34)
PmergA028	2	119	n (s)	NG_012510.1	Centromere protein I (CENPI), RefSeqGene on chromosome X	*Homo sapiens*	7.00E-11	91 (50/55)
PmergA068	1	103	x	XP_002105023.1	GD18147	*Drosophila simulans*	2.00E-06	96 (27/28)
PmergA077	2	132	n (s)	NM_212883.1	Alkylglycerol monooxygenase (agmo) mRNA	*Danio rerio*	8.00E-05	92 (35/38)
PmergB092	2	161	n (s)	AC122324.4	BAC clone RP23-333I5 from chromosome 3	*Mus musculus*	9.00E-18	88 (72/82)
PmergB107	4	134	n (s)	AL845365.1	Clone DKEY-150H13 in linkage group 20 contains five CpG islands	*Danio rerio*	8.00E-30	86 (103/120)
PmergAB129	2	122	n (s)	AF146420.1	Microsatellite Sgl7159INRA	*Silurus glanis*	1.00E-30	95 (86/91)
PmergAB141	29	427	x	XP_002000771.1	GI10411	*Drosophila mojavensis*	2.00E-09	94 (30/32)

When no homology found with a BLASTx [**x**] against non-redundant sequences in the public database, BLASTn optimised for highly similar sequences (megablast) [**n (h)**] was conducted against sequences in database. If no similarity obtained from n (h), then BLASTn optimised for somewhat similar sequences (blastn) [**n (s)**] was performed. If multiple significant similarities matched with a single cDNA (sequence consensus), only the highest scoring hit was included in the table. Libraries were listed in the contigs names. PmergA represented clones from library A (250 bp), PmergB represented clones from library B (200 bp) and PmergAB symbolised clones from libraries A and B. q/s: number of identical amino acids (nucleotides) between query and subjects sequences/number of amino acids (nucleotides) for alignment. A similarity was considered significant at E-value < 10^{-5}.

6.3.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification with bunyavirus primers and PCR amplification with parvovirus primers

Primary PCR with MoV24F/MoV25R primers produced an approximate 300 bp amplicon from 1 out of 20 samples examined (Table 6.2). In the nested PCR, this sample also had an approximate 300 bp amplicon size. However, sequencing results failed to show any similarity to Mourilyan virus or other viral sequences, but similarity with zebra fish DNA (BX248086). Primers MoV210F/MoV439R generated expected amplicons from 2 samples and other different amplicon sizes from several samples. However, once again sequencing results showed no similarity to viral sequences. Primers UUKV2558F/UUKV3205R and TosV2667F/TosV3064R produced no amplicons.

Table 6.2. PCR products of primers used to amplify presumptive bunyavirus and parvovirus in the lymphoid organ of hatchery population of *Penaeus merguiensis*.

Primer name	Approximate PCR amplicon size (number of samples)
MoV24F/MoV25R	300 bp (1)
MoV148F/MoV149R	300 bp (1)
MoV210F/MoV439R	100 bp (2), 200 bp (2), 250 bp (2), 300 bp (3), 600 bp (2), 900 bp (1) and 1500 bp (1)
UUKV2558F/UUKV3205R	-
TosV2667F/TosV3064R	-
HPV140F/HPV140R	140 bp (4), 200 bp (4), and 250 bp (5)

Interestingly, when HPV140F/HPV140R primers were applied, three bands with approximate amplicon sizes of 140 bp, 200 bp and 250 bp appeared in the electrophoretic gel (Figure 6.3) from DNA templates of hatchery population. Whilst, from DNA templates of wild caught population only two bands with approximate ampilicons sizes of 200 bp (4 samples) and 250 bp (5 samples) were observed in the electrophoretic gel. Since the 140 bp was the expected amplicon size for these primers, then three of these four bands (from DNA templates of hatchery population) were extracted from the gel, purified, cloned and sequenced. The sequencing results

revealed that the transcripts had 100% ($5e^{-42}$) nucleotide similarity to Australian *P. merguiensis* densovirus (DQ458718).

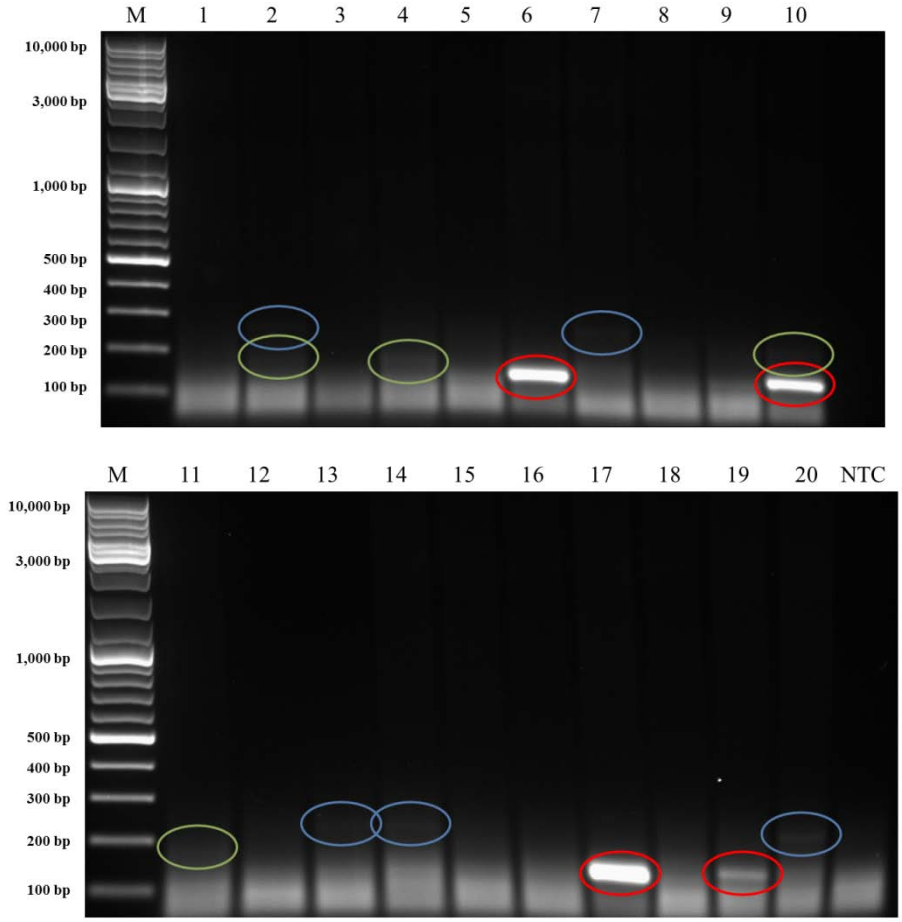

Figure 6.3.PCR using HPV140F and HPV140R primers produced approximate 140 bp (red highlight), 200 bp (green highlight) and 250 bp (blue highlight) amplicon sizes. Lane M = 10 kb DNA ladder (GeneRuler^{TM}, Fermentas, Canada). NTC: non-template control.

6.4. Discussion

6.4.1. Suppression subtractive hybridization

The suppression subtractive hybridisation technique was applied to investigate the aetiology of spheroid formation and the expression of differential genes in the lymphoid organ of Australian banana prawn, *P. merguiensis*. Sequenced clones from the lymphoid organ cDNA SSH libraries were clustered into several functional groups representing genes that have different functionalities. Functional activities of

each gene will be discussed based on the published reports derived from invertebrates in particular crustacean work and some information may be extracted from studies on vertebrates in case the relevant information is limited from invertebrate studies. More information on the roles of these differential gene expressions can be seen on Appendix 3. This study may provide a better understanding on the mechanism of virus and host genes interacting during viral infection at molecular level.

6.4.1.1. Defence related to immunity

Interestingly, heat shock protein 70 (HSP70) was the most frequently encountered clones within the group of immune related genes. Heat shock proteins are highly conserved intracellular protein, found in all organisms and categorised according to their molecule weight such as HSP10, HSP40, HSP60, HSP70, HSP90, HSP100 and HSP110. They play an essential role as molecular chaperones by mediating folding, translocation, regulation, secretion and degradation of other proteins. These proteins react to various stressors including temperature, energy, ions, osmolytes, gases and toxic materials (Feder and Hofmann, 1999; Brenner and Wainberg, 2001; Jolesch *et al.*, 2011). Significant increase in HSP immuno-reactivity was observed in hyperthermally and hypoosmotically stressed crustacea (Cimino *et al.*, 2002). Among the HSPs, HSP70 is the most inducible protein to temperature (Jolesch *et al.*, 2011). Pathogens such as viruses and bacteria can also activate HSPs (Brenner and Wainberg, 2001; Wang *et al.*, 2006; Pongsomboon *et al.*, 2008; Rungrassamee *et al.*, 2010). Increased level of HSP70 at high temperature was suggested to be associated with reduction of viral load in penaeid prawns (de la Vega *et al.*, 2006; Lin *et al.*, 2011).

Gamma-interferon-inducible lysosomal thiol reductase (GILT) is constitutively expressed in most antigen-presenting cells catalysing disulfide bond reduction and has maximum reductase activity in acid conditions (Hastings and Cresswell, 2011). In mammals, this protein is well known for its immunological function in enhancing MHC class II-restricted antigen processing and MHC class I-restricted epitopes derived from viral protein. GILT-mediated reduction is a critical factor for the

presentation of various antigens including melanocyte differentiation antigens and hen egg lysozyme (HEL). This protein also plays a role in regulation of cellular redox state by inducing the expression and stability of superoxidase dismutase 2 and decreasing the level of reactive oxygen species which corresponds with decreased cellular proliferation (Hastings and Cresswell, 2011). In penaeid prawns, GILT has been isolated and characterised in *P. monodon* (*Pm*GILT). It was found that this protein was significantly up-regulated in the lymphoid organ of *P. monodon* experimentally injected with both lipopolysaccharide (LPS) and WSSV (Kongton *et al.*, 2011).

Anti-lipopolysaccharide factor (ALF) is a member of antimicrobial peptides (AMPs) and plays an important role in innate immune mechanism. Anti-lipopolysaccharide factor showed a strong activity against fungus, both Gram-positive and negative bacteria including vibrios,the bacterial pathogen of prawns (de la Vega *et al.*, 2008; Somboonwiwat *et al.*, 2008; Tharntada *et al.*, 2008; Ponprateep *et al.*, 2009; Jaree *et al.*, 2012; Ponprateep *et al.*, 2012). The ALF has binding ability to lipoteichoic acid (LTA) and lipopolysaccharide (LPS), the major cell wall components of Gram-positive and negative bacteria, respectively (Somboonwiwat *et al.*, 2008). This peptide may bind to bacterial cell wall components leading to membrane permeabilization by forming transmembrane pore, inducing the leakage of cytoplasmic contents of the bacteria and then causing cell death (Jaree *et al.*, 2012). This antimicrobial peptide has also been shown to have immunological function against viral infection in penaeid prawns (de la Vega *et al.*, 2008; Antony *et al.*, 2011; Ponprateep *et al.*, 2012). Taken together, these results suggested the immunological functioning of the lymphoid organ in viral infected prawns involving humoral factors including HSP70, GILT and ALF.

6.4.1.2. Proteases and inhibitors

Two forms of proteases were detected in the SSH libraries, cathepsin B and metallothionein. Cathepsin B is a lysosomal cysteine protease, a unique member of the papain superfamily and is ubiquitously expressed in many types of cells (Mort and Buttle, 1997; Turk *et al.*, 2012). In aquatic invertebrates, only few studies have

been conducted concerning sequencing analysis and functional activity of cathepsin B. In larvae of the clam *Meretrix meretrix*, the cathepsin B was suggested to be involved in digestion and larval growth (Wang *et al.*, 2008). The cathepsin B of deepwater prawn *Pandalus borealis* showed extracellular activities and suggested also to function as a digestive protease (Aoki *et al.*, 2003). In *P. vannamei*, this enzyme was predicted not only to participate in intracellular protein hydrolysis but also in extracellular food protein hydrolysis (Stephens *et al.*, 2012). However, many aspects of cathepsin B including its immunological roles in aquatic invertebrates, particularly in penaeid prawns, are waiting to be investigated.

Metallothionein (MT) is cysteine rich metal-binding polypeptide with a low molecular weight and high metal content (Roesijadi, 1992). This peptide plays a primary role in regulation of essential metals such as copper (Cu) and zinc (Zn) and detoxification of nonessential metals such cadmium (Cd), mercury (Hg) and silver (Ag) (Roesijadi, 1992; Pourang *et al.*, 2004; Amiard *et al.*, 2006). Metallothionein has also an antioxidant defence mechanism against oxidative stress (Amiard *et al.*, 2006; Moltó *et al.*, 2007; Maret, 2009). The synthesis of MT can be induced by a variety of endogenous and exogenous factor such as hormones, cytokines, alkylating agents, pharmaceuticals, alcohols, irradiation, heavy metals and infection (Ilbäck *et al.*, 2004; Pourang *et al.*, 2004).

The high inducibility of this protein by heavy metals in vertebrates as well as in invertebrates, suggests that this peptide has a potential use as bioindicator for toxic metal contamination (bioaccumulation) in organisms (Bainy, 2000; Pourang *et al.*, 2004; Amiard *et al.*, 2006). In humans, this protein has been considered as a potential biomarker for a number of diseases (Maret, 2009). Furthermore, it was found that MT was up-regulated in several organs in mice infected with viral disease (Ilbäck *et al.*, 2004). However, the regulatory mechanism of this protein in crustacea due to viral infection is unknown.

6.4.1.3. Structural and cytoskeletal related molecules

Transcripts having homology with actin family were identified within the group of structural and cytoskeletal-related proteins. Actin participates in muscle contraction and is involved in cellular polarity, cellular trafficking, cell motility, cell shape, adhesion, cytokenesis, and endocytosis. Actin also plays an important role in signal transduction, transcription and chromatin remodelling (Hild *et al.*, 2010). A broad variety of actins identified in the lymphoid organ of *P. chinensis* injected with *V. anguillarum* suggested that actins may have a complex roles in the LO especially in the phagocytic response to foreign substances (Zhang *et al.*, 2010). Viral-actin interaction in penaeid prawns has been reported in several published works. According to Wu et al. (2007) phagocytosis of viral particles is actin-dependent. These authors proposed that in WSSV-experimentally infected *P. japonicus*, rearrangement of actin was induced by PjRab GTPase resulting in the formation of actin stress fibers. The PjRab may interact with actin cytoskeleton to bind to viral protein via intracellular virus recognition in regulation of phagocytosis.

In addition, structural protein VP26 of white spot syndrome virus and capsid proteins VP1, VP2 and VP3 of Taura syndrome virus (TSV) were able to bind to actin (Xie and Yang, 2005; Senapin and Phongdara, 2006). Furthermore, actins were found to be up regulated in penaeid prawns experimentally infected with WSSV and TSV suggesting defensive role of this protein against viral infections (Chongsatja *et al.*, 2007; Leu *et al.*, 2007; Reyes *et al.*, 2007; Pongsomboon *et al.*, 2011). In contrast, in YHV experimentally infected prawns, actins were reported to be down-regulated (Bourchookarn *et al.*, 2008). These authors predicted that the down regulation of actin may link to polymerization process, DNase imbalance or progressive cell death. This discrepancy may result from differences of infection stage, physiological state of the animals and expressed actin isoforms in different tissues. Perhaps, the changes in these gene transcriptions are just to build the scaffolding for the growth of the lymphoid organ spheroids.

6.4.1.4. Synthesis, processing and regulation proteins

Within the group of synthesis, processing and regulation related-proteins, eukaryotic translation initiation factor 6 (eIF6) and eukaryotic translation elongation factor 1-δ (EF1-δ) were identified. Translation initiation factors are soluble proteins facilitating the translation initiation of mRNA (Sonenberg and Dever, 2003; Passmore *et al.*, 2007; Mitchell and Lorsch, 2008). The eIF6 plays an important role in regulation of ribosome biogenesis and translation which mediates continuum between the maturation of large ribosomal subunit 60S in the nucleus and translation in cytoplasm. This protein also has an anti-association factor that prevents the association between 40S and 60S ribosomal subunits in the absence of mRNA (Miluzio *et al.*, 2009).

A study by Wang *et al*. (2009) showed that the eIF6 of *P. chinensis* (*Fc*-eIF6) was up regulated in WSSV-infected prawns. The *Fc*-eIF6 bound to the HIV transactivating response RNA-binding protein (*Fc*-TRBP) homolog. The TRBP is an integral component of a Dicer-containing complex that interacts with argonaute 2 (Ago2) which is a catalytic engine of the multi-protein complex RNA-induced silencing complex (RISC) (Chendrimada *et al.*, 2005). Wang *et al.*(2009) proposed that the interaction of *Fc*-eIF6 and *Fc*-TRBP was mediated by the RISC and may have an important role in defence mechanism against viral infections through RNAi pathway.

The elongation factor 1-δ is a subunit of EF-1 protein complex that mediates peptide chain elongation during translation of mRNA (Riis *et al.*, 1990). This factor has catalytic exchange-activity and bind to EF1-α and EF-1γ (Sanders *et al.*, 1993). The EF1-δ also interacts with human homologue of Sina (seven in absentia), SIAH-1, an E3 ubiquitin ligase which has an important role in cell cycle regulation, tumorigenesis and some neurodegenerative diseases (Wu *et al.*, 2011). The EF1-δ has been implicated in cell cycle regulation (Xiao *et al.*, 1998; Boulben *et al.*, 2003), cell proliferation and differentiation (Zou *et al.*, 2011), cell transformation and tumorigenesis (Joseph *et al.*, 2004; Ogawa *et al.*, 2004) and viral infection (Kawaguchi *et al.*, 1997). However, activities and functional roles of this protein in crustacea are waiting to be explored.

Transcripts that have similarity to calreticulin (CRT) precursor of *P. chinensis* was represented in the SHH libraries. Calreticulin is a protein in the endoplasmic reticulum (ER) that is involved in many cellular functions including protein folding, modulator of Ca^{2+} homoestasis, cell adhesion, regulation of gene expression, nuclear export and adipogenesis. This protein has also been implicated in vasostatin, wound healing, cardiogenesis, tumorigenesis, immunogenic cell death, neurodegenerative diseases and autoimmune diseases (Qiu and Michalak, 2009). Calreticulin is differentially expressed under variety of physiological and pathological conditions (Qiu and Michalak, 2009). For example, in decapod crustacea, the expression of calreticulin genes was associated with moulting cycles (Luana *et al.*, 2007) and was up regulated under hyperthermal stress (Luana *et al.*, 2007; Visudtiphole *et al.*, 2010). Over expression of CRT was also detected in the haemocytes of green mud carb, *Scylla paramamosain* injected with lipopolysaccharide (Chen *et al.*, 2010) and WSSV infected prawns and crayfish (Wang *et al.*, 2006; Luana *et al.*, 2007; Wang *et al.*, 2007a; Liu *et al.*, 2011a). The induction of CRT may be related to calcium cycling during WSSV infection (Wang *et al.*, 2006).

Protein disulfide isomerase (PDI) is a member of thioredoxin (Trx) superfamily that has oxidase and isomerase activities. This protein has an essential function as chaperone, inhibiting the aggregation of misfolded proteins with or without disulfides bonds (Wilkinson and Gilbert, 2004). In penaeid prawns, PDI genomes have been characterised in *P. vannamei* and two types of PDI (*Fc*PDI1 and *Fc*PDI2) have been identified in *P. chinensis*(Vargas-Albores *et al.*, 2009; Ren *et al.*, 2011).Like calreticulin, this protein was also expressed differentially under a variety of inducers. The PDI has been shown to be down regulated under osmotic stress (de la Vega *et al.*, 2007a). However, increase transcript level of PDI was observed in vibrio-challenged penaeid prawns (Vargas-Albores *et al.*, 2009; Ren *et al.*, 2011). The induction of PDI following microbial challenge may be associated with denaturing and refolding of disulfide bridges (Ren *et al.*, 2011). The up regulation of PDI was also found in penaeids infected with YHV (Bourchookarn *et al.*, 2008; Prapavorarat *et al.*, 2010), TSV (Chongsatja *et al.*, 2007) and WSSV (Wang *et al.*, 2006; Wang *et al.*, 2007a; Ren *et al.*, 2011). The presence of these two proteins

folding in SHH libraries suggests that they may be involved in folding and assembly of viral proteins in the ER (Doms *et al.*, 1993). The budding of viral protein may require host proteins for folding and modification, whilst physiological state and defence mechanisms of the host cells were activated for viral eradication (Bourchookarn *et al.*, 2008).

Another up regulated protein in the lymphoid organ of *P. merguiensis* was ubiquitin. These proteins are multifunctional in biological process including proteosomal and lysosomal proteolysis, intracellular trafficking, DNA repair, protein-protein interaction, signal transduction and cellular signalling pathways (Pickart and Eddins, 2004; Deshaise and Joazeiro, 2009; Schaefer *et al.*, 2012). Ubiquitin-like modifier may be involved in regulation of nucleocytoplasmic transport and cell cycle progression (Pickart and Eddins, 2004). Despite their variable roles in controlling cellular function, ubiquitins have also been suggested to be involved in pathological process such as inflammation, cancer and metastasis, cytokines-induced apoptosis, and interferon α/β-mediated response to viral infection (Pickart and Eddins, 2004; Schaefer *et al.*, 2012).

In crustacea, up regulation of ubiquitin was observed in prawns and redclaw crayfish infected with WSSV (Wang *et al.*, 2005; Wang *et al.*, 2006; Liu *et al.*, 2011a) suggesting it has a certain role in viral immune-reactivity. *P. chinensis*' ubiquitin (rFcUbc) could reduce the mortality of WSSV-infected prawns by inhibiting replication of WSSV through ubiquitination of WSSV RING domains (Chen *et al.*, 2011a). However, some viruses can also use a ubiquitination pathway of the host to infiltrate the host's defence line. This process involves an E3 ligase that is encoded by either virus or host genes as described in WSSV-infected prawns (Wang *et al.*, 2005; He *et al.*, 2009). It has been shown that RING motifs of WSSV222, WSSV249, and WSSV403 interact with ubiquitin of WSSV-infected penaeid prawns. These RINGs may function as an E3 ligase that is involved in sequestering ubiquitin of the host and in anti-apoptosis regulation by ubiquitin-mediated degradation of tumour suppressor-like protein (TSL) in prawn for WSSV replication and pathogenesis (Wang *et al.*, 2005; He and Kwang, 2008; He *et al.*, 2009).

