QUANTITATIVE HISTOPATHOLOGY AND EPIDEMIOLOGY OF PRAWN VIRAL DISEASES

Thesis submitted by

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Abstract

The interaction of prawns, viruses and the environment in disease development at individual and population levels was studied using histological and statistical analyses based on quantitative models and simple quantitative methods.

At the individual level, quantitative histopathology was utilized to differentiate monodon baculovirus (MBV) in the digestive tissues (the midgut or MG and hepatopancreas or HP) of Australian *Penaeus monodon* and *Penaeus merguiensis*. It seems MBV in *P. monodon* was less damaging than in *P. merguiensis.* Indeed, *P. monodon* sustained significantly lower severity of MBV infection (mean±SE of severity index (SI) = 1.6 ± 0.1) and proportion of abnormal cells (R_{ac} = 34 ± 3%) with a significantly higher proportion of infected nuclei (R_{in} = 64 ± 3%); while *P. merguiensis* had significantly higher SI (2.1 ± 0.1) and R_{ac} (76 ± 5%) with significantly lower R_{in} (46 ± 3%). Besides, the diameter of MBV occlusion bodies (OBs) in *P. monodon* (5.4 ± 0.3 µm*)* was significantly larger than that in *P. merguiensis* (3.5 ± 0.4 µm). Also, the number of MBV OBs per nuclei in *P. monodon* was moderately correlated to the OB diameter (R^2 = 0.34, p << 0.05), while in *P. merguiensis* it was weakly correlated (R^2 = 0.12, p << 0.05). Perhaps, *P. monodon* and MBV mutually determined the size of the OBs, whereas *P. merguiensis* mostly influenced the size of the OBs. Tissue tropism of Australia-type MBV in the AMG of *P. merguiensis* was reported for the first time based on MBV prevalence (91± 6%), SI (2.4± 0.1), R_{cc} (91±3%) and significant dominance of the final stage of nuclear change. Overall, the HP of both penaeid species was more tolerant to MBV than the AMG since the HP contained significantly larger OBs and higher proportion of nuclei with the early stage of MBV infection compared to that in the AMG.

For the first time a transect method was applied to analyse lymphoidal changes in *P. monodon*. This method was simple and cost effective since it detected the changes of the length of lymphoidal tubules and spheroids using a micrometer and a low-power light microscope. Two novel quantitative models were developed and tested here, i.e. the spheroid-total length ratio (STLR) and morphotype (MT) score. The results show that the ratio of spheroid length and morphotypes of the spheroids continually changed in a successive fashion from an early infection type (morphotype A) to a terminal type (morphotype C) in chronic or acute experimental infection by an insect *Autographa californica* nuclear polyhydrosis virus (AcNPV). Overall, this simple technique was sensitive and reliable to detect lymphoidal changes in the prawns.

At the population level, risk factor analysis was applied relative to a declining prawn production in a farm with no written records of prawn diseases. Statistics were employed to investigate any possible correlation between the food budget (proxy measure of prawn health) and 10 parameters of daily water quality (265 working days from 18 crops); the biomass (proxy measure of prawn health) to 5 parameters of prawn biology, 6 parameters of farming management and 1 parameter of production (29 crops within 9 month data span); and 2 parameters of histology (35 sub-adult *P. monodon*). Coherence, analogy and histology show that high salinity (33 ppt), low temperature (26 ^{o}C), young age (< 107 days), high stocking density (> 20 individuals/m^2), systemic bacterial infection (46% prevalence) and muscle necrosis (91% prevalence) were the potential risk factors of declining prawn production in the farm. Non-systemic and minor cellular changes associated with suspected viruses suggest that viral disease was very likely not a risk to the prawn production.

Since molecular techniques can be used to confirm the diagnosis by histology, the histological diagnosis of MBV was compared to non-isotope *in situ* hybridization (NISH) and polymerase chain reaction (PCR) assays. Molecular diagnosis of MBV appears to encounter technical difficulties, which were clarified in terms of possible causes and procedural modifications.

Quantitative histopathology, statistical epidemiology and simple, low-costs, time-efficient and robust quantitative methods in this research may be suitable for places like Indonesia lacking sophisticated diagnostic facilities.