Signal peptidase has a role in releasing exported proteins from the membranes so they can reach their correct cellular or extracellular compartment (Dalbey *et al.*, 1997). Sec11A is a member of type I signal peptidase. In the Archaeon *Haloferax volcanii*, sec11A may be expressed differentially as a function of growth stage or in response to environmental changes (Fine *et al.*, 2006). The ER signal peptidase complex (SPC) have been implicated in the processing of polyproteins of some viruses such as hantavirus, hepatitis C virus, influenza C virus, rubella virus, flavivirus, and foamy virus. These viruses contain internal signal peptides that are most likely cleaved by the ER SPC. Mutagenesis of predicted signal peptidase substrate specificity elements may block viral infectivity (Paetzel *et al.*, 2002). However, study on regulation of this peptide in crustacea is sparse.

6.4.1.5. Energy and metabolism factors

Mitochondria, double-membrane organelles regulate many critical cellular processes and homeostasis that are closely related to the cellular metabolic networks. Possibly, the most important role mitochondria play is in cellular energy metabolism (Chinnery and Schon, 2003; Picard *et al.*, 2011; Cloonan and Choi, 2012). Mitochondrial proteins have been shown to be involved in pattern recognition receptors (PRRs) of innate immunity such as Toll-like receptors (TLR), nuclear oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid inducible gene (RIG-I)-like receptors (RLRs) signalling pathways (Takeuchi and Akira, 2010; Ohta and Nishiyama, 2011; Cloonan and Choi, 2012). For an example, over expression of mitochondrial antiviral signalling protein (MAVS), a mitochondrial membrane protein induced type I interferon such as IFN-α and IFN-β via the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon regulatory factors (IRF3 and IRF7) leading to inhibition of viral infection. In contrast, knock down of MAVS expression through RNAi eliminated the activation of NF-κB, IRF3 and IRF7, thus inhibited the expression of interferons, allowing virus to replicate (Seth *et al.*, 2005; Scott, 2009). The MAVS do not only interact with retinoic acid inducible gene (RIG-I) to induce antiviral and anti-inflammatory responses, but also associate with peroxisomes, autophagosomes and

the endoplasmic reticulum (ER) to regulate apoptotic and metabolic functions. Other mitochondrial proteins including stimulator of interferon gene (STING), receptor for globular head domain of complement component (gC1qR), NOD like receptor X1 (NLRX1) and translocase of the mitochondrial outer membrane 70 (TOM70) could also activate RLR signalling pathways (Ohta and Nishiyama, 2011; Cloonan and Choi, 2012). Since the RLR signalling pathway congregates in mitochondria, it is more likely that viruses will target mitochondrial processes to evade this innate immune signalling pathway (Ohta and Nishiyama, 2011).

Alteration of metabolic pathways related to mitochondria and mitochondrial enzymes has been reported in WSSV-infected prawns (Mohankumar and Ramasamy, 2006; Chen *et al.*, 2011b). The Warburg effect (high rate of glycolytic response followed by increased lactate fermentation even under aerobic conditions in the cells) was observed in the early stage (12 h) of WSSV infection (Chen *et al.*, 2011b). Consumption of glucose and concentration of plasma lactate increased during viral replication. The activity of glucose-6-phosphate dehydrogenase (G6PDH), a key enzyme of the pentose phosphate pathway, also increased in the WSSV-infected animals. In the late stage of infection (24 h), changes related to the cell death were observed including induction of mitochondrial membrane permeabilization (MMP) and oxidative stress, decrease of glucose consumption and disruption of energy production (Chen *et al.*, 2011b). The Warburg effect is associated with mitochondria and partly mediated by the voltage-dependent anion channel (VDAC). The VDACs are also involved in cell death via its association with MMP. The induction of VDAC expression in the late stage of infection suggests a loss of mitochondrial membrane potential leading to MMP and cell death (Chen *et al.*, 2011b). Silencing of VDAC significantly down regulated the expression of this protein in the haemocytes, heart and lymphoid organ of the prawns and delayed WSSV infection suggesting its involvement in viral pathogenesis (Wang *et al.*, 2010).

Expression of outer membrane mitochondrial enzymes which are involved in the oxidation of glucose via the tricarboxylc acid (TCA) cycle and in production of ATP such as succinate dehydrogenase (SDH), malate dehydrogenase (MDH), α-

ketoglutarate dehydrogenase (KGDH), isocitrate dehydrogenase (ICDH), was suppressed from 24 h onward WSSV post-infection. This suggested that oxidation of glucose via TCA cycle declined because WSSV-infected prawns reduced feeding or WSSV infection may induce excessive production of free radicals (Mohankumar and Ramasamy, 2006). Similarly, the activities of inner membrane mitochondrial enzymes, NADH dehydrogenase and cytochrome C oxidase also declined suggesting cardiolipin was not available for their functional activity due to phospholipid degradation in prawns infected with WSSV (Mohankumar and Ramasamy, 2006).

NADH dehydrogenase subunit 5 and cytochrome oxidase subunits I (COI) and II (COII) were also identified within the group of energy and metabolism genes. NADH dehydrogenase (complex I), cytochrome reductase (complex III) and cytochrome oxidase (complex IV) are mitochondrial enzymes of transport electron chains that transfer electrons from NADH to O_2 (Weiss *et al.*, 1991). The complex I is the main entry point for electrons in respiratory chain (Kerscher *et al.*, 2008). This enzyme acts as a coupling element between the oxidation of NADH and the reduction of ubiquinone to generate a proton gradient for ATP synthesis (Sarkar *et al.*, 2005). The homologous NADH dehydrogenase subunits are significantly different in size in different species. Genes encoded NADH dehydrogenase subunit 2 (ND2), ND4 and ND5 have significant homologies suggesting that they may originate from the same ancestral genes (Weiss *et al.*, 1991; Kerscher *et al.*, 2008). The NADH dehydrogenase subunit 5 was found to be up regulated in the intestinal epithelial cell line Int407 of humans upon infection with *Vibrio cholerae*. The adherence, motility and virulence of *V. cholerae* may be the cause of ND5 induction (Sarkar *et al.*, 2005). The expression of this enzyme was also up regulated in penaeid prawns infected with microbes (He *et al.*, 2004), WSSV and YHV (He *et al.*, 2005; Leu *et al.*, 2007; Prapavorarat *et al.*, 2010).

Cytochrome oxidase involves the terminal oxidative step of the respiratory chain (Brunori and Wilson, 1982; Capaldi *et al.*, 1983; Denis, 1986). It has a crucial role in energy production as a traditional site for ATP synthesis. It also plays a role in energy transduction via a proton pumping mechanism (Brunori and Wilson, 1982).

In crustacea, few studies have shown the modulation of this enzyme due to infectious diseases. Cytochrome oxidase subunit I was observed to be up regulated in WSSV-infected haematopoietic tissue stem cells of redclaw crayfish (Liu *et al.*, 2011a). Up regulation of COI and COII was recorded in experimentally infected *P. monodon* with WSSV and YHV (Leu *et al.*, 2007; Prapavorarat *et al.*, 2010; Pongsomboon *et al.*, 2011). The expression of COI and COII were also induced in *P. monodon* upon injection with *V. harveyi*(Pongsomboon *et al.*, 2008; Nayak *et al.*, 2010).

The induction of genes related to energy production in the SSH libraries suggest that there is a high energy demand in viral-infected cells. This may be related to immunological response of the cells against viral infection as well as for other energy-dependent biological process. At the same time, this may also provide energy yield for viruses when they take over the host's machinery for replication.

6.4.1.6. Ribosomal proteins

A ribosome is the macromolecular machinery where proteins are synthesised in all living cells (Klein *et al.*, 2004; Brodersen and Nissen, 2005; Hu and Li, 2007; Perry, 2007). Some ribosomal proteins not only play crucial role in protein biosynthesis within the ribosomes (ribosomal functions), but also involved in other cellular process (extraribosomal functions) such as translational regulation and posttranslational modification, RNA splicing, DNA replication, DNA repairing, DNA topoisomerase activation, transcriptional regulation, nuclear transport and signal transduction. Ribosomal proteins are suggested to regulate cell growth and proliferation, development, cell apoptosis, tumour suppressor genes and proto-oncogenes. They may also involve in tumourigenesis and have antiviral activity (Wool, 1996; Brodersen and Nissen, 2005; Wilson and Nierhaus, 2005; Lai and Xu, 2007; Lindström, 2009; Warner and McIntosh, 2009).

Alteration of ribosomal protein genes are commonly found in the screening of differentially expressed genes in genetic diseases and cancer in humans (Lai and Xu, 2007). Similarly, up and down regulation of ribosomal protein genes were also frequently detected in cDNA libraries of crustacea challenged with a variety of

inducers. For examples, expression of ribosomal protein S24 was induced in the haemocytes during the long recovery phase from hyperthermic stress of *P. monodon* (de la Vega *et al.*, 2007b). Ribosomal proteins P2, S20, L11 and L32 in the haemocytes (Pongsomboon *et al.*, 2011) and ribosomal proteins P1, S27, L17, and L21 in the LO (Pongsomboon *et al.*, 2008) were up regulated in *V. harveyi*-infected *P. monodon*. In addition, 40S ribosomal protein S12 was induced in the lymphoid organ of *P. chinensis* infected with *V. anguillarum* (Zhang *et al.*, 2010). Moreover, ribosomal protein S6 serine/threonin kinase was induced in the gills of *P. vannamei* injected with dsRNA (Robalino *et al.*, 2007a).

Alteration of ribosomal proteins has also been reported in crustacea infected with viruses. In YHV-infected *P. vannamei*, several ribosomal proteins were expressed in the gills cDNA SSH libraries (Junkunlo *et al.*, 2010). The 40S ribosomal protein S23, ribosomal proteins P2, S24, L3, L8, L11, L18, L30 and L32 were up regulated in the haemocytes of *P. monodon* infected with YHV (Prapavorarat *et al.*, 2010; Pongsomboon *et al.*, 2011). Up regulation of ribosomal proteins including small subunit ribosomal protein S3, S5, S19, S20, S23, S25, S26, S28 and large subunit ribosomal protein L5A, L8, L10A, L11, L18A, L19, L21, L28, L34, L35A, L37, L37A and L38 were observed in the haemocytes of penaeid prawns infected with WSSV (Rojtinnakorn *et al.*, 2002; Pongsomboon *et al.*, 2011). In addition, expression levels of ribosomal protein L7 and L10A were significantly increased in *P. monodon* postlarvae infected with WSSV (Leu *et al.*, 2007). The expression of ribosomal proteins L7, S23e and acidic ribosomal protein P1 was up regulated in the hepatopancreas of WSSV-resistant *P. vannamei*(Zhao *et al.*, 2007).

However, down regulation of ribosomal protein was also observed in penaeids infected with WSSV. For instance, ribosomal protein S17, S21 and L14 were significantly decreased in WSSV-infected *P. monodon* postlarvae (Leu *et al.*, 2007). Ribosomal proteins L3, L23, 35A and rpL19 were down regulated in WSSV-resistant *P. vannamei* (Zhao *et al.*, 2007). A broad variety of ribosomal protein genes identified in penaeids suggests that these proteins may be selectively expressed in different tissues and diseases. The over expression of these proteins

may be associated with cell proliferation, while their down regulation is associated with maturation and differentiation of cells (Lai and Xu, 2007).

6.4.1.7. Other and unknown sequences

Several other sequences and many unknown sequences were also identified in cDNA SSH libraries. The presence of unknown sequences suggested that these genes were identified for the first time and had no similarity with known sequences in the GenBank database. A broad variety of unknown genes in the lymphoid organ may have important roles in the immune functions of this organ. Therefore, further studies are needed to uncover the exact roles and regulatory mechanism of these genes in penaeid prawn's immunity against viral diseases which is related to spheroid formation in the lymphoid organ.

6.4.2. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification with bunyavirus primers and PCR amplification with parvovirus primers

RT-PCR amplification and sequencing results showed that the formation of the lymphoid organ spheroid cells had no association with viruses of the Family Bunyaviridae. However, PCR amplification using hepatopancreatic parvovirus 140 (HPV140) primers produced interesting results. HPV140F primer is at nucleotide positions 4380 to 4403 and HPV140R primer at position 4500 to 4519 at ORF3 and encodes the structural protein (VP1) of PmergDNV (La Fauce *et al.*, 2007a). A previous study revealed that *P. merguiensis* densovirus (PmergDNV), the Australian strain of HPV commonly infected cultured and wild *P. merguiensis* (La Fauce *et al.*, 2007a; La Fauce *et al.*, 2007b).

The presence of only four positive samples on the PCR amplification suggested that in chronically infected animals, either PmergDNV was undetected due to low viral load in the remaining samples or this virus has been eliminated during spheroid cell development by an unknown mechanism (Hasson *et al.*, 1999b). Hasson *et al.*(1999a) who used *in situ* hybridization believed that in the chronic phase of viral infection, rapid development of massive spheroid cells within the LO only contained

low grade to moderate number of virions in infected foci. In addition, according to Tang and Lightner (2011), HPV is an enteric virus targeting hepatopancreatic and intestinal cells of penaeid prawns. Thus, PmergDNV could be an enteric virus that spills over into the open circulatory system of the entire systemic tissues including the LO of the animals and becomes widespread. Indeed, systemic circulating PmergDNV has been found in the mud crab, *Scylla serrata*(Owens *et al.*, 2010). Furthermore, another reported parvovirus of penaeid prawns, lymphoidal parvo-like virus (LPV) that has been found in *P. merguiensis, P. monodon* and *P. esculentus* also infected systemic tissues including antennal gland, nerve cord and lymphoid organ (Owens *et al.*, 1991). Based on the molecular evidence presented here and the principle of Ockham's razor (the law of parsimony), it is highly probable that LPV recorded by Owens *et al.*(1991) was indeed systemic PmergDNV.

To conclude, the massive development of spheroid cells in the lymphoid organ of *P. merguiensis* from the hatchery population seems most likely to be due to defence mechanism to PmergDNV infection. As a result, the health status of these two populations of banana prawn was remarkably different; causing differential gene expression between populations, with some genes being induced in the hatchery population. The up regulation of these genes implicated their involvement in the immune responses. Cell to cell communication may occur between these genes which is associated with their biological activities parallel with their immune-reactivity in response to the viral infection within the LO of penaeid prawns. All in all, the current investigation has provided some valuable evidence on the up regulated genes in the lymphoid organ that may play crucial roles in viral defence responses in penaeid prawns. This could be used for further research on the host-viral interaction leading to a new immune-intervention approach that may help to circumvent the catastrophe of viral diseases in penaeid prawn industry.

CHAPTER 7

GENERAL DISCUSSION

Hypertrophied nuclei with marginated chromatin but without Cowdry type A intranuclear inclusion bodies were detected in the gills of a redclaw crayfish C. *quadricarinatus* population, while these changes were undetected in non-hypertrophied nuclei population (Chapter 4). Meanwhile, spheroid cells were present in the lymphoid organ of banana prawn *P. merguiensis* from hatchery population, but these changes were absent in the wild population (Chapter 6). To investigate the possible viral aetiology these two cellular changes and to profile the differentially expressed genes related to these possible viral aetiology, suppression subtractive hybridization (SSH) was applied in the current studies (Chapter 4 and 6). This PCR-based cDNA subtraction method may provide a better insight into the host and pathogens interaction at the molecular level.

Sequence with homology to parvovirus genes or any other viral genes was not identified in the SHH libraries. Similar results were also found in previous studies when penaeid prawns and crayfish were experimentally infected with WSSV (Wang *et al.*, 2006; Zhao *et al.*, 2007; Zeng and Lu, 2009; James *et al.*, 2010; Liu *et al.*, 2011a). In these studies WSSV genomes could not be profiled in the SHH libraries but differentially expressed genes were. However, in the other studies on penaeids experimentally infected with WSSV (Reyes *et al.*, 2007; García *et al.*, 2009) and YHV (Junkunlo *et al.*, 2010; Prapavorarat *et al.*, 2010) both host genes and viral genes were identified. These discrepancies may indicate the variability of the cDNA used in profiling viral genomes using this PCR-based cDNA subtraction technique (see below). In addition, this is the first study attempting to profile a viral genome in a naturally infected crustacea where there might be a balance between virus and host using the SSH method.

There are several reasons that could explain the absence of viral genomes from the SSH libraries in both experiments. Firstly, the two populations of redclaw crayfish could be infected with the same virus (Chapter 4) and similar case may also occur in the two populations of *P. merguiensis* (Chapter 6). However, in the redclaw the histopathological changes appeared only in the hypertrophied nuclei population, while they were absent in the non-hypertrophied nuclei population. In the banana

prawn, the spheroid cells formed only in hatchery population, while these changes were not observed in the wild population. When the SSH was performed, the abundance of the viral transcripts in the gills of the redclaw crayfish or in the lymphoid organ of the prawns may not have differed to any great extent between the populations in both cases, thereby excluding the viral genes from the SSH libraries (de Lorgeril *et al.*, 2005). If this is the case, specific pathogen free (SPF) *C. quadricarinatus* and *P. merguiensis* should be used as driver cDNAs, but they do not exist in Australia.

Secondly, the hypertrophied nuclei population of *C. quadricarinatus* and the hatchery population of *P. merguiensis* may only be infected by virus but the concentration of viral genes in the tester cDNAs was too low to be expressed using SSH. Thirdly, the poly(A) tail on the mRNA of the parvovirus may be too short for this SSH technology to be successful. The complementary DNA synthetic primer of this protocol contains four poly(T)s at the first 5´ end. Thus, the target gene should also have at least four or more poly(A)s in the tail in order to be amplified with this method. However, studies on parvovirus indicate that this should not be the case. Most parvoviruses if not all, have more than four poly(A)s (Tattersall *et al.*, 2008). Finally, the virus causing these cellular changes may not have a poly(A) tail, therefore it could not be expressed in the SSH libraries. This is why the RT-PCRs for bunyavirus were attempted and further studies are necessary to determine which hypothesis is more likely (see below).

Even though viral genomes cannot be detected the SSH libraries of both trials, multi-transcripts were identified being up regulated in the hypertrophied nuclei population of *C. quadricarinatus* (Chapter 4) and hatchery population of *P. merguiensis* (Chapter 6). These genes represented immune-related genes, synthesis, processing and regulation-related proteins, proteases and inhibitors, structural and cytoskeletal related proteins, energy and metabolism factors, and ribosomal proteins, which are all known to be involved in biological process and immune response against infectious pathogens and mostly play an important part in antiviral defence mechanisms (Table 7.1). However, there are surprisingly few common genes

Table 7.1. Genes that maybe involved in a range of functional activities identified in cDNA suppression subtractive hybridization (SSH) libraries of the gills of *Cherax quadricarinatus* and lymphoid organ (LO) of *Penaeus merguiensis*. Putative functions against pathogens are assigned based on the up regulation of the genes reported in the published work as indicated. Plus (+) indicates the presence of the gene in the SSH library. Dash (—) indicates the absence of the gene in the SHH library. ND: no data available in the published literature.