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LIST OF ABBREVIATIONS

ABW	Average body weight
AcNPV	*Autographa californica* nuclear polyhedrosis virus
AMG	Anterior mid-gut
AR	Attributable risk
BBV	Bennettae baculovirus
BMNV	Baculoviral midgut gland necrosis virus
BP	Baculovirus penaei
BWF	Body weight fed
DNA	Deoxyribonucleic acid
d.p.i	Day post injection
DIG	Dixogenin-11-dUTP
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbant assay
FA	Fluorescent antibody
FCR	Food conversion ratio
FLW	Feed last week (the amount of feed in the last week before sampling)
GAV	Gill-associated virus
HHNBV	Hypodermal and hematopoietic necrosis baculovirus
HP	Hepatopancreas
h.p.i	Hour post injection
HPV	Hepatopancreatic parvo-like virus
ICTV	International Committee on Taxonomy of Viruses
IGR	Individual growth rate
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
LO	Lymphoid organ
LOS	Lymphoid organ spheroid
LOV	Lymphoid organ virus
LOVV	Lymphoidal organ vacuolization virus
LPV	Lympoidal parvo-like virus

MbSNPV	*Metapenaeus bennettae* single nucleocapsid polyhedrosis virus
MBV	Monodon baculovirus
MG	Mid gut
MNPV	Many nucleocapsid polyhedrosis virus
MT score	Morphotype score
NISH	Non-isotope *in situ* hybridization
OB	Occlusion body
OR	Odds ratio
PBV	Plebejus baculovirus
PCR	Polymerase chain reaction
PjNOB I	*Penaeus japonicus* non occluded baculovirus I
PL	Postlarvae
PmSNPV	*Penaeus monodon* single nucleocapsid polyhedrosis virus
PvSNPV	*Penaeus vannamei* single nucleocapsid polyhedrosis virus
PWG	Pond weight gain
R_{cc}	Ratio of cytolytic cells
R_{in}	Ratio of infected nuclei
RNA	Ribonucleic acid
RV-PJ	Rod-shaped nuclear virus of *Penaeus japonicus*
SC	Standing crop
SEM	Scanning electron microscopy
SEMBV	Systemic ectodermal and mesodermal baculovirus
SI	Severity index
SMV	Spawner-isolated mortality virus
SN	Spheroid number
SNPV	Single nucleocapsid polyhedrosis virus
SPF	Specific-pathogen free
SPR	Specific-pathogen resistance
SR	Survival rate
STL	Spheroid-total length
STLR	Spheroid-total length ratio

TEM	Transmission electron microscopy
TRF	Total running feed
TSV	Taura syndrome virus
WSBV	White spot syndrome baculoviruses
WSSV	White spot syndrome virus
YHV	Yellow-head virus

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CHAPTER 1
GENERAL INTRODUCTION

1.1. Overview

Infectious diseases in animals or humans arise from a dynamic and complex interaction between the host, pathogens and environment (Thrusfield, 1986; Weber and Rutala, 2001). This paradigm also transpires in the commercial culture of the penaeid prawns which have been inundated by infectious diseases (Lightner *et al*., 1992).

In this thesis, a special interest was given to monodon baculovirus (MBV) because of its' extensive biogeography and strain varieties found in a wide array of wild and cultured penaeids (Lightner *et al*., 1992; Lightner, 1996; Spann and Lester, 1997; Ramasamy *et al*., 2000). In fact, of the twenty-three known penaeid viruses to date, 13 are baculoviruses (Lightner, 1996).

MBV was suspected of being the primary cause of the collapse of Taiwanese prawn aquaculture in 1988 (Liao *et al*., 1992). However, subsequent analysis shows that the environmental and management factors also contributed to the collapse (Liao *et al*., 1992; Flegel, 1997). A high prevalence of MBV does not mean that MBV is virulent to penaeids (Lightner *et al*., 1992). This was confirmed by Flegel and Pasharawipas (1998) who found that under optimal environmental conditions MBV infection does not cause adverse effects on the hosts. Likewise, with double separation and washing of eggs (management factors), MBV infection can be eliminated in most hatcheries in Asia and Australia (Asian Shrimp News, 1989, 1990; Owens, 1997). So, it is clear that MBV-related diseases in prawn aquaculture conform to the paradigm of complex interactions between multiple factors of hosts, pathogens and the environment (including anthropogenic factors).