Genes	*cDNA SSH libraries*		Putative functional activities		
	Gills (*Cherax quadricarinatu*)	*LO* (*Penaeus merguiensis*)	Viruses	Bacteria	Biological process
Heat shock protein (HSP)	—	+	(Brenner and Wainberg, 2001; de la Vega *et al.*, 2006; Wang *et al.*, 2006)	(Rungrassamee *et al.*, 2010)	(Cimino *et al.*, 2002; Jolesch *et al.*, 2011)
Gamma-interferon-inducible lysosomal thiol reductase (GILT)	—	+	(Kongton *et al.*, 2011)	(Kongton *et al.*, 2011)	(Hastings and Cresswell, 2011)
Serum amyloid A (SAA) protein	+	—	(Cray *et al.*, 2009)	(Cray *et al.*, 2009)	(Santiago-Cardona *et al.*, 2003; Cray *et al.*, 2009)
Anti-lipopolysaccharide factor (ALF)	+	+	(Liu *et al.*, 2006; de la Vega *et al.*, 2008; Antony *et al.*, 2011)	(de la Vega *et al.*, 2008; Somboonwiwat *et al.*, 2008)	ND
Crustin	+	—	ND	(Donpudsa *et al.*, 2010b)	(Amparyup *et al.*, 2008)
Lysozyme	+	—	(He *et al.*, 2005; Pan *et al.*, 2005; Mai and Wang, 2010)	(Fenouil and Roch, 1991; Hikima *et al.*, 2003; Senapin and Phongdara, 2006; Burge *et al.*, 2007; Yao *et al.*, 2008)	ND
Cytosolic manganese superoxide dismutase (cMnSOD)	+	—	ND	ND	(Marchand *et al.*, 2009; Li *et al.*, 2010b; Lin *et al.*, 2010)
Cathepsin	—	+	ND	ND	(Mort and Buttle, 1997; Aoki *et al.*, 2003; Wang *et al.*, 2008; Stephens *et al.*, 2012; Turk *et al.*, 2012)
Kazal-type serine proteinase inhibitor	+	—	(Donpudsa *et al.*, 2010a)	(Donpudsa *et al.*, 2009; Li *et al.*, 2009b)	—
Metallothionein (MT)	—	+	(Ilbäck *et al.*, 2004)	ND	(Roesijadi, 1992; Pourang *et al.*, 2004; Amiard *et al.*, 2006)
Calreticulin precursor (CRT)	—	+	(Wang *et al.*, 2006; Wang *et al.*, 2007a; Liu *et al.*, 2011a)	(Chen *et al.*, 2010)	(Luana *et al.*, 2007; Qiu and Michalak, 2009)
Chaperonin	+	—	ND	(He *et al.*, 2004; Pongsomboon *et al.*, 2008)	(Levy-Rimler *et al.*, 2002; Valpuesta *et al.*, 2002; Dobocan *et al.*, 2009)

Genes	*cDNA SSH libraries*		Putative functional activities		
	Gills (*Cherax quadricarinatu*)	***LO* (*Penaeus merguiensis*)**	**Viruses**	**Bacteria**	**Biological process**
Eukaryotic translation elongation factor (eEF)	—	+	(Kawaguchi *et al.*, 1997)	ND	(Riis *et al.*, 1990; Xiao *et al.*, 1998; Boulben *et al.*, 2003; Wu *et al.*, 2011; Zou *et al.*, 2011)
Eukaryotic translation initiation factor (eIF)	+	+	(Wang *et al.*, 2009)	ND	(Sonenberg and Dever, 2003; Hernández and Vazquez-Pianzola, 2005; Low *et al.*, 2007; Passmore *et al.*, 2007; Mitchell and Lorsch, 2008; Miluzio *et al.*, 2009; Surakasi and Kim, 2010)
Protein disulfide isomerase (PDI)	—	+	(Wang *et al.*, 2006; Chongsatja *et al.*, 2007; Bourchookarn *et al.*, 2008; Prapavorarat *et al.*, 2010)	(Vargas-Albores *et al.*, 2009; Ren *et al.*, 2011)	(Wilkinson and Gilbert, 2004)
Translationally-controlled tumor protein (TCTP)	+	—	(Bangrak *et al.*, 2004; Graidist *et al.*, 2006; Tonganunt *et al.*, 2008)	ND	(Bommer and Thiele, 2004; Loongyai *et al.*, 2007b; Chen *et al.*, 2009)
Ubiquitin (Ubq)	—	+	(Wang *et al.*, 2005; Wang *et al.*, 2006; Chen *et al.*, 2011a; Liu *et al.*, 2011a)	ND	(Deshaise and Joazeiro, 2009; Picard *et al.*, 2011; Schaefer *et al.*, 2012)
Signal peptidase complex (SPC)	—	+	(Paetzel *et al.*, 2002)	ND	(Dalbey *et al.*, 1997; Paetzel *et al.*, 2002; Fine *et al.*, 2006)
ATP synthase	+	—	ND	ND	(Wagner *et al.*, 2009)
Cytochrome *c* oxidase (CO)	+	+	(Leu *et al.*, 2007; Prapavorarat *et al.*, 2010; Liu *et al.*, 2011a; Pongsomboon *et al.*, 2011)	(Pongsomboon *et al.*, 2008; Nayak *et al.*, 2010)	(Brunori and Wilson, 1982; Capaldi *et al.*, 1983; Denis, 1986; Belevich *et al.*, 2010)
Mitochondrion	—	+	(Mohankumar and Ramasamy, 2006; Chen *et al.*, 2011b)	ND	(Chinnery and Schon, 2003; Picard *et al.*, 2011; Cloonan and Choi, 2012)
NADH dehydrogenase	+	+	(He *et al.*, 2005; Leu *et al.*, 2007; Prapavorarat *et al.*, 2010)	(He *et al.*, 2004)	(Weiss *et al.*, 1991; Sarkar *et al.*, 2005; Kerscher *et al.*, 2008; Belevich *et al.*, 2010)
Putative DEAD box ATP-dependent RNA helicase	+	—	ND	ND	(Jankowsky, 2011)

Genes	*cDNA SSH libraries*		Putative functional activities		
	Gills (*Cherax quadricarinatu*)	***LO* (*Penaeus merguiensis*)**	**Viruses**	**Bacteria**	**Biological process**
Insulin-like growth factor binding protein (IGFBP)	+	—	ND	ND	(Clemmons, 1997; Hwa *et al.*, 1999; Navarro *et al.*, 1999; Castellanos *et al.*, 2008)
Allergen	+	—	ND	ND	(Leung *et al.*, 1996; Yu *et al.*, 2003; Lopata *et al.*, 2010)
Tubulin	+	—	ND	ND	(Murphy, 1991; Dutcher, 2001)
Actin	—	+	(Chongsatja *et al.*, 2007; Leu *et al.*, 2007; Wu *et al.*, 2007; Pongsomboon *et al.*, 2011)	(Zhang *et al.*, 2010)	(Hild *et al.*, 2010)
Laminin receptor	+	—	(Fülöp and Larbi, 2002; Senapin and Phongdara, 2006; Busayarat *et al.*, 2011; Liu *et al.*, 2011a)	(Fülöp and Larbi, 2002; Senapin and Phongdara, 2006)	(Fülöp and Larbi, 2002; Nelson *et al.*, 2008)
Putative elastin A	+	—	ND	ND	(Lapis and Tímár, 2002; Duca *et al.*, 2004; Foster, 2004; Muiznieks *et al.*, 2010)
Ribosomal proteins	+	—	(Rojtinnakorn *et al.*, 2002; Leu *et al.*, 2007; Prapavorarat *et al.*, 2010; Pongsomboon *et al.*, 2011)	(Pongsomboon *et al.*, 2008; Zhang *et al.*, 2010; Pongsomboon *et al.*, 2011)	(Klein *et al.*, 2004; Brodersen and Nissen, 2005; Wilson and Nierhaus, 2005; Hu and Li, 2007; Perry, 2007)
Secretory eggshell protein precursor (SEP)	+	—	ND	ND	(Chen *et al.*, 1992; Bae *et al.*, 2007)
Centromere protein (CENP)	—	+	ND	ND	(Cleveland *et al.*, 2003; Fritzler *et al.*, 2011; Pauleau and Erhardt, 2011)
Alkylglycerol monooxygenase (agmo)	—	+	ND	ND	(Koetting *et al.*, 1987; Kötting *et al.*, 1987)
Microsatellite	—	+	ND	ND	(Li *et al.*, 2004; Chistiakov *et al.*, 2006; Chen *et al.*, 2011c)

up regulated in the two trials that the aetiologies have very little in common or perhaps one could conclude the hosts respond to infection very differently. Therefore, comparative studies are very weak when transposing information from a crayfish host to a penaeid host.

The possibility of the viral aetiology of the hypertrophied nucleic in *C. quadricarinatus* (Chapter 5) and spheroids in *P. merguiensis* (Chapter 6) due to virus with no poly(A) tail, was investigated using primers designed from related-genus Phlebovirus of family Bunyaviridae. However, the results showed that these changes had no association with bunyaviruses rejecting the last supposition. Using HPV140F/HPV140R primers to amplify the expected viral genomes, the PCR produced no amplicon in populations of *C. quadricarinatus* (Chapter 5) eliminating the possibility of the presence PmergDNV or related sequences in the redclaw populations. Interestingly in *P. merguiensis*, the PCR and sequencing results revealing PmergDNV was present in the hatchery population, while it was absent in the wild population (Chapter 6). This suggested that the formation of spheroid cells in the lymphoid organ of the prawns was related to PmergDNV infection and these cellular changes may be formed as defensive response against this viral infection. This result does not support the first supposition that both populations of *P. merguiensis* were infected with the same virus, but allows the suggestion of the possibility that only the hatchery population of prawns had an overflow of the virus into the systemic circulation. Therefore, the absence of viral (PmergDNV) transcripts from cDNA SSH libraries is most likely due to the low viral load in the LO of the tester population.

Further investigation was conducted to uncover the cause of the nuclear changes in the gills of *C. quadricarinatus* (Chapter 5) using several parvovirus primers for PCR amplifications. Surprisingly, instead of identifying the exogenous viral sequences, the presence of endogenous Brevidensovirus-like elements (EBreVEs) were reported for the first time in *C. quadricarinatus*. Nine elements that can be assembled into four consensus sequences were found in the redclaw from different sources suggesting the widespread nature of these elements in *C. quadricarinatus*

populations in northern Queensland, Australia. Astonishingly, an identical Fragment 2 (128 bp) obtained from 170 bp amplicon was identified in *P. merguiensis* from the hatchery population (Figure 7.1) (see Section 5.3.2.2). Possibly the insertion of this element may have occurred before the separation of the Astacidea (*C. quadricarinatus*) and Dendrobranchiata (*P. merguiensis*), or perhaps these two species have been infected with the same ancestral virus after the separation from ancient origin million years ago.

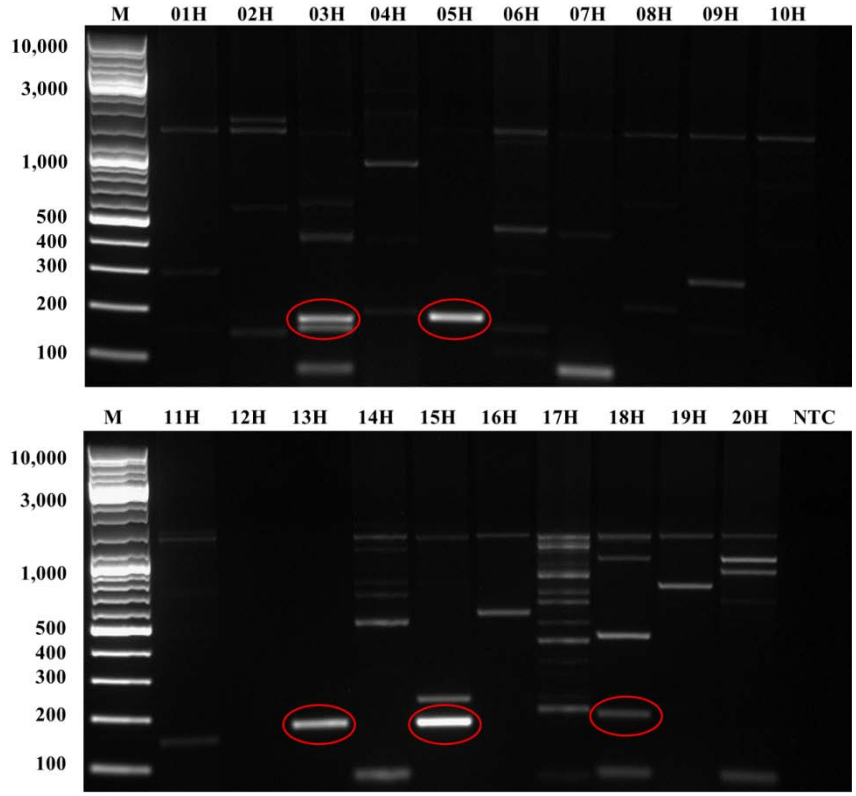

Figure 7.1.PCR amplification with QPF1/QPR1 primers showing 170 bp amplicon (red oval) from hatchery population of *Penaeus merguiensis*. Three amplicons (05H, 13H and 15H) were extracted, purified and subjected to cloning and sequencing. This amplicon yielded a 128 bp fragment (Fragment 2) that shared 82% nucleotide similarities with *Cherax quadricarinatus* clone epi15_C6 mRNA sequences (DQ847767) and it also shared 61% amino acid identity to NS1 of IHHNV (AAY59892). PCR products are electrophorosed on a 1.2% agarose/GelRed. M: 10 kbp DNA marker (SM0333 Fermentas), NTC: non-template control.

The most remarkable feature of these elements is that they are located in the same region relative to IHNNV sequences and most likely are originated from the non-structural protein of ancestral virus. Even though definitive insertion site could not be determined, probably most of these elements, if not all, are randomly inserted

within the mobile elements (microsatellites) of the host genomes. In addition, the presence of these endogenous virus-like elements may have immunological function for the host through RNAi pathway (Maori *et al.*, 2007; Flegel, 2009) against infection of the more closely related exogenous viruses. Therefore, if the endogenous viral sequences provide a protective function to the host in an RNAi manner, it is no wonder why, in the challenge experiment, animals injected with extracted gills (from animals that had hypertrophied nuclei with marginated chromatin) did not develop these nuclear changes or show any signs of diseases (Chapter 4). In other words, the pathological changes cannot be transmitted to animals with these endogenous sequences (non-hypertrophied nuclei population).

To sum up, in the current studies, suppression subtractive hybridisation was unable to provide unequivocal evidence of viral aetiology in naturally viral infected crustacea. This indicates that in elucidating viral genomes in viral infected animals, SSH may only work when the viral load is high such as in experimental trials, but this technique may not be suitable when the viral load is low such as in naturally viral infected animals. In addition, specific pathogen free crustacea may become a better choice to be used as a cDNA driver when the cDNA tester is generated from naturally viral infected animals to construct SSH libraries, but they are unavailable in Australia. Furthermore, different approaches such as isolation of nucleic acids from purified viruses should be taken into account in order to discover the viral aetiology of the nuclear changes in *C. quadricarinatus*.

Despite the absence of viral gene detection and therefore a conclusion of an idiopathic aetiology for signet ring changes in *C. quadricarinatus,* differentially expressed genes have been elucidated in cDNA libraries of crustacea using the PCR-based cDNA subtraction technique. These genes may have a potential role in immune response against invading pathogens in particular, viral infection. Moreover, the presence of endogenous virus-like elements (EBreVE) in these decapod crustacea may also offer beneficial effect to the host. Taken together, these studies have provided an insight into the host-viral interaction at the molecular level. This knowledge may contribute to future research on crustacean immunity into

establishing a holistic approach to combat the devastating impact of infectious diseases, in particular viral pathogens, in order to maintain production in crustacean aquaculture.

REFERENCES

Abdu, U., Davis, C., Khalaila, I. and Sagi, A. (2002) The vitellogenin cDNA of *Cherax quadricarinatus* encodes a lipoprotein with calcium binding ability, and its expression is induced following the removal of the androgenic gland in a sexually plastic system. *Gen Comp Endocrinol***127**: 263-272

Ai, H.-S., Liao, J.-X., Huang, X.-D., Yin, Z.-X., Weng, S.-P., Zhao, Z.-Y., Li, S.-D., Yu, X.-Q. and He, J.-G. (2009) A novel prophenoloxidase 2 exists in shrimp hemocytes. *Dev Comp Immunol***33**: 59-68

Alday-Sanz, V., Roque, A. and Turnbull, J.F. (2002) Clearing mechanisms of *Vibrio vulnificus* biotype I in the black tiger shrimp *Penaeus monodon*. *Dis Aquat Org***48**: 91-99

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J. and Rainbow, P.S. (2006) Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. *Aquat Toxicol***76**: 160-202

Amparyup, P., Donpudsa, S. and Tassanakajon, A. (2008) Shrimp single WAP domain (SWD)-containing protein exhibits proteinase inhibitory and antimicrobial activities. *Dev Comp Immunol***32**: 1497-1509

Amparyup, P., Wiriyaukaradecha, K., Charoensapsri, W. and Tassanakajon, A. (2010) A clip domain serine proteinase plays a role in antibacterial defense but is not required for prophenoloxidase activation in shrimp. *Dev Comp Immunol***34**: 168-176

Anantasomboon, G., Poonkhum, R., Sittidilokratna, N., Flegel, T.W. and Withyachumnarnkul, B. (2008) Low viral loads and lymphoid organ spheroids are associated with yellow head virus (YHV) tolerance in whiteleg shrimp *Penaeus vannamei*. *Dev Comp Immunol***32**: 613-626

Anantasomboon, G., Sriurairatana, S., Flegel, T.W. and Withyachumnarnkul, B. (2006) Unique lesions and viral-like particles found in growth retarded black tiger shrimp *Penaeus monodon* from East Africa. *Aquaculture***253**: 197-203

Andersen, J.T. and Baatrup, E. (1988) Ultrastructural localization of mercury accumulations in the gills, hepatopancreas, midgut, and antennal glands of the brown shrimp, *Crangon crangon*. *Aquat Toxicol***13**: 309-324

Andrade, T.P.D., Redman, R.M. and Lightner, D.V. (2008) Evaluation of the preservation of shrimp samples with Davidson's AFA fixative for infectious myonecrosis virus (IMNV) *in situ* hybridization. *Aquaculture***278**: 179-183

Anggraeni, M.S. (1998) The origin and the role of lymphoid organ spheroid cells in the giant tiger prawn, *Penaeus monodon*. MSc thesis, James Cook University.

Anggraeni, M.S. and Owens, L. (2000) The haemocytic origin of lymphoid organ spheroid cells in the penaeid prawn *Penaeus monodon*. *Dis Aquat Org***40**: 85-92

Angthong, P., Watthanasurorot, A., Klinbunga, S., Ruangdej, U., Söderhäll, I. and Jiravanichpaisal, P. (2010) Cloning and characterization of a melanization

inhibition protein (PmMIP) of the black tiger shrimp, *Penaeus monodon*. *Fish Shellfish Immunol***29**: 464-468

Antony, S.P., Philip, R., Joseph, V. and Singh, I.S.B. (2011) Anti-lipopolysaccharide factor and crustin-III, the anti-white spot virus peptides in *Penaeus monodon*: Control of viral infection by up-regulation. *Aquaculture***319**: 11-17

Aoki, H., Ahsan, M.N. and Watabe, S. (2003) Molecular cloning and characterization of cathepsin B from the hepatopancreas of northern shrimp *Pandalus borealis*. *Comp Biochem Physiol B***134**: 681-694

Arts, J.A.J. (2006) Immune defence of white spot syndrome virus infected shrimp, *Penaeus monodon*. PhD thesis, Wageningen University.

Ascenso, R., Leite, R., Afonso, R. and Cancela, M. (2007) Suppression subtractive hybridization: A rapid and inexpensive detection methodology for up-regulated *Perkinsus olseni* genes. *Afr J Biochem***1**: 024 - 028

Assavalapsakul, W. and Panyim, S. (2012) Molecular cloning and tissue distribution of the Toll receptor in the black tiger shrimp, *Penaeus monodon*. *Genet Mol Res***11** 484-493

Bachali, S., Jager, M., Hassanin, A., Schoentgen, F., Jolles, P., Fiala-Medioni, A. and Deutsch, J.S. (2002) Phylogenetic analysis of invertebrate lysozyme and the evolution of lysozyme activity. *J Mol Evol***54**: 652-664

Bachere, E. (2003) Anti-infectious immune effectors in marine invertebrate: potential tools for disease control in larviculture. *Aquaculture***237**: 427 - 438

Bae, Y.-A., Kim, S.-H., Cai, G.-B., Lee, E.-G., Kim, T.-S., Agatsuma, T. and Kong, Y. (2007) Differential expression of *Paragonimus westermani* eggshell proteins during the developmental stages. *Int J Parasitol***37**: 295-305

Bainy, A.C.D. (2000) Biochemical responses in penaeids caused by contaminants. *Aquaculture***191**: 163-168

Bangrak, P., Graidist, P., Chotigeat, W. and Phongdara, A. (2004) Molecular cloning and expression of a mammalian homologue of a translationally controlled tumor protein (TCTP) gene from *Penaeus monodon* shrimp. *J Biotechnol***108**: 219-226

Bangrak, P., Graidist, P., Chotigeat, W., Supamattaya, K. and Phongdara, A. (2002) A syntenin-like protein with postsynaptic density protein (PDZ) domains produced by black tiger shrimp *Penaeus monodon* in response to white spot syndrome virus infection. *Dis Aquat Org***49**: 19-25

Barrientos, A., Barros, M.H., Valnot, I., Rötig, A., Rustin, P. and Tzagoloff, A. (2002) Cytochrome oxidase in health and disease. *Gene***286**: 53-63

Bejarano, E.R., Khashoggi, A., Witty, M. and Lichtenstein, C. (1996) Integration of multiple repeats of geminiviral DNA into the nuclear genome of tobacco during evolution. *Proc Natl Acad Sci* **93**: 759-764

Belevich, I., Gorbikova, E., Belevich, N.P., Rauhamäki, V., Wikström, M. and Verkhovsky, M.I. (2010) Initiation of the proton pump of cytochrome c oxidase. *Proc Natl Acad Sci***107**: 18469-18474

Bell, T.A. and Lightner, D.V. (1988) A Handbook of Normal Penaeid Shrimp Histology, 114 pp. The World Aquaculture Society, Baton Rauge, Louisiana.

Belyi, V.A., Levine, A.J. and Skalka, A.M. (2010a) Sequences from ancestral single-stranded DNA viruses in vertebrate genomes: the parvoviridae and Circoviridae are more than 40 to 50 million years old. *J Virol***84**: 12458-12462

Belyi, V.A., Levine, A.J. and Skalka, A.M. (2010b) Unexpected inheritance: multiple integrations of ancient bornavirus and ebolavirus/marburgvirus sequences in vertebrate genomes. *PLoS Pathog***6**: e1001030

Benveniste, R.E. and Todaro, G.J. (1974) Evolution of C-type viral genes: inheritance of exogenously acquired viral genes. *Nature***252**: 456-459

Biswas, G., Korenaga, H., Nagamine, R., Kono, T., Shimokawa, H., Itami, T. and Sakai, M. (2012) Immune stimulant effects of a nucleotide-rich baker's yeast extract in the kuruma shrimp, *Marsupenaeus japonicus*. *Aquaculture***366-367**: 40-45

Bommer, U.-A. and Thiele, B.-J. (2004) The translationally controlled tumour protein (TCTP). *Int J Biochem Cell Biol***36**: 379-385

Bonami, J.R., Lightner, D.V., Redman, R.M. and Poulos, B.T. (1992) Partial characterisation of a togavirus (LOVV) associated with histopathological changes of the lymphoid organ of penaeid shrimps. *Dis Aquat Org***14**: 145 - 152

Bonami, J.R., Mahy, B.W.J. and Regenmortel, M.H.V.v. (2008) Shrimp Viruses, Encyclopedia of Virology, pp. 567-576. Academic Press, Oxford.