For example, MBV infection in *Penaeus monodon* varies with host factors (e.g., age, species, stress and genetics), human intervention and natural conditions (e.g., culture

density, sudden change in water salinity, increasing gill and surface fouling by epicomensals, and increasing numbers and changing composition of *Vibrio* communities in water) (Lightner *et al*., 1983; Baticados *et al*., 1991; Fulks and Main, 1992; Natividad and Lightner, 1992; Lightner, 1996; Ramasamy *et al*., 2000; Sung *et al*., 2001). The interaction becomes more complex with a cocktail of MBV and other pathogenic viruses such as yellow head virus (YHV), white spot syndrome virus (WSSV) (Wongterasupaya *et al.*, 1995; Mohan *et al.*, 1998) and hepatopancreatic parvo-like virus (HPV) (Flegel *et al.*, 1999).

From the perspective of the host, the multifactorial nature of prawn diseases can be seen from two different levels, i.e. individual and population levels.

At the individual level, the interplay between a prawn and pathogen needs sensitive, accurate and specific diagnostic tools for each particular pathogen. In prawn virology, advanced molecular diagnostics have developed rapidly since the end of 1980s (Vaughan, 1996; Benzie 1998; Cunningham, 2002). Yet, the conventional diagnosis of prawn viruses still relies on histology.

At the population level, the interactions between host (e.g. prawns), pathogens (e.g. MBV) and environment (e.g. water quality) in disease development fall into the field of epidemiology (Thrusfield, 1986). Epidemiology focuses on the patterns of distribution, and their determinants, of health or diseases in an animal or human population (Campbell, 1983; Dawson-Saunders and Trapp, 1994; Christie *et al.*, 1997).

Furthermore, from the perspective of diagnostic techniques, the application of molecular diagnosis in prawn aquaculture is problematic since most of technicians and farmers lack training (Liao, 1989; Fulks and Main, 1992), and results from different molecular laboratories may vary (Cunningham, 2002). Hence, most of diagnoses of diseases in finfish and shellfish still rely on histological techniques (Cunningham, 2002). Although histology is not senstive for detecting viral material in a prawn in early stages of infection (Lightner, 1996), time-consuming and mostly retrospective (Chanratchakool *et al*., 1998),

this technique is powerful, simple, cost-effective and robust in studying prawn viruses at advanced stages. It is most likely that histology will be used for a long time, if not forever, especially in cases where molecular techniques are not yet readily available. Besides, with histology, *in situ* identification can be done on more than one pathogen or histopathological conditions in the same sample.

The histological techniques can be empowered with the application of statistical analyses because the statistical method is 'specially adapted to the elucidation of the quantitative data affected by a multiplicity of causes' (Peller, 1967). In fact, quantitative logical reasoning is widely used in all branches of sciences, including animal medicine, animal pathology and animal health (Peller, 1967). Therefore, the interactions of multifactorial causes of prawn diseases, at individual and population levels, can be explained with quantitative analyses, including analyses of histopathology using numerical expressions (quantitative histopathology).

In third world countries, where expertise, facilities and funding for molecular diagnosis of prawn viruses are scarce, quantitative analyses based on conventional histology and epidemiology may fill the methodological gap. This is especially true for Indonesia where prawn virology, prawn histopathology and aquatic epidemiology are foreign, not only for the farmers and technicians, but also for university students and researchers in fisheries, aquaculture, aquatic animal biology or ecology. In those cases, the gaps between molecular advances and basic sciences of prawn viruses are exceedingly large. On the other hand, in western countries, molecular diagnosis of finfish and shellfish diseases is barely used by the government regulators due to the lack of laboratory standards and the fear of false positives or false alarms (Cunningham, 2002). Thus, it is not hard to imagine that molecular diagnosis of aquatic animal diseases will not become the basis of any regulation at all in third world countries. Fortunately, advanced statistical courses, computerized-statistical software, computers and a large number of highly qualified statisticians in universities and government agencies are widely available in Indonesia, or other third world countries. Thus, quantitative analyses of prawn viruses or other aquatic animal diseases, supported by advanced statistics, may bridge the

knowledge and scientific gaps, and help the governments to improve the analytical and scientific power and the quality of strategies and regulations in aquatic disease management without advanced diagnostic infrastructures. In that context, the current study is significantly appropriate and tremendously relevant to pursue.

1.2. General Hypothesis

Prawn diseases have multifactorial causes, i.e. host factors, pathogens and environment. It is hypothesized that the interactions between prawns (hosts), viruses (pathogens) and environment (water quality and human intervention) in the development of diseases and host's reactions to the diseases can be expounded using quantitative histopathology and statistical epidemiology (risk factor analysis).

1.3. General Research Paradigms

This study follows the paradigm that statistics can explicate the interactions between multifactorial causes of diseases. Accordingly, statistical analyses were applied here to understand the interactions between prawns (hosts), viruses (especially MBV) and the external environment. Two areas of interest in this thesis were the interaction between prawns and baculoviruses, and between prawns, baculoviruses and environment. Both cases involved analyses of coherence (consistency with known theories and facts in prawn biology and virology) and analogy (comparison with other cases in the past).

The first interest involved statistical analyses of MBV morphometry and prawn histopathology related to baculoviral infection. Their interactions were addressed in Chapter 4 and 5 of this thesis.

In Chapter 4, the focus was on the differentiation of MBV from two Australian prawn species (*Penaeus monodon* and *Penaeus merguiensis*). Advanced MBV infection can be easily detected with routine histology and low-power light microscope since this occluded virus has prominent eosinophilic occlusion bodies with diameters between 0.1

to 20 µm (Lightner *et al*., 1992). Besides, this virus only infects the digestive gland (hepatopancreas) and tracks (e.g. midgut) of the penaeids (Lightner, 1996). Moreover, MBV infection rarely causes cellular necrosis or apoptosis in cultured penaeids (Fegan *et al*., 1991; Spann and Lester, 1996; Flegel and Pasharawipas, 1998). This phenomenon provides opportunities to observe and quantify the cellular responses to MBV infection. Overall, this particular study was the first study focused on the differences of MBV in two Australian penaeids using simple and robust quantitative analyses.

In Chapter 5, the non-specific defence of prawns to viral infections was quantified based on the histopathological changes in the lymphoid organ in *P. monodon*. Here, *Autographa californica* nuclear polyhedrosis virus (AcNPV), an insect baculovirus, was used in infection trials since there was no tissue culture for MBV. AcNPV was considered appropriate to substitute MBV in this study because AcNPV has been widely used as the basis for the development of gene probes and polymerase chain reaction (PCR) for MBV (Vickers *et al*., 1992; Belcher and Young, 1998; Hsu *et al*., 2000). The analyses in this chapter specifically focused on lymphoid organ spheroids (clumps of haemocytes). For the first time an analysis of the lymphoid organ was conducted using a transect method. This method was simple, yet it was cost effective since it eliminated the need for specific gene probes for the viruses (Hasson *et al*., 1999 b), or computer software and hardware, digital microscope and image-capture video (Anggraeni and Owens, 2000).

The second interest was the extensive interaction of prawn, pathogens and environment because disease always arises from the interaction between these three factors. In Chapter 6, statistical epidemiology and histopathology were applied in combination to identify risk factors relative to prawn production of a prawn farm. In this particular case, MBV was diagnosed by private pathology laboratories as the pathogenic factor since the pattern of the declining production in the farm closely resembled the collapse of prawn production in Taiwan in 1988 related to MBV infection (Dr. Leigh Owens, Discipline of Microbiology and Immunology JCU, *pers. comm*.). With no farm record of prawn diseases, statistics was employed to investigate the potential risk factors. This study also

explored the possible correlations of diseases and internal pathology of prawns to the pathogens and environment based on coherence, analogy and strength of association.

Since MBV detection in the early stages needs sensitive and specific diagnostic tools, Chapter 7 illustrates a brief comparison between histological analyses (Chapter 4) and advanced molecular techniques, i.e. non-isotope *in situ* hybridization (NISH) and polymerase chain reaction (PCR) assays, in the diagnosis of MBV. This accentuates the importance of advanced molecular diagnosis as the counterpart of quantitative histopathology and epidemiology of prawn viruses. From a prospective point of view, when it is commonly available and affordable in Indonesia, or other third world countries, molecular diagnosis can validate and refine the results of quantitative histopathology and statistical epidemiology of prawns or other aquatic animals.