Boonyaratpalin, S., Supamattaya, K., Kasornchandra, J., Direkbusarocom, S., Aekpanithanpong, U. and Chantanachooklin, C. (1993) Non-occluded baculo-like virus, the causative agent of yellow head disease in the black tiger shrimp (*Penaeus monodon*). *Gyobyo Kenkyu***28**: 103 - 109

Boulben, S., Monnier, A., Le Breton, M., Morales, J., Cormier, P., Bellé, R. and Mulner-Lorillon, O. (2003) Sea urchin elongation factor 1d (EF1d) and evidence for cell cycle-directed localization changes of a sub-fraction of the protein at M phase. *Cell Mol Life Sci***60**: 2178-2188

Bourchookarn, A., Havanapan, P.-O., Thongboonkerd, V. and Krittanai, C. (2008) Proteomic analysis of altered proteins in lymphoid organ of yellow head virus infected *Penaeus monodon*. *Biochim Biophys Acta* **1784**: 504-511

Bowater, R.O., Wingfield, M., Fisk, A., Condon, K.M.L., Reid, A., Prior, H. and Kulpa, E.C. (2002) A parvo-like virus in cultured redclaw crayfish *Cherax quadricarinatus* from Queensland, Australia. *Dis Aquat Org***50**: 79-86

Brenner, B.G. and Wainberg, Z. (2001) Heat shock proteins: novel therapeutic tools for HIV-infection? *Exp Opin Biol Ther***1**: 67-77

Brodersen, D.E. and Nissen, P. (2005) The social life of ribosomal proteins. *FEBS Journal***272**: 2098-2108

Brunori, M. and Wilson, M.T. (1982) Cytochrome oxidase. *Trends Biochem Sci***7**: 295-299

Burge, E.J., Burnett, L.E. and Burnett, K.G. (2009) Time-course analysis of peroxinectin mRNA in the shrimp *Litopenaeus vannamei* after challenge with *Vibrio campbellii*. *Fish Shellfish Immunol***27**: 603-609

Burge, E.J., Madigan, D.J., Burnett, L.E. and Burnett, K.G. (2007) Lysozyme gene expression by hemocytes of Pacific white shrimp, *Litopenaeus vannamei*, after injection with *Vibrio*. *Fish Shellfish Immunol***22**: 327-339

Burgents, J.E., Burnett, L.E., Stabb, E.V. and Burnett, K.G. (2005) Localization and bacteriostasis of *Vibrio* introduced into the Pacific white shrimp, *Litopenaeus vannamei*. *Dev Comp Immunol***29**: 681-691

Busayarat, N., Senapin, S., Tonganunt, M., Phiwsaiya, K., Meemetta, W., Unajak, S., Jitrapakdee, S., Lo, C.-F. and Phongdara, A. (2011) Shrimp laminin receptor binds with capsid proteins of two additional shrimp RNA viruses YHV and IMNV. *Fish Shellfish Immunol***31**: 66-72

Byers, R.J., Hoyland, J.A., Dixon, J. and Freemont, A.J. (2000) Subtractive hybridization - genetic takeaways and the search for meaning. *Int J Exp Pathol***81**: 391-404

Cao, J.-X., Yin, G.-L. and Yang, W.-J. (2006) Identification of a novel male reproduction-related gene and its regulated expression patterns in the prawn, *Macrobrachium rosenbergii*. *Peptides***27**: 728-735

Capaldi, R.A., Malatesta, F. and Darley-Usmar, V.M. (1983) Structure of cytochrome c oxidase. *Biochim Biophys Acta* **726**: 135-148

Carulli, J.P., Artinger, M., Swain, P.M., Root, C.D., Chee, L., Tulig, C., Guerin, J., Osborne, M., Stein, G., Lian, J. and Lomedico, P.T. (1998) High throughput analysis of differential gene expression. *J Cell Biochem***72**: 286-296

Cassarino, D.S. and Bennett Jr, J.P. (1999) An evaluation of the role of mitochondria in neurodegenerative diseases: mitochondrial mutations and oxidative pathology, protective nuclear responses, and cell death in neurodegeneration. *Brain Res Rev***29**: 1-25

Castellanos, M., Jiménez-Vega, F. and Vargas-Albores, F. (2008) Single IB domain (SIBD) protein from *Litopenaeus vannamei*, a novel member for the IGFBP family. *Comp Biochem Physiol D***3**: 270-274

Chaikeeratisak, V., Somboonwiwat, K., Wang, H.-C., Lo, C. and Tassanakajon, A. (2012) Proteomic analysis of differentially expressed proteins in the lymphoid organ of *Vibrio harveyi* infected *Penaeus monodon*. *Mol Biol Rep***39**: 6367-6377

Chang, C.-C., Yeh, M.-S., Lin, H.-K. and Cheng, W. (2008) The effect of *Vibrio alginolyticus* infection on caspase-3 expression and activity in white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol***25**: 672-678

Chantanachookin, C., Boonyaratpalin, S., Kasornchandra, J., Direkbusarakom, S., Ekpanithanpong, U., Supamataya, K., Sriurairatana, S. and Flegel, T.W. (1993) Histology and ultrastructure reveal a new granulosis-like virus in *Penaeus monodon* affected by yellow-head disease. *Dis Aquat Org***17**: 145 - 157

Chen, A.-J., Wang, S., Zhao, X.-F., Yu, X.-Q. and Wang, J.-X. (2011a) Enzyme E2 from Chinese white shrimp inhibits replication of white spot syndrome virus and ubiquitinates Its RING domain proteins. *J Virol***85**: 8069-8079

Chen, D., He, N., Lei, K. and Xu, X. (2009) Genomic organization of the translationally controlled tumor protein (TCTP) gene from shrimp *Marsupenaeus japonicus*. *Mol Biol Rep***36**: 1135-1140

Chen, D., He, N. and Xu, X. (2008) Mj-DWD, a double WAP domain-containing protein with antiviral relevance in *Marsupenaeus japonicus*. *Fish Shellfish Immunol***25**: 775-781

Chen, F.-Y., Liu, H.-P., Bo, J., Ren, H.-L. and Wang, K.-J. (2010) Identification of genes differentially expressed in hemocytes of *Scylla paramamosain* in response to lipopolysaccharide. *Fish Shellfish Immunol***28**: 167-177

Chen, I.T., Aoki, T., Huang, Y.-T., Hirono, I., Chen, T.-C., Huang, J.-Y., Chang, G.-D., Lo, C.-F. and Wang, H.-C. (2011b) White spot syndrome virus induces metabolic changes resembling the Warburg effect in shrimp hemocytes in the early stage of Infection. *J Virol***85**: 12919-12928

Chen, L.-l., Rekosh, D.M. and LoVerde, P.T. (1992) *Schistosoma mansoni* p48 eggshell protein gene: characterization, developmentally regulated expression and comparison to the p14 eggshell protein gene. *Mol Biochem Parasitol***52**: 39-52

Chen, M., Tan, Z. and Zeng, G. (2011c) Microsatellite is an important component of complete Hepatitis C virus genomes. *Infect Genet Evol***11**: 1646-1654

Chendrimada, T.P., Finn, K.J., Ji, X., Baillat, D., Gregory, R.I., Liebhaber, S.A., Pasquinelli, A.E. and Shiekhattar, R. (2007) MicroRNA silencing through RISC recruitment of eIF6. *Nature***447**: 823-828

Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K. and Shiekhattar, R. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature***436**: 740-744

Cheng, W. and Chen, J.C. (2001) Effect of intrinsic and extrinsic factors on the haemocyte profile of the prawn, *Macrobrachium rosenbergii*. *Fish Shellfish Immunol***11**: 53 - 63

Chiba, S., Kondo, H., Tani, A., Saisho, D., Sakamoto, W., Kanematsu, S. and Suzuki, N. (2011) Widespread Endogenization of Genome Sequences of Non-Retroviral RNA Viruses into Plant Genomes. *PLoS Pathog***7**: e1002146

Chinnery, P.F. and Schon, E.A. (2003) Mitochondria. *J Neurol Neurosurg Psych***74**: 1188-99

Chistiakov, D.A., Hellemans, B. and Volckaert, F.A.M. (2006) Microsatellites and their genomic distribution, evolution, function and applications: A review with special reference to fish genetics. *Aquaculture***255**: 1-29

Chong, Y.C. and Loh, H. (1984) Hepatopancreas chlamidial and parvoviral infections of farmed marine prawns in Singapore. *Sing Vet J***9**: 51-56

Chongsatja, P.-o., Bourchookarn, A., Lo, C.F., Thongboonkerd, V. and Krittanai, C. (2007) Proteomic analysis of differentially expressed proteins in *Penaeus vannamei* hemocytes upon Taura syndrome virus infection. *Proteomics***7**: 3592-3601

Chou, P.-H., Chang, H.-S., Chen, I.T., Lee, C.-W., Hung, H.-Y. and Han-Ching Wang, K.C. (2011) *Penaeus monodon* Dscam (PmDscam) has a highly diverse cytoplasmic tail and is the first membrane-bound shrimp Dscam to be reported. *Fish Shellfish Immunol***30**: 1109-1123

Chou, P.-H., Chang, H.-S., Chen, I.T., Lin, H.-Y., Chen, Y.-M., Yang, H.-L. and Wang, K.C.H.-C. (2009) The putative invertebrate adaptive immune protein *Litopenaeus vannamei* Dscam (LvDscam) is the first reported Dscam to lack a transmembrane domain and cytoplasmic tail. *Dev Comp Immunol***33**: 1258-1267

Cimino, E.J., Owens, L., Bromage, E. and Anderson, T.A. (2002) A newly developed ELISA showing the effect of environmental stress on levels of hsp86 in *Cherax quadricarinatus* and *Penaeus monodon*. *Comp Biochem Physiol A***132**: 591-598

Clavero-Salas, A., Sotelo-Mundo, R.R., Gollas-Galván, T., Hernández-López, J., Peregrino-Uriarte, A.B., Muhlia-Almazán, A. and Yepiz-Plascencia, G. (2007) Transcriptome analysis of gills from the white shrimp *Litopenaeus vannamei* infected with White Spot Syndrome Virus. *Fish Shellfish Immunol***23**: 459-472

Clemmons, D.R. (1997) Insulin-like growth factor binding proteins and their role in controlling IGF actions. *Cytokine Growth Factor Rev***8**: 45-62

Cleveland, D.W., Mao, Y. and Sullivan, K.F. (2003) Centromeres and Kinetochores: From Epigenetics to Mitotic Checkpoint Signaling. *Cell***112**: 407-421

Clontech Laboratories, I. (2008) PCR-Select™ cDNA Subtraction Kit User Manual, 44 pp. Clontech Laboratories, Inc., Mountain View, California.

Cloonan, S.M. and Choi, A.M.K. (2012) Mitochondria: commanders of innate immunity and disease? *Curr Opin Immunol***24**: 32-40

Cock, J.M., Sterck, L., Rouze, P., Scornet, D., Allen, A.E., Amoutzias, G., Anthouard, V., Artiguenave, F., Aury, J.-M., Badger, J.H., Beszteri, B., Billiau, K., Bonnet, E., Bothwell, J.H., Bowler, C., Boyen, C., Brownlee, C., Carrano, C.J., Charrier, B., Cho, G.Y., Coelho, S.M., Collen, J., Corre, E., Da Silva, C., Delage, L., Delaroque, N., Dittami, S.M., Doulbeau, S., Elias, M., Farnham, G., Gachon, C.M.M., Gschloessl, B., Heesch, S., Jabbari, K., Jubin, C., Kawai, H., Kimura, K., Kloareg, B., Kupper, F.C., Lang, D., Le Bail, A.,

Leblanc, C., Lerouge, P., Lohr, M., Lopez, P.J., Martens, C., Maumus, F., Michel, G., Miranda-Saavedra, D., Morales, J., Moreau, H., Motomura, T., Nagasato, C., Napoli, C.A., Nelson, D.R., Nyvall-Collen, P., Peters, A.F., Pommier, C., Potin, P., Poulain, J., Quesneville, H., Read, B., Rensing, S.A., Ritter, A., Rousvoal, S., Samanta, M., Samson, G., Schroeder, D.C., Segurens, B., Strittmatter, M., Tonon, T., Tregear, J.W., Valentin, K., von Dassow, P., Yamagishi, T., Van de Peer, Y. and Wincker, P. (2010) The Ectocarpus genome and the independent evolution of multicellularity in brown algae. *Nature***465**: 617-621

Consortium, I.H.G.S. (2001) Initial sequencing and analysis of the human genome. *Nature***409**: 860-921

Costa, A.M., Buglione, C.C., Bezerra, F.L., Martins, P.C.C. and Barracco, M.A. (2009) Immune assessment of farm-reared *Penaeus vannamei* shrimp naturally infected by IMNV in NE Brazil. *Aquaculture***291**: 141-146

Cowley, J.A., McCulloch, R.J., Rajendran, K.V., Cadogan, L.C., Spann, K.M. and Walker, P.J. (2005a) RT-nested PCR detection of Mourilyan virus in Australian *Penaeus monodon* and its tissue distribution in healthy and moribund prawns. *Dis Aquat Org***66**: 91-104

Cowley, J.A., McCulloch, R.J., Spann, K.M., Cadogan, L.C. and Walker, P.J. (2005c) Preliminary molecular and biological chracterization of Mourilyan virus (MoV): A new bunya-related virus of penaeid prawns. Diseases in Asian Aquaculture V, 113-124.

Crawford, A.C., Kricker, J.A., Anderson, A.J., Richardson, N.R. and Mather, P.B. (2004) Structure and function of a cellulase gene in redclaw crayfish, *Cherax quadricarinatus*. *Gene***340**: 267-274

Cray, C., Zaias, J. and Altman, N.H. (2009) Acute phase response in animals: A review. *Comp Med***59**: 517-526

Crochu, S., Cook, S., Attoui, H., Charrel, R.N., De Chesse, R., Belhouchet, M., Lemasson, J.-J., de Micco, P. and de Lamballerie, X. (2004) Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of *Aedes* spp. mosquitoes. *J Gen Virol***85**: 1971-1980

Cudmore, S., Reckmann, I. and Way, M. (1997) Viral manipulations of the actin cytoskeleton. *Trends Microbiol***5**: 142-148

Cui, J. and Holmes, E.C. (2012) Endogenous RNA viruses of plants in insect genomes. *Virology***427**: 77-79

Dalbey, R.E., Lively, M.O., Bron, S. and Dijl, J.M.V. (1997) The chemistry and enzymology of the type I signal peptidases. *Protein Sci***6**: 1129-1138

Dall, W. and Smith, D.M. (1981) Ionic regulation of four species of penaeid prawn. *J Exp Mar Biol Ecol***55**: 219-232

Dang, L.T., Koyama, T., Shitara, A., Kondo, H., Aoki, T. and Hirono, I. (2010) Involvement of WSSV–shrimp homologs in WSSV infectivity in kuruma shrimp: *Marsupenaeus japonicus*. *Antiviral Res***88**: 217-226

de la Vega, E., Degnan, B.M., Hall, M.R. and Wilson, K.J. (2007a) Differential expression of immune-related genes and transposable elements in black tiger shrimp (*Penaeus monodon*) exposed to a range of environmental stressors. *Fish Shellfish Immunol***23**: 1072-1088

de la Vega, E., Hall, M.R., Degnan, B.M. and Wilson, K.J. (2006) Short-term hyperthermic treatment of *Penaeus monodon* increases expression of heat shock protein 70 (HSP70) and reduces replication of gill associated virus (GAV). *Aquaculture***253**: 82-90

de la Vega, E., Hall, M.R., Wilson, K.J., Reverter, A., Woods, R.G. and Degnan, B.M. (2007b) Stress-induced gene expression profiling in the black tiger shrimp *Penaeus monodon*. *Physiol Genomics***31**: 126-138

de la Vega, E., O'Leary, N.A., Shockey, J.E., Robalino, J., Payne, C., Browdy, C.L., Warr, G.W. and Gross, P.S. (2008) Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): A broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. *Mol Immunol***45**: 1916-1925

de Lorgeril, J., Saulnier, D., Janech, M.G., Gueguen, Y. and Bachere, E. (2005) Identification of genes that are differentially expressed in hemocytes of the Pacific blue shrimp (*Litopenaeus stylirostris*) surviving an infection with *Vibrio penaeicida*. *Physiol Genomics***21**: 174-183

Delaroque, N., Maier, I., Knippers, R. and Muller, D.G. (1999) Persistent virus integration into the genome of its algal host, *Ectocarpus siliculosus* (Phaeophyceae). *J Gen Virol***80**: 1367-70

Denis, M. (1986) Structure and function of cytochrome-c oxidase. *Biochimie***68**: 457-470

Deshaise, R.J. and Joazeiro, C.A.P. (2009) RING domain E3 ubiquitin ligases. *Annu Rev Biochem***78**: 399-434

Diatchenko, L., Lau, Y.F., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D. and Siebert, P.D. (1996) Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci USA* **93**: 6025-6030

Diatchenko, L., Lukyanov, S., Lau, Y.-F.C. and Siebert, P.D. (1999) Suppression subtractive hybridization: A versatile method for identifying differentially expressed genes. *Methods Enzymol***303**: 349-380

Dobocan, M.C., Sadvakassova, G. and Congote, L.F. (2009) Chaperonin 10 as an endothelial-derived differentiation factor: Role of glycogen synthase kinase-3. *J Cell Physiol***219**: 470-476

Dominguez, R. and Holmes, K.C. (2011) Actin structure and function. *Annu Rev of Biophys***40**: 169-186

Doms, R.W., Lamb, R.A., Rose, J.K. and Helenius, A. (1993) Folding and assembly of viral membrane proteins. *Virology***193**: 545-562

Dong, B. and Xiang, J.-H. (2007) Discovery of genes involved in defense/immunity functions in a haemocytes cDNA library from *Fenneropenaeus chinensis* by ESTs annotation. *Aquaculture***272**: 208-215

Donpudsa, S., Ponprateep, S., Prapavorarat, A., Visetnan, S., Tassanakajon, A. and Rimphanitchayakit, V. (2010a) A Kazal-type serine proteinase inhibitor SPIPm2 from the black tiger shrimp *Penaeus monodon* is involved in antiviral responses. *Dev Comp Immunol***34**: 1101-1108

Donpudsa, S., Rimphanitchayakit, V., Tassanakajon, A., Söderhäll, I. and Söderhäll, K. (2010b) Characterization of two crustin antimicrobial peptides from the freshwater crayfish *Pacifastacus leniusculus*. *J Invertebr Pathol***104**: 234-238

Donpudsa, S., Tassanakajon, A. and Rimphanitchayakit, V. (2009) Domain inhibitory and bacteriostatic activities of the five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp *Penaeus monodon*. *Dev Comp Immunol***33**: 481-488

Doughtie, D.G. and Rao, K.R. (1983) Ultrastructural and histological study of degenerative changes in the antennal glands, hepatopancreas, and midgut of grass shrimp exposed to two dithiocarbamate biocides. *J Invertebr Pathol***41**: 281-300

Doughtie, D.G. and Rao, K.R. (1984) Histopathological and ultrastructural changes in the antennal gland, midgut, hepatopancreas, and gill of grass shrimp following exposure to hexavalent chromium. *J Invertebr Pathol***43**: 89-108

Duangsuwan, P., Phoungpetchara, I., Tinikul, Y., Poljaroen, J., Wanichanon, C. and Sobhon, P. (2008a) Histological and three dimensional organizations of lymphoid tubules in normal lymphoid organ of *Penaeus monodon*. *Fish Shellfish Immunol***24**: 426-435

Duangsuwan, P., Tinikul, Y., Chotwiwatthanakun, C., Vanichviriyakit, R. and Sobhon, P. (2008b) Changes in the histological organization and spheroid formation in lymphoid organ of *Penaeus monodon* infected with yellow head virus. *Fish Shellfish Immunol***25**: 560-569

Duca, L., Floquet, N., Alix, A.J.P., Haye, B. and Debelle, L. (2004) Elastin as a matrikine. *Crit Rev Oncol/Hematol***49**: 235-244

Duguid, J.R. and Dinauer, M.C. (1990) Library subtraction of in vitro cDNA libraries to identify differentially expressed genes in scrapie infection. *Nucleic Acids Res***18**: 2789-2792

Duguid, J.R., Rohwer, R.G. and Seed, B. (1988) Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization of a cDNA library. *Proc Natl Acad Sci USA***85**: 5738-5742

Dutcher, S.K. (2001) The tubulin fraternity: alpha to eta. *Curr Opin Cell Biol***13**: 49-54

Edgerton, B., Webb, R. and Wingfield, M. (1997) A systemic parvo-like virus in the freshwater crayfish *Cherax destructor*. *Dis Aquat Org***29**: 73-78

Edgerton, B.F., Webb, R., Anderson, I.G. and Kulpa, E.C. (2000) Description of a presumptive hepatopancreatic reovirus, and a putative gill parvovirus, in the freshwater crayfish *Cherax quadricarinatus*. *Dis Aquat Org***41**: 83-90

Efthymiadis, A., Shao, H., Hübner, S. and Jans, D.A. (1997) Kinetic characterization of the human retinoblastoma protein bipartite nuclear localization sequence (NLS) *in vivo* and *in vitro:* a comparison with the SV40 large T-antigen NLS. *J Biol Chem***272**: 22134-22139

Egusa, S., Takahashi, Y., Itami, T. and Momoyama, K. (1988) Histopathology of vibriosis in the kuruma prawn *Penaeus japonicus* Bate. *Fish Pathol***23**: 59 - 65

Elliott, R.M. (2008) Bunyaviruses: General Features. In: B.W.J. Mahy and M.H.V. van Regenmortel (Eds), Encyclopedia of Virology (Third Edition), pp. 390-399. Academic Press, Oxford.