A general discussion in Chapter 8 sums up the findings of this research in light of synthesising the effects of infections by baculoviruses (i.e, MBV and AcNPV) on the digestive and immune systems of the prawns. Also, the usefulness, reliability and validity of the simple quantitative methods and models in this study were assessed in view of possible applications of the results, the roles of statistics in empowering quantitative analyses of prawn histopathology and epidemiology, and hypotheses for further research and development. Finally, the results were analysed in the context of their usefulness for regions lacking the capabilities for advanced diagnosis of prawn viral diseases.

CHAPTER 2

QUANTITATION IN THE CHARACTERIZATION, DIAGNOSIS, PATHOLOGY AND EPIDEMIOLOGY OF BACULOVIRUSES (PARTICULARLY MBV)

2.1. Introduction

Since the end of 1980s, the study of prawn diseases has reflected the advance of molecular biology, especially with the application of genetic-based technologies like gene probes and polymerase chain reaction (PCR) (Vaughan, 1996). These technologies increase the diagnostic sensitivity, accuracy and speed (Benzie, 1998), although the application of each molecular method contains inherent weaknesses (Cunningham, 2002).

While the molecular technologies are developing quickly, the number of pathogens and diseases is also increasing in parallel with a rapid worldwide expansion of penaeid prawn aquaculture in the last two decades (Bower *et al.*, 1994; Lightner, 1996). One of the challenges in the diagnosis of prawn viruses is the need of trained technicians and farmers (Liao, 1989; Fulks and Main, 1992). Economic constrains make training in the diagnosis of prawn viruses not a priority for low capital farmers and governments. Even in Europe, molecular diagnostics still lag behind in their applications at farm levels and in developing regulations dealing with diseases in finfish and shellfish due to a lack of funding and expertise (Cunningham, 2002). Under such conditions, development of inexpensive, practical and user-friendly approaches may compensate for under-trained technicians and farmers, particularly in the third world countries. Here, quantitative approaches may bridge the gap between the advance of molecular approaches and the lack of resources, while molecular methods can be used to validate the finding of the quantitaive methods.

The term “quantitative approaches” in this thesis refers to the use of numerical expressions in presenting and analyzing information. In the field of human biology and medicine, Peller (1967) used the term *medical statistics* to include all quantitative observations in the sciences of human health (embryology, normal gross and microscopic

anatomy, physiology, pathology and therapy). Hence, statistics is the core of quantitative approaches.

A British statistician, G. U. Yule, (cited by Peller, 1967) stated that statistics 'deal with data affected by a multiplicity of causes'. Thus, the statistical method is 'specially adapted to the elucidation of the quantitative data affected by a multiplicity of causes'. According to K. Freudenberg (cited by Peller, 1967) the aim and purpose of medical statistics are to: "(1) collect reliable medical observations, (2) explain them by means of dividing the material into its components, and (3) find rules for the most probable course of events". Indeed, a great emphasis in methodology to approach any quantitative problem has given rise to a school of mathematical statisticians such as K. Pearson, G. U. Yule, 'Student' (pseudonym), R. A. Fisher, and J. Neyman (Peller, 1967). They contributed quantitative logical reasoning to all branches of sciences, including animal medicine, animal pathology and animal health.

Since the causes of diseases in animals or human are multifactorial (Thrusfield, 1986; Weber and Rutala, 2001), the investigation of animal diseases focuses more on the population rather than on the individual (Thrusfield, 1986). This approach is called epidemiology (from the Greek "*epi*" (upon) and "*demos*" people) (Dawson-Saunders & Trapp, 1994). It refers to the study of the patterns or distribution, and their determinants, of health or diseases in an animal or human population (Campbell, 1983; Dawson-Saunders & Trapp, 1994; Christie *et al.*, 1997).

Based on several proposed models of infectious diseases in humans, Weber & Rutala (2001) showed the host factors, characteristics of the infectious agent and environmental influences interact in a complex interplay to produce an infectious disease. The similar interaction also occurs in prawn aquaculture, including infection of MBV in *P. monodon* (section 1.1.). Therefore, this thesis reviews the quantitative approaches in prawn virology with a special, but not sole, emphasis on economically important viruses in cultured penaeids, especially baculoviruses (rod-shaped viruses), as the major model for the interactions of statistics and prawn viruses. Baculoviruses are chosen as the viral

model because they comprise the majority of prawn viruses known to date (Lightner, 1996).