Emerman, M. and Malik, H.S. (2010) Paleovirology—modern consequences of ancient viruses. *PLoS Biol***8**: e1000301

Fagutao, F.F., Yasuike, M., Santos, M.D., Ruangpan, L., Sangrunggruang, K., Tassanakajon, A., Takahashi, Y., Ueno, R., Kondo, H., Hirono, I. and Aoki, T. (2009) Differential gene expression in black tiger shrimp, *Penaeus monodon*, following administration of oxytetracycline and oxolinic acid. *Dev Comp Immunol***33**: 1088-1092

Fall, J., Kono, T., Tanekhy, M., Itami, T. and Sakai, M. (2010) Expression of innate immune-related genes of Kuruma shrimp, *Marsupenaeus japonicus*, after challenge with *Vibrio nigripulchritudoAfr J Microbiol Res***4**: 2426-2433

Fang, D.-A., Wang, Q., He, L., Wang, J. and Wang, Y. (2012) Characterization of heat shock protein 70 in the red claw crayfish (*Cherax quadricarinatus*): Evidence for its role in regulating spermatogenesis. *Gene***492**: 138-147

FAO. (2012a) FAO Yearbook 2010, Fishery and Aquaculture Statistics. Food and Agriculture Organisation of the United Nations, Rome.

FAO. (2012b) FAO Yearbook 2010, Fishery and Aquaculture Statistics: Aquaculture Production. Food and Agriculture Organization of the United Nation, Rome.

FAO. (2012c) Fishery Statistical Colections: Global Aquaculture Production. Fisheries and Aquaculture Department, Food and Agriculture Organization of the United Nations, Rome.

FAO. (2012d) The State of World Fisheries and Aquaculture. FAO Fisheries and Aquaculture Department, Food and Agriculture Organization of the United Nations, Rome.

Feder, M.E. and Hofmann, G.E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annu Rev Physiol***61**: 243-282

Fenouil, E. and Roch, P. (1991) Evidence and characterization of lysozyme in six species of freshwater crayfish from astacidae and cambaridae families. *Comp Biochem Physiol B***99**: 43-49

Feschotte, C. (2010) Bornavirus enters the genome. *Nature***463**: 39-40

Feschotte, C. and Gilbert, C. (2012) Endogenous viruses: insights into viral evolution and impact on host biology. *Nat Rev Genet***13**: 283-296

Fine, A., Irihimovitch, V., Dahan, I., Konrad, Z. and Eichler, J. (2006) Cloning, expression, and purification of functional sec11a and sec11b, type I signal peptidases of the archaeon *Haloferax volcanii*. *J Bacteriol***188**: 1911-1919

Flegel, T. (2009) Hypothesis for heritable, anti-viral immunity in crustaceans and insects. *Biol Dir***4**: 32

Flegel, T. and Sritunyalucksana, K. (2011) Shrimp Molecular Responses to Viral Pathogens. *Mar Biotechnol***13**: 587-607

Flegel, T.W. (2007) Update on viral accommodation, a model for host-viral interaction in shrimp and other arthropods. *Dev Comp Immunol***31**: 217-231

Flegel, T.W. (2012) Historic emergence, impact and current status of shrimp pathogens in Asia. *J Invertebr Pathol***110**: 166-173

Flegel, T.W., Lightner, D.V., Lo, C.F. and Owens, L. (2008) Shrimp diseases control: past, present and future. In: M.G. Bondad-Reantaso, C.V. Mohan, M. Crumlish and R.P. Subasinghe (Eds), Diseases in Asian Aquaculture. Vol. VI. Fish Health Section, Asian Fisheries Society, Manila.

Flegel, T.W. and Pasharawipas, T. (1998) Active Viral Accommodation: A New Concept for Crustacean Response to Viral Pathogens. In: T.W. Flegel (Ed), Advances in Shrimp Biotechnology. National Centre for Genetic Engineering and Biotechnology, Bangkok.

Fort, P., Albertini, A., Van-Hua, A., Berthomieu, A., Roche, S., Delsuc, F., Pasteur, N., Capy, P., Gaudin, Y. and Weill, M. (2012) Fossil rhabdoviral sequences integrated into Arthropod genomes: Ontogeny, evolution, and potential functionality. *Mol Biol Evol***29**: 381-390

Foster, J.A. (2004) Elastin. In: J.L. William and M.D. Lane (Eds), Encyclopedia of Biological Chemistry, pp. 10-12. Elsevier, New York.

Frank, A.C. and Wolfe, K.H. (2009) Evolutionary capture of viral and plasmid DNA by yeast nuclear chromosomes. *Eukaryot Cell***8**: 1521-1531

Fraser, C.A. and Owens, L. (1996) Spawner-isolated mortality virus from Australian *Penaeus monodon*. *Dis Aquat Org***27**: 141-148

Freire, C.A., Onken, H. and McNamara, J.C. (2008) A structure-function analysis of ion transport in crustacean gills and excretory organs. *Comp Biochem Physiol A***151**: 272-304

Fritzler, M.J., Rattner, J.B., Luft, L.M., Edworthy, S.M., Casiano, C.A., Peebles, C. and Mahler, M. (2011) Historical perspectives on the discovery and elucidation of autoantibodies to centromere proteins (CENP) and the emerging importance of antibodies to CENP-F. *Autoimmun Rev***10**: 194-200

Fülöp, T. and Larbi, A. (2002) Putative role of 67 kDa elastin-laminin receptor in tumor invasion. *Semin Cancer Biol***12**: 219-229

Gandin, V., Miluzio, A., Barbieri, A.M., Beugnet, A., Kiyokawa, H., Marchisio, P.C. and Biffo, S. (2008) Eukaryotic initiation factor 6 is rate-limiting in translation, growth and transformation. *Nature***455**: 684-8

García, J.C., Reyes, A., Salazar, M. and Granja, C.B. (2009) Differential gene expression in White Spot Syndrome Virus (WSSV)-infected naïve and previously challenged Pacific white shrimp *Penaeus* (*Litopenaeus*) *vannamei*. *Aquaculture***289**: 253-258

Ghorbel, M.T. and Murphy, D. (2011) Suppression Subtractive Hybridization. In: A. Merighi (Ed), Neuropeptides: Methods and Protocols, Vol. 789. pp. 237-259.

Gifford, R. and Tristem, M. (2003) The evolution, distribution and diversity of endogenous retroviruses. *Virus Genes***26**: 291-315

Gonçalves-Soares, D., Seiffert, W.Q., Schlindwein, A.D., Toledo-Silva, G., Zanette, J., Marques, M.R.F. and Bainy, A.C.D. (2012) Identification of differentially transcribed genes in shrimp *Litopenaeus vannamei* exposed to osmotic stress and challenged with WSSV virus. *Comp Biochem Physiol D***7**: 73-81

Graidist, P., Fujise, K., Wanna, W., Sritunyalucksana, K. and Phongdara, A. (2006) Establishing a role for shrimp fortilin in preventing cell death. *Aquaculture***255**: 157-164

Griffith, D.R.W. and Wigglesworth, J.M. (1993) Growth rhytms in the shrimp *Penaeus vannamei* and *P. schimitti*. *Mar Biol***115**: 295-299

Griffiths, D. (2001) Endogenous retroviruses in the human genome sequence. *Gen Biol***2**: reviews1017.1 - reviews1017.5

Gross, P.S., Bartlett, T.C., Browdy, C.L., Chapman, R.W. and Warr, G.W. (2001) Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific white shrimp, *Litopenaeus vannamei*, and the Atlantic white shrimp, *L. setiferus*. *Dev Comp Immunol***25**: 565-577

Gui, L., Wang, B., Li, F.-H., Sun, Y.-M., Luo, Z. and Xiang, J.-H. (2012) Blocking the large extracellular loop (LEL) domain of FcTetraspanin-3 could inhibit the infection of white spot syndrome virus (WSSV) in Chinese shrimp, *Fenneropenaeus chinensis*. *Fish Shellfish Immunol***32**: 1008-1015

Gurskaya, N.G., Diatchenko, L., Chenchik, A., Siebert, P.D., Khaspekov, G.L., Lukyanov, K.A., Vagner, L.L., Ermolaeva, O.D., Lukyanov, S.A. and Sverdlov, E.D. (1996) Equalizing cDNA subtraction based on selective suppression of polymerase chain reaction: Cloning of jurkat cell transcripts induced by phytohemaglutinin and phorbol 12-myristate 13-acetate. *Anal Biochem***240**: 90-97

Han-Ching Wang, K.C., Kondo, H., Hirono, I. and Aoki, T. (2010) The *Marsupenaeus japonicus* voltage-dependent anion channel (MjVDAC) protein is involved in white spot syndrome virus (WSSV) pathogenesis. *Fish Shellfish Immunol***29**: 94-103

Hara, E., Kato, T., Nakada, S., Sekiya, S. and Oda, K. (1991) Subtractive cDNA cloning using oligo(dt)30-latex and PCR: isolation of cDNA clones specific

to undifferentiated human embryonal carcinoma cells. *Nucleic Acids Res***19**: 7097-7104

Hara, E., Yamaguchi, T., Tahara, H., Tsuyama, N., Tsurui, H., Ide, T. and Oda, K. (1993) DNA-DNA subtractive cDNA cloning using oligo(dT)30-latex and PCR: Identification of cellular genes which are overexpressed in senescent human diploid fibroblasts. *Anal Biochem***214**: 58-64

Harper, G., Hull, R., Lockhart, B. and Olszewski, N. (2002) Viral sequences integrated into plant genomes. *Ann Rev Phytopathol***40**: 119-119

Hasson, K.W., Lightner, D.V., Mohney, L.L., Redman, R.M., Poulos, B.T. and White, B.M. (1999a) Taura syndrome virus (TSV) lesion development and the disease cycle in the Pacific white shrimp *Penaeus vannamei*. *Dis Aquat Org***36**: 81-93

Hasson, K.W., Lightner, D.V., Mohney, L.L., Redman, R.M. and White, B.M. (1999b) Role of lymphoid organ spheroids in chronic Taura syndrome virus (TSV) infections in *Penaeus vannamei*. *Dis Aquat Org***38**: 93-105

Hasson, K.W., Lightner, D.V., Poulos, B.T., Redman, R.M., White, B.L., Brock, J.A. and Bonami, J.R. (1995) Taura syndrome in *Penaeus vannamei*: Demonstration of a viral etiology. *Dis Aquat Org***23**: 115-126

Hastings, K.T. and Cresswell, P. (2011) Disulfide reduction in the endocytic pathway: Immunological functions of gamma-interferon-inducible lysosomal thiol reductase. *Antioxid Redox Signaling***15**: 657-668

Hauton, C. and Smith, V.J. (2007) Adaptive immunity in invertebrates: A straw house without a mechanistic foundation. *BioEssays***29**: 1138-1146

Hawkins, T.B., Dantzer, J., Peters, B., Dinauer, M., Mockaitis, K., Mooney, S. and Cornetta, K. (2011) Identifying viral integration sites using SeqMap 2.0. *Bioinformatics*

He, F. and Kwang, J. (2008) Identification and characterization of a new E3 ubiquitin ligase in white spot syndrome virus involved in virus latency. *Virol J***5**: 151

He, F., Syed, S.M., Hameed, A.S.S. and Kwang, J. (2009) Viral ubiquitin ligase WSSV222 is required for efficient white spot syndrome virus replication in shrimp. *J Gen Virol***90**: 1483-1490

He, N., Liu, H. and Xu, X. (2004) Identification of genes involved in the response of haemocytes of *Penaeus japonicus* by suppression subtractive hybridization (SSH) following microbial challenge. *Fish Shellfish Immunol***17**: 121-128

He, N., Qin, Q. and Xu, X. (2005) Differential profile of genes expressed in hemocytes of white spot syndrome virus-resistant shrimp (*Penaeus japonicus*) by combining suppression subtractive hybridization and differential hybridization. *Antivir Res***66**: 39-45

Hedrick, S.M., Cohen, D.I., Nielsen, E.A. and Davis, M.M. (1984) Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature***308**: 149-153

Hernández, G., Lalioti, V., Vandekerckhove, J., Sierra, J.M. and Santarén, J.F. (2004) Identification and characterization of the expression of the translation initiation factor 4A (eIF4A) from *Drosophila melanogaster*. *Proteamics* **4**: 316-326

Hernández, G. and Vazquez-Pianzola, P. (2005) Functional diversity of the eukaryotic translation initiation factors belonging to eIF4 families. *Mech Dev***122**: 865-876

Hikima, S., Hikima, J.-i., Rojtinnakorn, J., Hirono, I. and Aoki, T. (2003) Characterization and function of kuruma shrimp lysozyme possessing lytic activity against *Vibrio* species. *Gene***316**: 187-195

Hild, G., Bugyi, B. and Nyitrai, M. (2010) Conformational dynamics of actin: Effectors and implications for biological function. *Cytoskeleton***67**: 609-629

Holmes, E.C. (2011) The evolution of endogenous viral elements. *Cell Host Microbe***10**: 368-377

Homvises, T., Tassanakajon, A. and Somboonwiwat, K. (2010) *Penaeus monodon* SERPIN, PmSERPIN6, is implicated in the shrimp innate immunity. *Fish Shellfish Immunol***29**: 890-898

Horie, M., Honda, T., Suzuki, Y., Kobayashi, Y., Daito, T., Oshida, T., Ikuta, K., Jern, P., Gojobori, T., Coffin, J.M. and Tomonaga, K. (2010) Endogenous non-retroviral RNA virus elements in mammalian genomes. *Nature***463**: 84-87

Hose, J.E., Martin, G.G., Tiu, S. and McKrell, N. (1992) Patterns of hemocyte production and release throughout the molt cycle in the penaeid shrimp *Sicyonia ingentis*. *Biol Bull***183**: 185-199

Hossler, F.E. (2003) Vascular corrosion casting. *Microsc Microanal***9**: 1578-1579

Hsiao, C.-Y. and Song, Y.-L. (2010) A long form of shrimp astakine transcript: Molecular cloning, characterization and functional elucidation in promoting hematopoiesis. *Fish Shellfish Immunol***28**: 77-86

Hu, H. and Li, X. (2007) Transcriptional regulation in eukaryotic ribosomal protein genes. *Genomics***90**: 421-423

Hu, S.-Y., Huang, J.-H., Huang, W.-T., Yeh, Y.-H., Chen, M.H.-C., Gong, H.-Y., Chiou, T.-T., Yang, T.-H., Chen, T.T., Lu, J.-K. and Wu, J.-L. (2006) Structure and function of antimicrobial peptide penaeidin-5 from the black tiger shrimp *Penaeus monodon*. *Aquaculture***260**: 61-68

Huang, S.-W., Lin, Y.-Y., You, E.-M., Liu, T.-T., Shu, H.-Y., Wu, K.-M., Tsai, S.-F., Lo, C.-F., Kou, G.-H., Ma, G.-C., Chen, M., Wu, D., Aoki, T., Hirono, I. and Yu, H.-T. (2011) Fosmid library end sequencing reveals a rarely known genome structure of marine shrimp *Penaeus monodon*. *BMC Genomics***12**: 242

Huang, T., Xu, D. and Zhang, X. (2012) Characterization of shrimp Drosha in virus infection. *Fish Shellfish Immunol***33**: 575-581

Huang, X., Li, Y., Niu, Q. and Zhang, K. (2007) Suppression Subtractive Hybridization (SSH) and its modifications in microbiological research. *Appl Microbiol Biotechnol***76**: 753-760

Hwa, V., Oh, Y. and Rosenfeld, R.G. (1999) The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev***20**: 761-87

Ilbäck, N.-G., Glynn, A.W., Wikberg, L., Netzel, E. and Lindh, U. (2004) Metallothionein is induced and trace element balance changed in target organs of a common viral infection. *Toxicology***199**: 241-250

Inada, M., Mekata, T., Sudhakaran, R., Okugawa, S., Kono, T., El Asely, A.M., Linh, N.T.H., Yoshida, T., Sakai, M., Yui, T. and Itami, T. (2010) Molecular cloning and characterization of the nitric oxide synthase gene from kuruma shrimp, *Marsupenaeus japonicus*. *Fish Shellfish Immunol***28**: 701-711

Inada, M., Sudhakaran, R., Kihara, K., Nishi, J., Yoshimine, M., Mekata, T., Kono, T., Sakai, M., Yoshida, T. and Itami, T. (2012) Molecular cloning and characterization of the NADPH oxidase from the kuruma shrimp, *Marsupenaeus japonicus*: Early gene up-regulation after *Vibrio penaeicida* and poly(I:C) stimulations in vitro. *Mol Cell Prob***26**: 29-41

Jaenisch, R. (1976) Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. *Proc Natl Acad Sci USA***73**: 1260-1264

James, R., Thampuran, N., Lalitha, K.V., Rajan, L.A. and Joseph, T.C. (2010) Differential gene expression profile of the hepatopancreas of white spot syndrome virus infected *Fenneropenaeus indicus* by suppression subtractive hybridization. *Fish Shellfish Immunol***29**: 884-889

Jankowsky, E. (2011) RNA helicases at work: binding and rearranging. *Trends Biochem Sci***36**: 19-29

Jans, D.A., Xiao, C.-Y. and Lam, M.H.C. (2000) Nuclear targeting signal recognition: a key control point in nuclear transport? *BioEssays***22**: 532-544

Jaree, P., Tassanakajon, A. and Somboonwiwat, K. (2012) Effect of the anti-lipopolysaccharide factor isoform 3 (ALFPm3) from *Penaeus monodon* on *Vibrio harveyi* cells. *Dev Comp Immunol***38**: 554-560

Ji, W., Wright, M., Cai, L., Flament, A. and Lindpaintner, K. (2002) Efficacy of SSH PCR in isolating differentially expressed genes. *BMC Genomics***3**: 1-7

Jiravanichpaisal, P. and Miyazaki, T. (1994) Histopathology, biochemistry, and pathogenicity of *Vibrio harveyi* infecting black tiger prawn *Penaeus monodon*. *J Aquat Anim Health***6**: 27 - 35

Jolesch, A., Elmer, K., Bendz, H., Issels, R.D. and Noessner, E. (2011) Hsp70, a messenger from hyperthermia for the immune system. *Eur J Cell Biol***91**: 48-52

Jolles, P. and Jolles, J. (1984) What's new in lysozyme research? Always a model system, today as yesterday. *Mol Cell Biochem***63**: 165-189

Jose, S., Jayesh, P., Sudheer, N.S., Poulose, G., Mohandas, A., Philip, R. and Bright Singh, I.S. (2012) Lymphoid organ cell culture system from *Penaeus monodon* (Fabricius) as a platform for white spot syndrome virus and shrimp immune-related gene expression. *J Fish Dis***35**: 321-334

Joseph, P., Lei, Y.-X. and Ong, T.-m. (2004) Up-regulation of expression of translation factors – a novel molecular mechanism for cadmium carcinogenesis. *Mol Cell Biochem***255**: 93-101

Junkunlo, K., Barvornpinyo, S., Flegel, T.W. and Sritunyalucksana, K. (2010) Characterization of putative immune genes in yellow head virus (YHV) infected shrimp (*Penaeus vannamei*) by suppression subtractive hybridization (SSH) technique. The 48th Kasetsart University Annual Conference, 3-5 March, 2010.

Junkunlo, K., Prachumwat, A., Tangprasittipap, A., Senapin, S., Borwornpinyo, S., Flegel, T.W. and Sritunyalucksana, K. (2012) A novel lectin domain-containing protein (LvCTLD) associated with response of the whiteleg shrimp *Penaeus* (*Litopenaeus*) *vannamei* to yellow head virus (YHV). *Dev Comp Immunol***37**: 334-341

Kaeodee, M., Pongsomboon, S. and Tassanakajon, A. (2011) Expression analysis and response of *Penaeus monodon* 14-3-3 genes to salinity stress. *Comp Biochem Physiol B***159**: 244-251

Kapoor, A., Simmonds, P. and Lipkin, W.I. (2010) Discovery and characterization of mammalian endogenous parvoviruses. *J Virol***84**: 12628-12635

Katzourakis, A. and Gifford, R.J. (2010) Endogenous viral elements in animal genomes. *PLoS Genet***6**: e1001191

Kawaguchi, Y., Bruni, R. and Roizman, B. (1997) Interaction of herpes simplex virus 1 alpha regulatory protein ICP0 with elongation factor 1delta: ICP0 affects translational machinery. *J Virol***71**: 1019-1024

Kerscher, S., Dröse, S., Zickermann, V. and Brandt, U. (2008) The Three Families of Respiratory NADH Dehydrogenases

Bioenergetics. *Results Probl Cell Differ***45**: 185-222

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J. and Gibson, L. (2008) Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture***274**: 1-14

Khalimonchuk, O. and Rödel, G. (2005) Biogenesis of cytochrome c oxidase. *Mitochondrion***5**: 363-388

Khimmakthong, U., Deachamag, P., Phongdara, A. and Chotigeat, W. (2011) Stimulating the immune response of *Litopenaeus vannamei* using the phagocytosis activating protein (PAP) gene. *Fish Shellfish Immunol***31**: 415-422

Kiss, C., Nishikawa, J., Dieckmann, A., Takada, K., Klein, G. and Szekely, L. (2003) Improved subtractive suppression hybridization combined with high

density cDNA array screening identifies differentially expressed viral and cellular genes. *J Virol Methods***107**: 195-203

Klein, D.J., Moore, P.B. and Steitz, T.A. (2004) The roles of ribosomal proteins in the structure assembly, and evolution of the large ribosomal subunit. *J Mol Biol***340**: 141-177

Koetting, J., Unger, C. and Eibl, H. (1987) A continuous assay forO-alkylglycerol monooxygenase (E.C. 1.14.16.5). *Lipids***22**: 824-830

Kondo, M., Itami, T., Takahashi, Y., Fujii, R. and Tomonaga, S. (1994) Structure and function of the lymphoid organ in the kuruma prawn. *Dev Comp Immunol***18 Supplement 1**: S109

Kongton, K., Phongdara, A., Tonganunt-Srithaworn, M. and Wanna, W. (2011) Molecular cloning and expression analysis of the interferon-γ-inducible lysosomal thiol reductase gene from the shrimp *Penaeus monodon*. *Mol Biol Rep***38**: 3463-3470

Kötting, J., Unger, C. and Eibl, H. (1987) Substrate specificity ofO-alkylglycerol monooxygenase (E.C. 1.14.16.5), solubilized from rat liver microsomes. *Lipids***22**: 831-835

Koyama, T., Asakawa, S., Katagiri, T., Shimizu, A., Fagutao, F., Mavichak, R., Santos, M., Fuji, K., Sakamoto, T., Kitakado, T., Kondo, H., Shimizu, N., Aoki, T. and Hirono, I. (2010) Hyper-expansion of large DNA segments in the genome of kuruma shrimp, *Marsupenaeus japonicus*. *BMC Genomics***11**: 141

Kuballa, A.V., Guyatt, K., Dixon, B., Thaggard, H., Ashton, A.R., Paterson, B., Merritt, D.J. and Elizur, A. (2007) Isolation and expression analysis of multiple isoforms of putative farnesoic acid O-methyltransferase in several crustacean species. *Gen Comp Endocrinol***150**: 48-58

Kunecki, M. and Nawrocka, A. (2001) Elastin–laminin receptor and abdominal aortic aneurysms. New subject to study? A review. *Pathol Biol***49**: 333-338

La Fauce, K. and Owens, L. (2012) RNA Interference with Special Reference to Combating Viruses of Crustacea. *Indian J Virol*: 1-18

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

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

La Fauce, K.A. and Owens, L. (2009) RNA interference reduces *Pmerg*DNV expression and replication in an *in vivo* cricket model. *J Invertebr Pathol***100**: 111-115

Labat-Moleur, F., Guillermet, C., Lorimier, P., Robert, C., Lantuejoul, S., Brambilla, E. and Negoescu, A. (1998) TUNEL apoptotic cell detection in tissue

sections: critical evaluation and improvement. *J Histochem Cytochem***46**: 327-334

Lai, M.-D. and Xu, J. (2007) Ribosomal proteins and colorectal cancer. *Curr Genomics***8**: 43-49

Lapis, K. and Tímár, J. (2002) Role of elastin-matrix interactions in tumor progression. *Semin Cancer Biol***12**: 209-217

Le Moullac, G., Le Groumellec, M., Ansquer, D., Froissard, S., Levy, P. and Aquacop. (1997) Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: protection against vibriosis. *Fish Shellfish Immunol***7**: 227 - 234

Leelatanawit, R., Klinbunga, S., Aoki, T., Hirono, I., Valyasevi, R. and Menasveta, P. (2008) Suppression subtractive hybridization (SSH) for isolation and characterization of genes to testicular development in the giant tiger shrimp *Penaeus monodon*. *BMB reports***41**: 796 - 802

Leu, J.-H., Chang, C.-C., Wu, J.-L., Hsu, C.-W., Hirono, I., Aoki, T., Juan, H.-F., Lo, C.-F., Kou, G.-H. and Huang, H.-C. (2007) Comparative analysis of differentially expressed genes in normal and white spot syndrome virus infected *Penaeus monodon*. *BMC Genomics***8**: 120

Leu, J.-H., Chen, Y.-C., Chen, L.-L., Chen, K.-Y., Huang, H.-T., Ho, J.-M. and Lo, C.-F. (2012) *Litopenaeus vannamei* inhibitor of apoptosis protein 1 (LvIAP1) is essential for shrimp survival. *Dev Comp Immunol***38**: 78-87

Leung, P.S.C., Chow, W.K., Duffey, S., Kwan, H.S., Gershwin, M.E. and Chu, K.H. (1996) IgE reactivity against a cross-reactive allergen in crustacea and mollusca: Evidence for tropomyosin as the common allergen. *J Allergy Clin Immunol***98**: 954-961

Levy-Rimler, G., Bell, R.E., Ben-Tal, N. and Azem, A. (2002) Type I chaperonins: not all are created equal. *FEBS Letters***529**: 1-5

Li, F., Wang, D., Li, S., Yan, H., Zhang, J., Wang, B., Zhang, J. and Xiang, J. (2010a) A Dorsal homolog (FcDorsal) in the Chinese shrimp *Fenneropenaeus chinensis* is responsive to both bacteria and WSSV challenge. *Dev Comp Immunol***34**: 874-883

Li, F., Yan, H., Wang, D., Priya, T.A.J., Li, S., Wang, B., Zhang, J. and Xiang, J. (2009a) Identification of a novel relish homolog in Chinese shrimp *Fenneropenaeus chinensis* and its function in regulating the transcription of antimicrobial peptides. *Dev Comp Immunol***33**: 1093-1101

Li, J., Chen, P., Liu, P., Gao, B., Wang, Q. and Li, J. (2010b) The cytosolic manganese superoxide dismutase cDNA in swimming crab *Portunus trituberculatus*: Molecular cloning, characterization and expression. *Aquaculture***309**: 31-37

Li, X.-C., Wang, X.-W., Wang, Z.-H., Zhao, X.-F. and Wang, J.-X. (2009b) A three-domain Kazal-type serine proteinase inhibitor exhibiting domain inhibitory

and bacteriostatic activities from freshwater crayfish *Procambarus clarkii*. *Dev Comp Immunol***33**: 1229-1238

Li, Y.-C., Korol, A.B., Fahima, T. and Nevo, E. (2004) Microsatellites within genes: Structure, function, and evolution. *Mol Biol Evol***21**: 991-1007

Lightner, D.V. and Brock, J.A. (1987) A lymphoma-like neoplasm arising from hematopoietic tissue in the white shrimp, *Penaeus vannamei* Boone (Crustacea: Decapoda). *J Invertebr Pathol***49**: 188-193

Lightner, D.V., Hendrick, R.P., Freyer, J.L., Chen, S.N., Liao, I.C. and Kou, G.H. (1987) A survey of cultured penaeid shrimp in Taiwan for viral and other important diseases. *Fish Pathol***22**: 127 - 140

Lightner, D.V. and Redman, R.M. (1985) A parvo-like virus disease of penaeid shrimp. *J Invertebr Pathol***45**: 47-53

Lightner, D.V., Redman, R.M., Pantoja, C.R., Tang, K.F.J., Noble, B.L., Schofield, P., Mohney, L.L., Nunan, L.M. and Navarro, S.A. (2012) Historic emergence, impact and current status of shrimp pathogens in the Americas. *J Invertebr Pathol***110**: 174-183

Lin, C.-L., Lee, J.-C., Chen, S.-S., Alan Wood, H., Li, M.-L., Li, C.-F. and Chao, Y.-C. (1999) Persistent Hz-1 virus infection in insect cells: evidence for insertion of viral DNA into host chromosomes and viral infection in a latent status. *J Virol***73**: 128-139

Lin, S.-C., Liou, C.-H. and Cheng, J.-H. (2000) The role of the antennal glands in ion and body volume regulation of cannulated *Penaeus monodon* reared in various salinity conditions. *Comp Biochem Physiol A***127**: 121-129

Lin, S.-J., Hsia, H.-L., Liu, W.-J., Huang, J.-Y., Liu, K.-F., Chen, W.-Y., Yeh, Y.-C., Huang, Y.-T., Lo, C.-F., Kou, G.-H. and Wang, H.-C. (2012) Spawning stress triggers WSSV replication in brooders via the activation of shrimp STAT. *Dev Comp Immunol***38**: 128-135

Lin, Y.-C., Lee, F.-F., Wu, C.-L. and Chen, J.-C. (2010) Molecular cloning and characterization of a cytosolic manganese superoxide dismutase (cytMnSOD) and mitochondrial manganese superoxide dismutase (mtMnSOD) from the kuruma shrimp *Marsupenaeus japonicus*. *Fish Shellfish Immunol***28**: 143-150

Lin, Y.-R., Hung, H.-C., Leu, J.-H., Wang, H.-C., Kou, G.-H. and Lo, C.-F. (2011) The role of aldehyde dehydrogenase and Hsp70 in suppression of white spot syndrome virus replication at high temperature. *J Virol***85**: 3517-3525

Lindström, M.S. (2009) Emerging functions of ribosomal proteins in gene-specific transcription and translation. *Biochem Biophys Res Commun***379**: 167-170

Littik, S.A.M. (2003) Quantitative histopathology and epidemiology of prawn viral diseases. PhD thesis, James Cook University.

Liu, C.-H., Yeh, S.-T., Cheng, S.-Y. and Chen, J.-C. (2004) The immune response of the white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio*infection in relation with the moult cycle. *Fish Shellfish Immunol***16**: 151-161

Liu, H.-p., Chen, R.-y., Zhang, Q.-x., Peng, H. and Wang, K.-j. (2011a) Differential gene expression profile from haematopoietic tissue stem cells of red claw crayfish, *Cherax quadricarinatus*, in response to WSSV infection. *Dev Comp Immunol***35**: 716-724

Liu, H., Fu, Y., Jiang, D., Li, G., Xie, J., Cheng, J., Peng, Y., Ghabrial, S.A. and Yi, X. (2010a) Widespread horizontal gene transfer from double-stranded RNA viruses to eukaryotic nuclear genomes. *J Virol***84**: 11876-11887

Liu, H., Fu, Y., Xie, J., Cheng, J., Ghabrial, S.A., Li, G., Peng, Y., Yi, X. and Jiang, D. (2011b) Widespread endogenization of densoviruses and parvoviruses in animal and human genomes. *J Virol***85**: 9863-9876

Liu, H., Jiravanichpaisal, P., Söderhäll, I., Cerenius, L. and Söderhäll, K. (2006) Antilipopolysaccharide factor Interferes with white spot syndrome virus replication in vitro and in vivo in the crayfish *pacifastacus leniusculus*. *J Virol***80**: 10365-10371

Liu, H., Söderhäll, K. and Jiravanichpaisal, P. (2009a) Antiviral immunity in crustaceans. *Fish Shellfish Immunol***27**: 79-88

Liu, K.-F., Yeh, M.-S., Kou, G.-H., Cheng, W. and Lo, C.-F. (2010b) Identification and cloning of a selenium-dependent glutathione peroxidase from tiger shrimp, *Penaeus monodon*, and its transcription following pathogen infection and related to the molt stages. *Dev Comp Immunol***34**: 935-944

Liu, Y., Li, F., Wang, B., Dong, B., Zhang, X. and Xiang, J. (2009b) A serpin from Chinese shrimp *Fenneropenaeus chinensis* is responsive to bacteria and WSSV challenge. *Fish Shellfish Immunol***26**: 345-351

Loker, E.S., Adema, C.M., Zhang, S.-M. and Kepler, T.B. (2004) Invertebrate immune system - not homogeneous, not simple, not well understood. *Immunol Rev***198**: 10 - 24

Loongyai, W., Avarre, J.-C., Cerutti, M., Lubzens, E. and Chotigeat, W. (2007a) Isolation and functional characterization of a new shrimp ovarian peritrophin with antimicrobial activity from *Fenneropenaeus merguiensis*. *Mar Biotechnol***9**: 624-637

Loongyai, W., Phongdara, A. and Chotigeat, W. (2007b) Cloning and expression of a TCTP homolog from the ovaries of banana prawn. *Mar Biol***150**: 455-462

Lopata, A.L., O'Hehir, R.E. and Lehrer, S.B. (2010) Shellfish allergy. *Clin Exp Allergy***40**: 850-858

Low, W.-K., Dang, Y., Bhat, S., Romo, D. and Liu, J.O. (2007) Substrate-dependent targeting of eukaryotic translation initiation factor 4A by pateamine A: Negation of domain-Linker regulation of activity. *Chem Biol***14**: 715-727

Lu, K.-Y., Sung, H.-J., Liu, C.-L. and Sung, H.-H. (2009) Differentially enhanced gene expression in hemocytes from *Macrobrachium rosenbergii* challenged *in vivo* with lipopolysaccharide. *J Invertebr Pathol***100**: 9-15

Lu, Y., Tapay, L.M., Loh, P.C., Brock, J.A. and Gose, R.B. (1995) Distribution of yellow-head virus in selected tissues and organs of penaeid shrimp *Penaeus vannamei*. *Dis Aquat Org***23**: 67-70

Luana, W., Li, F., Wang, B., Zhang, X., Liu, Y. and Xiang, J. (2007) Molecular characteristics and expression analysis of calreticulin in Chinese shrimp *Fenneropenaeus chinensis*. *Comp Biochem Physiol B***147**: 482-491

Lundin, C.G. (1997) Global attempts to address shrimp disease. The 2nd Asia-Pacific Marine Biotechnology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology, 1-23.

Ma, H., Wang, B., Zhang, J., Li, F. and Xiang, J. (2010) Multiple forms of alpha-2 macroglobulin in shrimp *Fenneropenaeus chinesis* and their transcriptional response to WSSV or Vibrio pathogen infection. *Dev Comp Immunol***34**: 677-684

Mai, W.-j. and Hu, C.-q. (2009a) Molecular cloning, characterization, expression and antibacterial analysis of a lysozyme homologue from *Fenneropenaeus merguiensis*. *Mol Biol Rep***36**: 1587-1595

Mai, W.-j. and Wang, W.-n. (2010) Protection of blue shrimp (*Litopenaeus stylirostris*) against the White Spot Syndrome Virus (WSSV) when injected with shrimp lysozyme. *Fish Shellfish Immunol***28**: 727-733

Mai, W. and Hu, C. (2009b) cDNA cloning, expression and antibacterial activity of lysozyme C in the blue shrimp (*Litopenaeus stylirostris*). *Prog Nat Sci***19**: 837-844

Malik, H.S., Henikoff, S. and Eickbush, T.H. (2000) Poised for contagion: Evolutionary origins of the infectious abilities of invertebrate retroviruses. *Genome Res***10**: 1307-1318

Maningas, M.B.B., Kondo, H., Hirono, I., Saito-Taki, T. and Aoki, T. (2008a) Essential function of transglutaminase and clotting protein in shrimp immunity. *Mol Immunol***45**: 1269-1275

Maningas, M.B.B., Koyama, T., Kondo, H., Hirono, I. and Aoki, T. (2008b) A peroxiredoxin from kuruma shrimp, *Marsupenaeus japonicus*, inhibited by peptidoglycan. *Dev Comp Immunol***32**: 198-203

Maori, E., Tanne, E. and Sela, I. (2007) Reciprocal sequence exchange between non-retroviruses and hosts leading to the appearance of new host phenotypes. *Virology***362**: 342-349

Marchand, J., Leignel, V., Moreau, B. and Chénais, B. (2009) Characterization and sequence analysis of manganese superoxide dismutases from Brachyura (Crustacea: Decapoda): Hydrothermal Bythograeidae versus littoral crabs. *Comp Biochem Physiol B***153**: 191-199

Maret, W. (2009) Fluorescent probes for the structure and function of metallothionein. *J Chromatogr B***877**: 3378-3383

Martin, G.G., Hose, J.E. and Kim, J.J. (1987) Structure of hematopoietic nodules in the ridgeback prawn, *Sicyonia ingentis*: Light and electron microscopic observations. *J Morphol* **192**: 193-204

Martin, G.G., Hose, J.E., Minka, G. and Rosenberg, S. (1996) Clearance of bacteria injected into the hemolymph of the ridgeback prawn, *Sicyonia ingentis* (Crustacea: Decapoda): role of hematopoietic tissue. *J Morphol***227**: 227-233

Martínez-Pérez, F., Zinker, S., Aguilar, G., Valdés, J. and Aréchiga, H. (2005) Circadian oscillations of RPCH gene expression in the eyestalk of the crayfish *Cherax quadricarinatus*. *Peptides***26**: 2434-2444

Máximo, V., Lima, J., Soares, P. and Sobrinho-simões, M. (2009) Mitochondria and cancer. *Virchows Archiv***454**: 481-95

Mekata, T., Okugawa, S., Inada, M., Yoshimine, M., Nishi, J., Kono, T., Sakai, M., Itami, T. and Sudhakaran, R. (2011) Class B scavenger receptor, Croquemort from kuruma shrimp *Marsupenaeus japonicus*: Molecular cloning and characterization. *Mol Cell Prob***25**: 94-100

Mekata, T., Sudhakaran, R., Okugawa, S., Inada, M., Kono, T., Sakai, M. and Itami, T. (2010) A novel gene of tumor necrosis factor ligand superfamily from kuruma shrimp, *Marsupenaeus japonicus*. *Fish Shellfish Immunol***28**: 571-578

Miluzio, A., Beugnet, A., Volta, V. and Biffo, S. (2009) Eukaryotic initiation factor 6 mediates a continuum between 60S ribosome biogenesis and translation. *EMBO Rep***10**: 459-465

Mitchell, S.F. and Lorsch, J.R. (2008) Should I stay or should I go? Eukaryotic translation initiation factors 1 and 1A control start codon recognition. *J Biol Chem***283**: 27345-27349

Mohankumar, K. and Ramasamy, P. (2006) Activities of membrane bound phosphatases, transaminases and mitochondrial enzymes in white spot syndrome virus infected tissues of *Fenneropenaeus indicus*. *Virus Res***118**: 130-135

Moltó, E., Bonzón-Kulichenko, E., Gallardo, N. and Andrés, A. (2007) MTPA: A crustacean metallothionein that affects hepatopancreatic mitochondrial functions. *Arch Biochem Biophys***467**: 31-40

Mort, J.S. and Buttle, D.J. (1997) Cathepsin B. *Int J Biochem Cell Biol***29**: 715-720

Moss, S.M., Doyle, R.W. and Lightner, D.V. (2005) Breeding shrimp for disease resistance: Challenges and opportunities for improvement. In: P. Walker, R. Lester and M.G. Bondad-Reantaso (Eds), Diseases in Asian Aquaculture V, pp. 379-393. Fish Health Section, Asian Fisheries Society, Manila.

Muiznieks, L.D., Weiss, A.S. and Keeley, F.W. (2010) Structural disorder and dynamics of elastin. *Biochem Cell Biol***88**: 239-250

Munir, S., Singh, S., Kaur, K. and kapur, V. (2004) Suppression subtractive hybridization coupled with microarray analysis to examine differential expression of genes in virus infected cells. *Biol Proced Online***6**: 94-104

Murphy, D.B. (1991) Functions of tubulin isoforms. *Curr Opin Cell Biol***3**: 43-51

Nadala, E.C.B., Lu, Y., Loh, P.C. and Brock, J.A. (1992) Infection of *Penaeus stylirostris* (Boone) with a rhabdovirus isolated from *Penaeus* spp. *Gyobyo Kenkyu***27**: 143 - 147

Nakamura, K. (1987) Lymphoid organ and its developmental property of larval prawn *Penaeus japonicus*. *Mem Fac Fish, Kagoshima Universty***36**: 215 - 220

Nakata, P.A. and McConn, M.M. (2002) Sequential subtractive approach facilitates identification of differentially expressed genes. *Plant Physiol Biochem***40**: 307-312

Navarro, I., Leibush, B., Moon, T.W., Plisetskaya, E.M., Baños, N., Méndez, E., Planas, J.V. and Gutiérrez, J. (1999) Insulin, insulin-like growth factor-I (IGF-I) and glucagon: the evolution of their receptors. *Comp Biochem Physiol B***122**: 137-153

Nayak, S., Ajay, K.M., Ramaiah, N., Meena, R.M. and Sreepada, R.A. (2011) Profiling of a few immune responsive genes expressed in postlarvae of *Fenneropenaeus indicus* challenged with *Vibrio harveyi* D3. *J Invertebr Pathol***107**: 168-172

Nayak, S., Singh, S.K., Ramaiah, N. and Sreepada, R.A. (2010) Identification of upregulated immune-related genes in *Vibrio harveyi* challenged *Penaeus monodon* postlarvae. *Fish Shellfish Immunol***29**: 544-549

Nelson, J., McFerran, N.V., Pivato, G., Chambers, E., Doherty, C., Steele, D. and Timson, D.J. (2008) The 67 kDa laminin receptor: Structure, function and role in disease. *Biosci Rep***28**: 33-48

Nomura, T. and Katunuma, N. (2005) Involvement of cathepsins in the invasion, metastasis and proliferation of cancer cells. *J Med Inves***52**: 1-9

O'Leary, N.A., Trent, H.F., III, Robalino, J., Peck, M.E.T., McKillen, D.J. and Gross, P.S. (2006) Analysis of multiple tissue-specific cDNA libraries from the Pacific whiteleg shrimp, *Litopenaeus vannamei*. *Integr Comp Biol***46**: 931-939

Ogawa, K., Utsunomiya, T., Mimori, K., Tanaka, Y., Tanaka, F., Inoue, H., Murayama, S. and Mori, M. (2004) Clinical significance of elongation factor-1 delta mRNA expression in oesophageal carcinoma. *Br J Cancer***91**: 282-286

Ohta, A. and Nishiyama, Y. (2011) Mitochondria and viruses. *Mitochondrion***11**: 1-12

Oka, M. (1969) Studies on *Penaeus orientalis* KISHINOUYE-VIII structure of the newly found lymphoid organ. *Bull Jpn Soc Sci Fish***35**: 245 - 250

Okugawa, S., Mekata, T., Inada, M., Kihara, K., Shiki, A., Kannabiran, K., Kono, T., Sakai, M., Yoshida, T., Itami, T. and Sudhakaran, R. (2012) The SOCS and STAT from JAK/STAT signaling pathway of kuruma shrimp *Marsupenaeus japonicus*: Molecular cloning, characterization and expression analysis. *Mol Cell Prob***27**: 6-14

Owens, L., Beer, S.D. and Smith, J. (1991) Lymphoidal parvovirus-like particles in Australian penaeid prawns. *Dis Aquat Org***11**: 129 - 134

Owens, L. and Cullen, B.R. (2004) Development of diagnostic capability for priority aquatic animal diseases of national significant: Spawner isolated mortality virus. James Cook University and Fisheries Research and Development Corperation. Townsville

Owens, L., Liessmann, L., La Fauce, K., Nyguyen, T. and Zeng, C. (2010) Intranuclear bacilliform virus and hepatopancreatic parvovirus (PmergDNV) in the mud crab Scylla serrata (Forskal) of Australia. *Aquaculture***310**: 47-51