2.2. History of the Taxonomy of Penaeid Viruses

A universal system of viral classification and nomenclature was created by the International Committee on Taxonomy of Viruses (ICTV), which was founded in 1963 (Ackermann & Berthiaume, 1995). In principle, the ICTV classifies viruses based on the following criteria : genome, morphology, physicochemical properties, proteins, lipids and carbohydrates, genome organization and replication, antigenic properties, and biological properties.

However, the taxonomy of prawn viruses has changed substantially with the development of advanced identification techniques. For example, using electron microscopy, Johnson (1984) classified prawn baculoviruses (rod-shaped viruses) into 4 (four) categories, i.e. subgroup A (nuclear polyhedrosis viruses with many virions embedded in a polyhedral occlusion bodies or OBs), subgroup B (granulosis viruses embedded singly, or rarely in pairs, in a small OB), and subgroups C and D (non-occluded baculoviruses lacking OB, and virions embedded in the nucleocytoplasm). Subgroups C and D cannot always be distinguished morphologically, while granulosis viruses have not been found in prawns. However, with the recent advance of molecular techniques, this classification is outdated and discarded.

Using advanced molecular techniques, Lightner (1996) grouped the families of the viruses of penaeid prawns into three families of DNA viruses (*Parvoviridae*, *Baculoviridae* and *Iridoviridae*) and five families of RNA viruses (*Picornaviridae*, *Reoviridae*, *Rhabdoviridae*, *Togaviridae* and one unknown type). Recently, ICTV has modified this classification (Mayo, 2002). Nevertheless, of twenty-three known penaeid viruses, 13 belong to family *Baculoviridae*, which are of the interest in this literature review. Baculoviruses are viruses with enveloped nucleocapsids, generally occluded, i.e.

virions become embedded in a proteinaceous occlusion body (OB) (Summers, 1977; Ackermann & Berthiaume, 1995).

Most of the known crustacean baculoviruses belong to the genus *Nucleopolyhedrovirus* (Bonami *et al.*, 1997), or the old Subgroup A of Johnson's classification. These viruses exhibit a polyhedral OB of 1 to 4 μm in size (Ackermann & Berthiaume, 1995), with two distinguished structural relationships with enveloped nucleocapsids (Summers, 1977). That is : (a) single nucleocapsid polyhedrosis viruses (SNPVs) in which enveloped single nucleocapsids contain many single occluded virions; and (b) many NPVs (MNPVs) in which more than one nucleocapsids are common to a viral envelope with many occluded bundles.

The outdated version of Lightner's classification (1996) divides penaeid baculoviruses found around the world into 4 groups (Table 2.1.), i.e.:
1. Baculovirus penaei type (BP-type) viruses or *Penaeus vannamei* SNPV (PvSNPV type sp.) since this viral type was initially found in *P. vannamei*.
2. Monodon baculovirus type (MBV-type viruses) or *Penaeus monodon* SNPV (PmSNPV type) as it was firstly found in *P. monodon*. Included as strains of MBV are bennettae baculovirus (BBV) and plebejus baculovirus (PBV).
3. Baculoviral midgut gland necrosis type (BMN type) or *Penaeus japonicus* non occluded baculovirus I (PjNOB I); I = first reported type of this genus of viruses.
4. White spot syndrome baculoviruses complex (WSBV-type viruses) or white spot syndrome viruses (WSSV). This type includes hypodermal and hematopoietic necrosis baculovirus (HHNBV), rod-shaped nuclear virus of *Penaeus japonicus* (RV-PJ), systemic ectodermal and mesodermal baculovirus (SEMBV) and white spot baculovirus (WSBV).

According to Lightner (1996), the first two types of the viruses are occluded, whereas the last two are not. However, because of their differences with the occluded viruses, especially in their lack of OBs, the ICTV classified the non-occluded baculoviruses of invertebrates to be unassigned viruses (Murphy *et al.*, 1995).