Owens, L. and McElnea, C. (2000) Natural infection of the redclaw crayfish *Cherax quadricarinatus* with presumptive spawner-isolated mortality virus. *Dis Aquat Org***40**: 219-223

Owens, L., McElnea, C., Snape, N., Harris, L. and Smith, M. (2003) Prevalence and effect of spawner-isolated mortality virus on the hatchery phases of *Penaeus monodon* and *P. merguiensis* in Australia. *Dis Aquat Org***53**: 101-106

Paetzel, M., Karla, A., Strynadka, N.C.J. and Dalbey, R.E. (2002) Signal Peptidases. *Chem Rev***102**: 4549-4580

Pamuru, R.R., Rosen, O., Manor, R., Chung, J.S., Zmora, N., Glazer, L., Aflalo, E.D., Weil, S., Tamone, S.L. and Sagi, A. (2012) Stimulation of molt by RNA interference of the molt-inhibiting hormone in the crayfish *Cherax quadricarinatus*. *Gen Comp Endocrinol***178**: 227-236

Pan, D., He, N., Yang, Z., Liu, H. and Xu, X. (2005) Differential gene expression profile in hepatopancreas of WSSV-resistant shrimp (*Penaeus japonicus*) by suppression subtractive hybridization. *Dev Comp Immunol***29**: 103-112

Park, J.H., Lee, Y.S., Lee, S. and Lee, Y. (1998) An infectious viral disease of penaeid shrimp newly found in Korea. *Dis Aquat Org***34**: 71-75

Passmore, L.A., Schmeing, T.M., Maag, D., Applefield, D.J., Acker, M.G., Algire, Mikkel A., Lorsch, J.R. and Ramakrishnan, V. (2007) The eukaryotic translation initiation factors eIF1 and eIF1A induce an open conformation of the 40S ribosome. *Mol Cell***26**: 41-50

Pauleau, A.-L. and Erhardt, S. (2011) Centromere regulation: New players, new rules, new questions. *Eur J Cell Biol***90**: 805-810

Perry, R.P. (2007) Balanced production of ribosomal proteins. *Gene***401**: 1-3

Phetrungnapha, A., Panyim, S. and Ongvarrasopone, C. (2011) A Tudor staphylococcal nuclease from *Penaeus monodon*: cDNA cloning and its involvement in RNA interference. *Fish Shellfish Immunol***31**: 373-380

Phongdara, A., Wanna, W. and Chotigeat, W. (2006) Molecular cloning and expression of caspase from white shrimp *Penaeus merguiensis*. *Aquaculture***252**: 114-120

Picard, M., Taivassalo, T., Gouspillou, G. and Hepple, R.T. (2011) Mitochondria: isolation, structure and function. *J Physiol***589**: 4413-4421

Pickart, C.M. and Eddins, M.J. (2004) Ubiquitin: structures, functions, mechanisms. *Biochim Biophys Acta***1695**: 55-72

Ploubidou, A. and Way, M. (2001) Viral transport and the cytoskeleton. *Curr Opin Cell Biol***13**: 97-105

Pongsomboon, S., Tang, S., Boonda, S., Aoki, T., Hirono, I. and Tassanakajon, A. (2011) A cDNA microarray approach for analyzing transcriptional changes in *Penaeus monodon* after infection by pathogens. *Fish Shellfish Immunol***30**: 439-446

Pongsomboon, S., Wongpanya, R., Tang, S., Chalorsrikul, A. and Tassanakajon, A. (2008) Abundantly expressed transcripts in the lymphoid organ of the black tiger shrimp, *Penaeus monodon*, and their implication in immune function. *Fish Shellfish Immunol***25**: 485-493

Ponprateep, S., Somboonwiwat, K. and Tassanakajon, A. (2009) Recombinant anti-lipopolysaccharide factor isoform 3 and the prevention of vibriosis in the black tiger shrimp, *Penaeus monodon*. *Aquaculture***289**: 219-224

Ponprateep, S., Tharntada, S., Somboonwiwat, K. and Tassanakajon, A. (2012) Gene silencing reveals a crucial role for anti-lipopolysaccharide factors from *Penaeus monodon* in the protection against microbial infections. *Fish Shellfish Immunol***32**: 26-34

Porkka, K.P. and Visakorpi, T. (2001) Detection of differentially expressed genes in prostate cancer by combining suppression subtractive hybridization and cDNA library array. *J Pathol***193**: 73-79

Pourang, N., Dennis, J.H. and Ghourchian, H. (2004) Tissue distribution and redistribution of trace elements in shrimp species with the emphasis on the roles of metallothionein. *Ecotoxicology***13**: 519-533

Prapavorarat, A., Pongsomboon, S. and Tassanakajon, A. (2010) Identification of genes expressed in response to yellow head virus infection in the black tiger shrimp, *Penaeus monodon*, by suppression subtractive hybridization. *Dev Comp Immunol***34**: 611-617

Preechaphol, R., Klinbunga, S., Khamnamtongand, B. and Menasveta, P. (2010) Isolation and characterization of genes functionally involved in ovarian development of the giant tiger shrimp *Penaeus monodon* by suppression subtractive hybridization (SSH). *Genet Mol Biol***33**: 676-685

Qiu, Y. and Michalak, M. (2009) Transcriptional control of the calreticulin gene in health and disease. *Int J Biochem Cell Biol***41**: 531-538

Rajendran, K.V., Cowley, J.A., McCulloch, R.J. and Walker, P.J. (2006) A TaqMan real-time RT-PCR for quantifying Mourilyan virus infection levels in penaeid shrimp tissues. *J Virol Methods***137**: 265-271

Rattanaporn, O. and Utarabhand, P. (2011) Molecular cloning of a C-type lectin with two CRD domains from the banana shrimp *Fenneropenaeus merguiensis*: Early gene up-regulation after *Vibrio harveyi* infection. *J Invertebr Pathol***106**: 196-204

Rebrikov, D.V., Britanova, O.V., Gurskaya, N.G., Lukyanov, K.A., Tarabykin, V.S. and Lukyanov, S.A. (2000) Mirror orientation selection (MOS): A method for eliminating false positive clones from libraries generated by suppression subtractive hybridization. *Nucleic Acids Res***28**: e90

Rebrikov, D.V., Desai, S.M., Siebert, P.D. and Lukyanov, S.A. (2004) Suppression Subtractive Hybridization. In: R.A. Shimkets (Ed), Gene Expression Profiling: Methods and Protocols, pp. 107-134. Vol. 258. Humana Press Inc., Totowa, NJ.

Ren, Q., Zhou, J., Sun, S.-S., Kang, C.-J., Zhao, X.-F. and Wang, J.-X. (2011) Molecular cloning and expression pattern analysis of two novel disulfide isomerases in shrimp. *Comp Biochem Physiol C***153**: 301-309

Reyes, A., Salazar, M. and Granja, C. (2007) Temperature modifies gene expression in subcuticular epithelial cells of white spot syndrome virus-infected *Litopenaeus vannamei*. *Dev Comp Immunol***31**: 23-29

Reynaud, Y., Saulnier, D., Mazel, D., Goarant, C. and Le Roux, F. (2008) Correlation between detection of a plasmid and high-level virulence of *Vibrio nigripulchritudo*, a pathogen of the shrimp *Litopenaeus stylirostris*. *Appl Environ Microbiol***74**: 3038-3047

Riis, B., Rattan, S.I., Clark, B.F. and Merrick, W.C. (1990) Eukaryotic protein elongation factors. *Trends Biochem Sci***15**: 420-424

Rittidach, W., Paijit, N. and Utarabhand, P. (2007) Purification and characterization of a lectin from the banana shrimp *Fenneropenaeus merguiensis* hemolymph. *Biochim Biophys Acta* **1770**: 106-114

Robalino, J., Almeida, J.S., McKillen, D., Colglazier, J., Trent, H.F., III, Chen, Y.A., Peck, M.E.T., Browdy, C.L., Chapman, R.W., Warr, G.W. and Gross, P.S. (2007a) Insights into the immune transcriptome of the shrimp *Litopenaeus vannamei*: Tissue-specific expression profiles and transcriptomic responses to immune challenge. *Physiol Genomics***29**: 44-56

Robalino, J., Bartlett, T.C., Chapman, R.W., Gross, P.S., Browdy, C.L. and Warr, G.W. (2007b) Double-stranded RNA and antiviral immunity in marine shrimp: Inducible host mechanisms and evidence for the evolution of viral counter-responses. *Dev Comp Immunol***31**: 539-547

Robalino, J., Browdy, C.L., Prior, S., Metz, A., Parnell, P., Gross, P. and Warr, G. (2004) Induction of Antiviral Immunity by Double-Stranded RNA in a Marine Invertebrate. *J Virol***78**: 10442-10448

Robinson, B.H. (2000) Human cytochrome oxidase deficiency. *Pediatr Res***48**: 581-585

Roch, P. (1999) Defense mechanism and disease prevention in farmed marine invertebrate. *Aquaculture***172**: 125 - 145

Rodríguez, J., Bayot, B., Amano, Y., Panchana, F., Blas, I.d., Alday, V. and Calderón, J. (2003) White spot syndrome virus infection in cultured *Penaeus*

vannamei (Boone) in Ecuador with emphasis on histopathology and ultrastructure. *J Fish Dis***26**: 439-450

Rodriguez, J. and Le Moullac, G. (2000) State of the art of immunological tools and health control of penaeid shrimp. *Aquaculture***191**: 109 - 119

Rodríguez, J., Ruiz, J., Maldonado, M. and Echeverría, F. (2012) Immunodetection of hemocytes, peneidins and α2-macroglobulin in the lymphoid organ of white spot syndrome virus infected shrimp. *Microbiol Immunol***56**: 562-571

Roesijadi, G. (1992) Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquat Toxicol***22**: 81-113

Roiz, D., Vazquez, A., Seco, M., Tenorio, A. and Rizzoli, A. (2009) Detection of novel insect flavivirus sequences integrated in Aedes albopictus (Diptera: Culicidae) in Northern Italy. *Virol J***6**: 93

Rojtinnakorn, J., Hirono, I., Itami, T., Takahashi, Y. and Aoki, T. (2002) Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. *Fish Shellfish Immunol***13**: 69-83

Romero, X., Turnbull, J.F. and Jiménez, R. (2000) Ultrastructure and cytopathology of a Rickettsia-like organism causing systemic infection in the redclaw crayfish, *Cherax quadricarinatus* (Crustacea: Decapoda), in Ecuador. *J Invertebr Pathol***76**: 95-104

Roubal, F.R., Paynter, J.L. and Lester, R.J.G. (1989) Electron microscopic observation of hepatopancreatic parvo-like virus (HPV) in the penaeid prawn, *Penaeus merguiensis* de Man, from Australia. *J Fish Dis***12**: 199-203

Rungrassamee, W., Leelatanawit, R., Jiravanichpaisal, P., Klinbunga, S. and Karoonuthaisiri, N. (2010) Expression and distribution of three heat shock protein genes under heat shock stress and under exposure to *Vibrio harveyi* in *Penaeus monodon*. *Dev Comp Immunol***34**: 1082-1089

Rusaini. (2006) The lymphoid organ in penaeids and its interaction with moulting. MSc thesis, James Cook University.

Rusaini and Owens, L. (2007) Validation of quantitative analysis of the LOS cells in prawns. *Agroland***14**: 72 - 76

Rusaini and Owens, L. (2010) Effect of moulting and lunar rhythms on the lymphoid organ spheroid (LOS) cells of the black tiger prawn (*Penaeus monodon*). *J Exp Mar Biol Ecol***389**: 6-12

Saksmerprome, V., Jitrakorn, S., Chayaburakul, K., Laiphrom, S., Boonsua, K. and Flegel, T.W. (2011) Additional random, single to multiple genome fragments of *Penaeus stylirostris* densovirus in the giant tiger shrimp genome have implications for viral disease diagnosis. *Virus Res***160**: 180-190

Saksmerprome, V., Puiprom, O., Noonin, C. and Flegel, T.W. (2010) Detection of infectious hypodermal and haematopoietic necrosis virus (IHHNV) in farmed Australian *Penaeus monodon* by PCR analysis and DNA sequencing. *Aquaculture***298**: 190-193

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J.C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thebault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J. and Boucher, C.A. (2002) Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature***415**: 497-502

Sanders, J., Raggiaschi, R., Morales, J. and Möller, W. (1993) The human leucine zipper-containing guanine-nucleotide exchange protein elongation factor-1δ. *Biochim Biophys Acta* **1174**: 87-90

Sangsuriya, P., Rojtinnakorn, J., Senapin, S. and Flegel, T.W. (2007) Characterization and tissue expression of apoptosis-related ALG-2 interacting protein Alix/AIP1 from the black tiger shrimp *Penaeus monodon*. *Fish Shellfish Immunol***23**: 485-492

Sangsuriya, P., Rojtinnakorn, J., Senapin, S. and Flegel, T.W. (2010) Identification and characterization of Alix/AIP1 interacting proteins from the black tiger shrimp, *Penaeus monodon*. *J Fish Dis***33**: 571-581

Santiago-Cardona, P.G., Berríos, C.A., Ramírez, F. and García-Arrarás, J.E. (2003) Lipopolysaccharides induce intestinal serum amyloid A expression in the sea cucumber *Holothuria glaberrima*. *Dev Comp Immunol***27**: 105-110

Sargent, T.D. and Dawid, I.B. (1983) Differential gene expression in the gastrula of *Xenopus laevis*. *Science***222**: 135-139

Sarkar, M., Das, S., Bandyopadhaya, A., Ray, K. and Chaudhuri, K. (2005) Upregulation of human mitochondrial NADH dehydrogenase subunit 5 in intestinal epithelial cells is modulated by *Vibrio cholerae* pathogenesis. *FEBS Lett***579**: 3449-3460

Schaefer, A., Nethe, M. and Hordijk, P.L. (2012) Ubiquitin links to cytoskeletal dynamics, cell adhesion and migration. *Biochem J***442**: 13-25

Schoenenberger, C.-A., Mannherz, H.G. and Jockusch, B.M. (2011) Actin: From structural plasticity to functional diversity. *Eur J Cell Biol***90**: 797-804

Scott, I. (2009) Mitochondrial factors in the regulation of innate immunity. *Microb Infec***11**: 729-736

Senapin, S., Phiwsaiya, K., Anantasomboon, G., Sriphaijit, T., Browdy, C.L. and Flegel, T.W. (2010) Knocking down a Taura syndrome virus (TSV) binding protein Lamr is lethal for the whiteleg shrimp *Penaeus vannamei*. *Fish Shellfish Immunol***29**: 422-429

Senapin, S. and Phongdara, A. (2006) Binding of shrimp cellular proteins to Taura syndrome viral capsid proteins VP1, VP2 and VP3. *Virus Res***122**: 69-77

Sequeira, T., Vilanova, M., Lobo-da-Cunha, A., Baldaia, L. and Arala-Chaves, M. (1995) Flow cytometric analysis of molt-related changes in hemocyte type in male and female *Penaeus japonicus*. *Biol Bull***189**: 376 - 380

Serrano-Pinto, V., Landais, I., Ogliastro, M.-H., Gutiérrez-Ayala, M., Mejía-Ruíz, H., Villarreal-Colmenares, H., García-Gasca, A. and Vázquez-Boucard, C. (2004) Vitellogenin mRNA expression in *Cherax quadricarinatus* during secondary vitellogenic at first maturation females. *Mol Reprod Dev***69**: 17-21

Seth, R.B., Sun, L., Ea, C.-K. and Chen, Z.J. (2005) Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF3. *Cell***122**: 669-682

Shao, M.Y., Zhang, Z.F., Kang, K.H., Chen, Z.T. and Kim, J.M. (2004) The study on the cytology and histochemistry of lymphoid organ spheroids in *Penaeus chinensis*. *Aquaculture***240**: 463-471

Shechter, A., Aflalo, E.D., Davis, C. and Sagi, A. (2005) Expression of the reproductive female-specific vitellogenin gene in endocrinologically induced male and intersex *Cherax quadricarinatus* crayfish. *Biol Reprod***73**: 72-79

Shechter, A., Tom, M., Yudkovski, Y., Weil, S., Chang, S.A., Chang, E.S., Chalifa-Caspi, V., Berman, A. and Sagi, A. (2007) Search for hepatopancreatic ecdysteroid-responsive genes during the crayfish molt cycle: from a single gene to multigenicity. *J Exp Biol***210**: 3525-3537

Shike, H., Dhar, A.K., Burns, J.C., Shimizu, C., Jousset, F.X., Klimpel, K.R. and Bergoin, M. (2000) Infectious Hypodermal and Hematopoietic Necrosis Virus of Shrimp Is Related to Mosquito Brevidensoviruses. *Virology***277**: 167-177

Simpson, A.J.G., Reinach, F.C., Arruda, P., Abreu, F.A., Acencio, M., Alvarenga, R., Alves, L.M.C., Araya, J.E., Baia, G.S., Baptista, C.S., Barros, M.H., Bonaccorsi, E.D., Bordin, S., Bove, J.M., Briones, M.R.S., Bueno, M.R.P., Camargo, A.A., Camargo, L.E.A., Carraro, D.M., Carrer, H., Colauto, N.B., Colombo, C., Costa, F.F., Costa, M.C.R., Costa-Neto, C.M., Coutinho, L.L., Cristofani, M., Dias-Neto, E., Docena, C., El-Dorry, H., Facincani, A.P., Ferreira, A.J.S., Ferreira, V.C.A., Ferro, J.A., Fraga, J.S., Franca, S.C., Franco, M.C., Frohme, M., Furlan, L.R., Garnier, M., Goldman, G.H., Goldman, M.H.S., Gomes, S.L., Gruber, A., Ho, P.L., Hoheisel, J.D., Junqueira, M.L., Kemper, E.L., Kitajima, J.P., Krieger, J.E., Kuramae, E.E., Laigret, F., Lambais, M.R., Leite, L.C.C., Lemos, E.G.M., Lemos, M.V.F., Lopes, S.A., Lopes, C.R., Machado, J.A., Machado, M.A., Madeira, A.M.B.N., Madeira, H.M.F., Marino, C.L., Marques, M.V., Martins, E.A.L., Martins, E.M.F., Matsukuma, A.Y., Menck, C.F.M., Miracca, E.C., Miyaki, C.Y., Monteiro-Vitorello, C.B., Moon, D.H., Nagai, M.A., Nascimento, A.L.T.O., Netto, L.E.S., Nhani, A., Nobrega, F.G., Nunes, L.R., Oliveira, M.A., de Oliveira, M.C., de Oliveira, R.C., Palmieri, D.A., Paris, A., Peixoto, B.R., Pereira, G.A.G., Pereira, H.A., Pesquero, J.B., Quaggio, R.B., Roberto, P.G., Rodrigues, V., de M. Rosa, A.J., de Rosa, V.E., de Sa, R.G., Santelli, R.V., Sawasaki, H.E., da Silva, A.C.R., da Silva, A.M., da Silva, F.R., Silva, W.A., da Silveira, J.F., *et al.* (2000) The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature***406**: 151-157

Smith, A.E. and Helenius, A. (2004) How viruses enter animal cells. *Science***304**: 237-242

Smith, V.J., Brown, J.H. and Hauton, C. (2003) Immunostimulation in crustaceans: does it really protect against infection? *Fish Shellfish Immunol***15**: 71-90

Soderhall, K. and Cerenius, L. (1992) Crustacean immunity. *Annu Rev Fish Dis*: 3-23

Somboonwiwat, K., Bachère, E., Rimphanitchayakit, V. and Tassanakajon, A. (2008) Localization of anti-lipopolysaccharide factor (ALFPm3) in tissues of the black tiger shrimp, *Penaeus monodon*, and characterization of its binding properties. *Dev Comp Immunol***32**: 1170-1176

Somnuk, S., Tassanakajon, A. and Rimphanitchayakit, V. (2012) Gene expression and characterization of a serine proteinase inhibitor PmSERPIN8 from the black tiger shrimp *Penaeus monodon*. *Fish Shellfish Immunol***33**: 332-341

Sonenberg, N. and Dever, T.E. (2003) Eukaryotic translation initiation factors and regulators. *Curr Opin Struct Biol***13**: 56-63

Soonthornchai, W., Rungrassamee, W., Karoonuthaisiri, N., Jarayabhand, P., Klinbunga, S., Söderhäll, K. and Jiravanichpaisal, P. (2010) Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by Vibrio harveyi. *Dev Comp Immunol***34**: 19-28

Soowannayan, C., Flegel, T.W., Sithigorngul, P., Slater, J., Hyatt, A., Cramerri, S., Wise, T., Crane, M.S.J., Cowley, J.A., McCulloch, R.J. and Walker, P.J. (2003) Detection and differentiation of yellow head complex viruses using monoclonal antibodies. *Dis Aquat Org***57**: 193-200

Soowannayan, C., Sithigorngul, P. and Flegel, T.W. (2002) use of a specific monoclonal antibody to determine tissue tropism of yellow head virus (YHV) of *Penaeus monodon* by *in situ* immunocytochemistry. *Fish Sci***68 Supplement 1**: 805-809

Soto-Rodriguez, S., Gomez-Gil, B. and Lozano, R. (2010) Bright-red syndrome in Pacific white shrimp *Litopenaeus vannamei* is caused by *Vibrio harveyi*. *Dis Aquat Org***92**: 11-19

Spann, K.M., Cowley, J.A., Walker, P.J. and Lester, R.J.G. (1997) A yellow-head-like virus from *Penaeus monodon* cultured in Australia. *Dis Aquat Org***31**: 169-179

Spann, K.M., Donaldson, R.A., Cowley, J.A. and Walker, P.J. (2000) Differences in the susceptibility of some penaeid prawn species to gill-associated virus (GAV) infection. *Dis Aquat Org***42**: 221-225

Spann, K.M., McCulloch, R.J., Cowley, J.A., East, I.J. and Walker, P.J. (2003) Detection of gill-associated virus (GAV) by *in situ* hybridization during acute and chronic infections of *Penaeus monodon* and *P. esculentus*. *Dis Aquat Org***56**: 1-10

Spann, K.M., Vickers, J.E. and Lester, R.J.G. (1995) Lymphoid organ virus of *Penaeus monodon* from Australia. *Dis Aquat Org***23**: 127-134

Speel, E.J.M., Hopman, A.H.N. and Komminoth, P. (1999) Amplification methods to increase the sensitivity of *in situ* hybridization: play CARD(S). *J Histochem Cytochem***47**: 281-288

Spiess, C., Meyer, A.S., Reissmann, S. and Frydman, J. (2004) Mechanism of the eukaryotic chaperonin: protein folding in the chamber of secrets. *Trends Cell Biol***14**: 598-604

Sriphaijit, T. and Senapin, S. (2007) High expression of a novel leucine-rich repeat protein in hemocytes and the lymphoid organ of the black tiger shrimp *Penaeus monodon*. *Fish Shellfish Immunol***22**: 264-271

Sritunyalucksana, K., Apisawetakan, S., Boon-nat, A., Withyachumnarnkul, B. and Flegel, T.W. (2006) A new RNA virus found in black tiger shrimp *Penaeus monodon* from Thailand. *Virus Res***118**: 31-38

Stephens, A., Rojo, L., Araujo-Bernal, S., Garcia-Carreño, F. and Muhlia-Almazan, A. (2012) Cathepsin B from the white shrimp *Litopenaeus vannamei*: cDNA sequence analysis, tissues-specific expression and biological activity. *Comp Biochem Physiol B***161**: 32-40

Sun, C., Xu, W.-T., Zhang, H.-W., Dong, L.-P., Zhang, T., Zhao, X.-F. and Wang, J.-X. (2011) An anti-lipopolysaccharide factor from red swamp crayfish, *Procambarus clarkii*, exhibited antimicrobial activities in vitro and in vivo. *Fish Shellfish Immunol***30**: 295-303

Supungul, P., Klibunga, S., Pichangkura, R., Jitrapakdee, S., Hirono, I., Aoki, T. and Tassanakajon, A. (2002) Identification of immune-related genes in haemocytes of black tiger shrimp (*Penaeus monodon*). *Mar Biotechnol***4**: 487 - 494

Supungul, P., Klinbunga, S., Pichyangkura, R., Hirono, I., Aoki, T. and Tassanakajon, A. (2004) Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach. *Dis Aquat Org***61**: 123-135

Supungul, P., Rimphanitchayakit, V., Aoki, T., Hirono, I. and Tassanakajon, A. (2010) Molecular characterization and expression analysis of a c-type and two novel muramidase-deficient i-type lysozymes from *Penaeus monodon*. *Fish Shellfish Immunol***28**: 490-498

Surakasi, V.P. and Kim, Y. (2010) Cloning and expression pattern of eukaryotic translation initiation factor 4A of the diamondback moth, *Plutella xylostella*. *J Asia-Pac Entomol***13**: 297-301

Suthianthong, P., Pulsook, N., Supungul, P., Tassanakajon, A. and Rimphanitchayakit, V. (2011) A double WAP domain-containing protein PmDWD from the black tiger shrimp *Penaeus monodon* is involved in the controlling of proteinase activities in lymphoid organ. *Fish Shellfish Immunol***30**: 783-790

Takeuchi, O. and Akira, S. (2010) Pattern Recognition Receptors and Inflammation. *Cell***140**: 805-820

Tang, K.F.J. and Lightner, D.V. (2006) Infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequences in the genome of the black tiger prawn *Penaeus monodon* from Africa and Australia. *Virus Res***118**: 185-191

Tang, K.F.J. and Lightner, D.V. (2011) Duplex real-time PCR for detection and quantification of monodon baculovirus (MBV) and hepatopancreatic parvovirus (HPV) in Penaeus monodon. *Dis Aquat Org***93**: 191-198

Tang, K.F.J., Navarro, S.A. and Lightner, D.V. (2007) PCR assay for discriminating between infectious hypodermal and hematopoietic necrosis virus (IHHNV) and virus-related sequences in the genome of *Penaeus monodon*. *Dis Aquat Org***74**: 165-170

Tang, K.F.J., Pantoja, C.J., Poulos, B.T., Redman, R.M. and Lightner, D.V. (2005) In situ hybridization demonstrates that *Litopenaeus vannamei*, *L. stylirostris* and *Penaeus monodon* are susceptible to experimental infection with infectious myonecrosis virus (IMNV). *Dis Aquat Org***63**: 261-265

Tang, K.F.J., Pantoja, C.R. and Lightner, D.V. (2008) Nucleotide sequence of a Madagascar hepatopancreatic parvovirus (HPV) and comparison of genetic variation among geographic isolates. *Dis Aquat Org***80**: 105-112

Tangprasittipap, A., Tiensuwan, M. and Withyachumnarnkul, B. (2010) Characterization of candidate genes involved in growth of black tiger shrimp *Penaeus monodon*. *Aquaculture***307**: 150-156

Tanne, E. and Sela, I. (2005) Occurrence of a DNA sequence of a non-retro RNA virus in a host plant genome and its expression: evidence for recombination between viral and host RNAs. *Virology***332**: 614-622

Tattersall, P., Mahy, B.W.J. and Regenmortel, M.H.V.v. (2008) Parvoviruses: General Features, Encyclopedia of Virology, pp. 90-97. Academic Press, Oxford.

Taylor, D. and Bruenn, J. (2009) The evolution of novel fungal genes from non-retroviral RNA viruses. *BMC Biol***7**: 88

Tharntada, S., Somboonwiwat, K., Rimphanitchayakit, V. and Tassanakajon, A. (2008) Anti-lipopolysaccharide factors from the black tiger shrimp, *Penaeus monodon*, are encoded by two genomic loci. *Fish Shellfish Immunol***24**: 46-54

Tonganunt, M., Nupan, B., Saengsakda, M., Suklour, S., Wanna, W., Senapin, S., Chotigeat, W. and Phongdara, A. (2008) The role of Pm-fortilin in protecting shrimp from white spot syndrome virus (WSSV) infection. *Fish Shellfish Immunol***25**: 633-637

Tonganunt, M., Saelee, N., Chotigeat, W. and Phongdara, A. (2009) Identification of a receptor for activated protein kinase C1 (Pm-RACK1), a cellular gene product from black tiger shrimp (*Penaeus monodon*) interacts with a protein, VP9 from the white spot syndrome virus. *Fish Shellfish Immunol***26**: 509-514

Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B. and Turk, D. (2012) Cysteine cathepsins: From structure, function and regulation to new frontiers. *Biochim Biophys Acta* **1824**: 68-88

Turnbull, J.F., Larkins, P.E., McPadden, C. and Matondang, R. (1994) A histopathological disease survey of cultured shrimp in North East Sumatera, Indonesia. *J Fish Dis***17**: 57-65

Unajak, S., Boonsaeng, V. and Jitrapakdee, S. (2006) Isolation and characterization of cDNA encoding Argonaute, a component of RNA silencing in shrimp (*Penaeus monodon*). *Comp Biochem Physiol B***145**: 179-187

Valpuesta, J.M., MartIn-Benito, J., Gómez-Puertas, P., Carrascosa, J.L. and Willison, K.R. (2002) Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT. *FEBS Letters***529**: 11-16

van de Braak, C.B.T., Botterblom, M.H.A., Liu, W., Taverne, N., van der Knaap, W.P.W. and Rombout, J.H.W.M. (2002a) The role of the haematopoietic tissue in haemocyte production and maturation in the black tiger shrimp (*Penaeus monodon*). *Fish Shellfish Immunol***12**: 253-272

van de Braak, C.B.T., Botterblom, M.H.A., Taverne, N., van Muiswinkel, W.B., Rombout, J.H.W.M. and van der Knaap, W.P.W. (2002b) The roles of haemocytes and the lymphoid organ in the clearance of injected *Vibrio* bacteria in *Penaeus monodon* shrimp. *Fish Shellfish Immunol***13**: 293-309

van Loo, N.-D., Fortunati, E., Ehlert, E., Rabelink, M., Grosveld, F. and Scholte, B.J. (2001) Baculovirus infection of nondividing mammalian cells: Mechanisms of entry and nuclear transport of capsids. *J Virol***75**: 961-970

van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carsten, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R. and Wickner, R.B., eds. (2000) Virus Taxonomy: Classification and Nomenclatur of Viruses. Seventh Report of the International Committee on Taxonomy of Virus. Academic Press, San Diego.

Vargas-Albores, F., Martínez-Martínez, A., Aguilar-Campos, J. and Jiménez-Vega, F. (2009) The expression of protein disulfide isomerase from Litopenaeus vannamei hemocytes is regulated by bacterial inoculation. *Comp Biochem Physiol D***4**: 141-146

Vatanavicharn, T., Pongsomboon, S. and Tassanakajon, A. (2012) Two plasmolipins from the black tiger shrimp, *Penaeus monodon* and their response to virus pathogens. *Dev Comp Immunol***38**: 389-394

Vatanavicharn, T., Supungul, P., Puanglarp, N., Yingvilasprasert, W. and Tassanakajon, A. (2009) Genomic structure, expression pattern and functional characterization of crustinPm5, a unique isoform of crustin from *Penaeus monodon*. *Comp Biochem Physiol B***153**: 244-252

Verli, F.D., Rossi-Schneider, T.R., Schneider, F.L., Yurgel, L.S. and de Souza, M.A.L. (2007) Vascular corrosion casting technique steps. *Scanning***29**: 128-132

Visudtiphole, V., Watthanasurorot, A., Klinbunga, S., Menasveta, P. and Kirtikara, K. (2010) Molecular characterization of calreticulin: A biomarker for

temperature stress responses of the giant tiger shrimp *Penaeus monodon*. *Aquaculture***308, Supplement 1**: S100-S108

Vogt, G. (2012) Ageing and longevity in the Decapoda (Crustacea): A review. *Zool Anz***251**: 1-25

Wagner, K., Rehling, P., Sanjuán Szklarz, L.K., Taylor, R.D., Pfanner, N. and van der Laan, M. (2009) Mitochondrial F1Fo-ATP synthase: The small subunits e and g associate with monomeric complexes to trigger dimerization. *J Mol Biol***392**: 855-861

Walker, P.J. and Mohan, C.V. (2009) Viral disease emergence in shrimp aquaculture: origins, impact and the effectiveness of health management strategies. *Rev Aquac***1**: 125-154

Wang, B., Li, F., Dong, B., Zhang, X., Zhang, C. and Xiang, J. (2006) Discovery of the genes in response to white spot syndrome virus (WSSV) infection in *Fenneropenaeus chinensis* through cDNA microarray. *Mar Biotechnol***8**: 491-500

Wang, C.S., Tang, K.F.J., Kou, G.H. and Chen, S.N. (1997) Light and electron microscopic evidence of white spot disease in the giant tiger shrimp, *Penaeus monodon* (Fabricius), and the kuruma shrimp, *Penaeus japonicus* (Bate), cultured in Taiwan. *J Fish Dis***20**: 323-331

Wang, D.-L., Zuo, D., Wang, L.-M., Sun, T., Wang, Q. and Zhao, Y.-L. (2012) Effects of white spot syndrome virus infection on immuno-enzyme activities and ultrastructure in gills of *Cherax quadricarinatus*. *Fish Shellfish Immunol***32**: 645-650

Wang, H.-C., Wang, H.-C., Leu, J.-H., Kou, G.-H., Wang, A.H.J. and Lo, C.-F. (2007a) Protein expression profiling of the shrimp cellular response to white spot syndrome virus infection. *Dev Comp Immunol***31**: 672-686

Wang, K.C.H.-C., Kondo, H., Hirono, I. and Aoki, T. (2010) The *Marsupenaeus japonicus* voltage-dependent anion channel (MjVDAC) protein is involved in white spot syndrome virus (WSSV) pathogenesis. *Fish Shellfish Immunol***29**: 94-103

Wang, S., Liu, N., Chen, A.-J., Zhao, X.-F. and Wang, J.-X. (2009) TRBP homolog interacts with eukaryotic initiation factor 6 (eIF6) in *Fenneropenaeus chinensis*. *J Immunol***182**: 5250-5258

Wang, S.M. and Rowley, J.D. (1998) A strategy for genome-wide gene analysis: Integrated procedure for gene identification. *Proc Natl Acad Sci USA***95**: 11909-11914

Wang, X., Liu, B., Wang, G., Tang, B. and Xiang, J. (2008) Molecular cloning and functional analysis of cathepsin B in nutrient metabolism during larval development in *Meretrix meretrix*. *Aquaculture***282**: 41-46

Wang, Y.-C., Chang, P.-S. and Chen, H.-Y. (2007b) Tissue expressions of nine genes important to immune defence of the Pacific white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol***23**: 1161-1177

Wang, Y., Li, J., Liu, P., Li, J., Zhang, Z., Chang, Z., He, Y. and Liu, D. (2011) The responsive expression of a caspase gene in Chinese shrimp *Fenneropenaeus chinensis* against pH stress. *Aquac Res***42**: 1214-1230

Wang, Y.T., Liu, W., Seah, J.N., Lam, C.S., Xiang, J.H., Korzh, V. and Kwang, J. (2002) White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp *Penaeus merguiensis*. *Dis Aquat Org***52**: 249-259

Wang, Z. and Brown, D.D. (1991) A gene expression screen. *Proc Natl Acad Sci USA***88**: 11505-11509

Wang, Z., Chua, H.K., Gusti, A.A.R.A., He, F., Fenner, B., Manopo, I., Wang, H. and Kwang, J. (2005) RING-H2 protein WSSV249 from white spot syndrome virus sequesters a shrimp ubiquitin-conjugating enzyme, PvUbc, for viral pathogenesis. *J Virol***79**: 8764-8772

Warner, J.R. and McIntosh, K.B. (2009) How common are extraribosomal functions of ribosomal proteins? *Mol Cell***34**: 3-11

Watthanasurorot, A., Söderhäll, K. and Jiravanichpaisal, P. (2012) A mammalian like interleukin-1 receptor-associated kinase 4 (IRAK-4), a TIR signaling mediator in intestinal innate immunity of black tiger shrimp (*Penaeus monodon*). *Biochem Biophys Res Commun***417**: 623-629

Weiss, H., Friedrich, T., Hofhaus, G. and Preis, D. (1991) The respiratory-chain NADH dehydrogenase (complex I) of mitochondria. *Eur J Biochem***197**: 563-576

Wieland, I., Bolger, G., Asouline, G. and Wigler, M. (1990) A method for difference cloning: gene amplification following subtractive hybridization. *Proc Natl Acad Sci USA***87**: 2720-2724

Wilkinson, B. and Gilbert, H.F. (2004) Protein disulfide isomerase. *Biochim Biophys Acta* **1699**: 35-44

Wilson, D.N. and Nierhaus, K.H. (2005) Ribosomal proteins in the spotlight. *Crit Rev Biochem Mol Biol***40**: 243-67

Winkler, H.H. (1990) Rickettsia Species (As Organisms). *Ann Rev Microbiol***44**: 131-153

Withers, P.C. (1992) Comparative Animal Physiology. Brooks/Cole - Thomson Learning, Pacific Grove, CA, USA.

Wonglapsuwan, M., Miyazaki, T., Loongyai, W. and Chotigeat, W. (2010) Characterization and biological activity of the ribosomal protein L10a of the white shrimp: *Fenneropenaeus merguiensis* De Man during vitellogenesis. *Mar Biotechnol***12**: 230-240

Wonglapsuwan, M., Phongdara, A. and Chotigeat, W. (2009) Dynamic changes in gene expression during vitellogenic stages of the white shrimp: *Fenneropenaeus merguiensis* de Man. *Aquac Res***40**: 633-643

Wool, I.G. (1996) Extraribosomal functions of ribosomal proteins. *Trends Biochem Sci***21**: 164-165

Wu, H., Shi, Y., Lin, Y., Qian, W., Yu, Y. and Huo, K. (2011) Eukaryotic translation elongation factor 1 delta inhibits the ubiquitin ligase activity of SIAH-1. *Mol Cell Biochem***357**: 209-215

Wu, J.L. and Muroga, K. (2004) Apoptosis does not play an important role in the resistance of 'immune' *Penaeus japonicus* against white spot syndrome virus. *J Fish Dis***27**: 15-21

Wu, W., Zong, R., Xu, J. and Zhang, X. (2007) Antiviral phagocytosis is regulated by a novel Rab-dependent complex in shrimp *Penaeus japonicus*. *J Prot Res***7**: 424-431

Xiao, H., Neuveut, C., Benkirane, M. and Jeang, K.-T. (1998) Interaction of the second coding exon of Tat with human EF-1δ delineates a mechanism for HIV-1-mediated shut-off of host mRNA translation. *Biochem Biophys Res Commun***244**: 384-389

Xie, X. and Yang, F. (2005) Interaction of white spot syndrome virus VP26 protein with actin. *Virology***336**: 93-99

Xie, Y., Li, F., Wang, B., Li, S., Wang, D., Jiang, H., Zhang, C., Yu, K. and Xiang, J. (2010) Screening of genes related to ovary development in Chinese shrimp *Fenneropenaeus chinensis* by suppression subtractive hybridization. *Comp Biochem Physiol D***5**: 98-104

Yang, C., Zhang, J., Li, F., Ma, H., Zhang, Q., Jose Priya, T.A., Zhang, X. and Xiang, J. (2008) A Toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to *Vibrio anguillarum* infection. *Fish Shellfish Immunol***24**: 564-574

Yao, C.-L., Wu, C.-G., Xiang, J.-H., Li, F., Wang, Z.-Y. and Han, X. (2008) The lysosome and lysozyme response in Chinese shrimp *Fenneropenaeus chinensis* to *Vibrio anguillarum* and laminarin stimulation. *J Exp Mar Biol Ecol***363**: 124-129

Yedery, R.D. and Reddy, K.V.R. (2009) Identification, cloning, characterization and recombinant expression of an anti-lipopolysaccharide factor from the hemocytes of Indian mud crab, *Scylla serrata*. *Fish Shellfish Immunol***27**: 275-284

Yeh, M.-S., Huang, C.-J., Cheng, J.-H. and Tsai, I.-H. (2007) Tissue-specific expression and regulation of the haemolymph clottable protein of tiger shrimp (*Penaeus monodon*). *Fish Shellfish Immunol***23**: 272-279

Yeh, M.-S., Huang, C.-J., Guo, C.-H., Liu, K.-F., Tsai, I.-H. and Cheng, W. (2012) Identification and cloning of a selenophosphate synthetase (SPS) from tiger shrimp, *Penaeus monodon*, and its transcription in relation to molt stages and following pathogen infection. *Dev Comp Immunol***36**: 21-30

Yeh, M.-S., Lai, C.-Y., Liu, C.-H., Kuo, C.-M. and Cheng, W. (2009a) A second proPO present in white shrimp *Litopenaeus vannamei* and expression of the proPOs during a *Vibrio alginolyticus* injection, molt stage, and oral sodium alginate ingestion. *Fish Shellfish Immunol***26**: 49-55

Yeh, M.-S., Liu, C.-H., Hung, C.-W. and Cheng, W. (2009b) cDNA cloning, identification, tissue localisation, and transcription profile of a transglutaminase from white shrimp, *Litopenaeus vannamei*, after infection by *Vibrio alginolyticus*. *Fish Shellfish Immunol***27**: 748-756

Yu, C.-J., Lin, Y.-F., Chiang, B.-L. and Chow, L.-P. (2003) Proteomics and Immunological Analysis of a Novel Shrimp Allergen, Pen m 2. *J Immunol***170**: 445-453

Yudkovski, Y., Glazer, L., Shechter, A., Reinhardt, R., Chalifa-Caspi, V., Sagi, A. and Tom, M. (2010) Multi-transcript expression patterns in the gastrolith disk and the hypodermis of the crayfish *Cherax quadricarinatus* at premolt. *Comp Biochem Physiol D***5**: 171-177

Yudkovski, Y., Shechter, A., Chalifa-Caspi, V., Auslander, M., Ophir, R., Dauphin-Villemant, C., Waterman, M., Sagi, A. and Tom, M. (2007) Hepatopancreatic multi-transcript expression patterns in the crayfish *Cherax quadricarinatus* during the moult cycle. *Insect Mol Biol***16**: 661-674

Zeng, Y. and Lu, C.-P. (2009) Identification of differentially expressed genes in haemocytes of the crayfish (*Procambarus clarkii*) infected with white spot syndrome virus by suppression subtractive hybridization and cDNA microarrays. *Fish Shellfish Immunol***26**: 646-650

Zhang, J., Li, F., Jiang, H., Yu, Y., Liu, C., Li, S., Wang, B. and Xiang, J. (2010) Proteomic analysis of differentially expressed proteins in lymphoid organ of *Fenneropenaeus chinensis* response to *Vibrio anguillarum* stimulation. *Fish Shellfish Immunol***29**: 186-194

Zhao, Z.-Y., Yin, Z.-X., Weng, S.-P., Guan, H.-J., Li, S.-D., Xing, K., Chan, S.-M. and He, J.-G. (2007) Profiling of differentially expressed genes in hepatopancreas of white spot syndrome virus-resistant shrimp (*Litopenaeus vannamei*) by suppression subtractive hybridisation. *Fish Shellfish Immunol***22**: 520-534

Zhdanov, V.M. (1975) Integration of viral genomes. *Nature***256**: 471-3

Zhixun, G., vande Braak, K. and Botterbloom, M. (2004) Distribution of bacteria injected in body of giant black shrimp, *Penaeus monodon*. *Chin J Oceanol Limnol***22**: 186-191

Zou, J., Yu, X.-F., Bao, Z.-J. and Dong, J. (2011) Proteome of human colon cancer stem cells: A comparative analysis. *World J Gastroenterol***17**: 1276-1285