Table 2. 1. Morphometric variations of penaeid baculoviruses in the world

Virus Names / Location	Nucleocapsids (nm)		Enveloped Virions (nm)		Reference
	Length	Diameter	Length	Diameter	
MBV-type:					Lightner (1996) Lester *et al.* (1987) Spann & Lester (1996)
1. MBV (general)	246 ± 15	42 ± 15	324 ± 33	75 ± 4	
2. PBV (Australia)	-	30 – 45	440	60	
3. BBV (Australia)	238-288	37-38	300-387	54-56	
BP-type:					Lightner (1996)
1. BP (Hawaii)	-	-	286	56	
2. BP (Ecuador)	-	-	337	79	
3. BP (Florida)	-	-	330	75	
WSSV-type:					
1. Asia/US	330-350	58-67	250-380	70-150	
2. China	-	-	360	120	
3. Asia	226	84	-	-	
4. Asia	216	54	275	83	
5. Thailand	201	89	276	121	
BMN (Asia & Australia)	250	36	310	72	

Half a decade later, van Hulten *et al.* (2001) proposed a new family name, *Nimaviridae*, (nima = thread) for the bacilliform WSSV based on its 292.976 kb long, circular, double-stranded DNA. In early 2002, the ICTV accepted the new family name, in which the species WSSV complex was grouped into genus *Whispovirus* (Mayo, 2002).

Therefore, the prawn viruses now are classified into four families of DNA viruses (*Parvoviridae*, *Baculoviridae*, *Iridoviridae* and *Nimaviridae*) and five families of RNA viruses (*Picornaviridae*, *Reoviridae*, *Rhabdoviridae*, *Togaviridae* and *Roniviridae*). Perhaps the taxonomy will change in the future since new penaeid viruses may arise in parallel with the growth of prawn aquaculture around the world.

2.3. Geographic Distribution and Host Range

2.3.1. PvSNPV or BP-type viruses

BP infects wild and cultured *P. duorarum, P. aztecus, Trachypeanaeus similis* and possibly *P. setiferus* in Florida and Mississippi, in wild and cultured *P. marginatus* in Oahu, Hawaii, and in hatchery-reared *P. aztecus* and *P. vannamei* in Florida and Texas (Lightner, 1996). BP is also found in cultured *P. vannamei* on the Pacific coast of central and south America, including Peru, Ecuador, Columbia, Panama, Costa Rica, and Honduras. In Brazil, BP occurs in imported *P. vannamei* and *P. penicillatus*, and in native *P. schmitti, P. paulensis* and *P. subtilis*.

2.3.2. PmSNPV or MBV-type viruses

The two best described strains of PmSNPV are MBV and PBV (Lightner, 1996). MBV infects penaeids from Indo-Pacific countries (People's Republic of China, Taiwan, Philippines, Malaysia, Singapore, Thailand, Sri Lanka, India, Indonesia, and Australia), the Middle East (Kuwait, Oman, and Israel), as well as the Eastern Hemisphere (Italy, Kenya, and Gambia in West Africa) (Lightner *et al*., 1990; Lightner, 1996).

Penaeids from Tahiti, Hawaii, Mexico, Ecuador, Brazil, Puerto Rico, and several southeastern U.S. states, also contain MBV (Lightner, 1996). Infected hosts include *P. monodon, P. merguiensis, P. semisulcatus, P. indicus, P. plebejus, P. penicillatus, P. esculentus* and *P. kerathus*, and possibly *P. vannamei*.

PBV was the first baculovirus to be found in an Australian cultured penaeid *P. plebejus* (Lester *et al.*, 1987; Lester and Paynter, 1990). Eventhough PBV morphologically resembles MBV, it has a distinct ultrastructure from MBV. Lester and Paynter (1990) could not transfer PBV from *P. plebejus* to *P. monodon.* Lightner (1996) argued these strains may reflect completely different viruses or a complex of viruses scattered in a wide range of geographical areas and hosts. For example, both MBV and PBV have round occlusion bodies and similar virion sizes, but differ in several histological and

ultrastructural features (Lester *et al.*, 1987). Among other things, PBV capsid is enveloped by 2 electron-dense zones, while MBV only by one. However, Ramasamy *et al*. (2000) found both single- and double layered MBV virions in the same population of *P. monodon* in India. Moreover, Doubrovsky *et al.* (1988) found MBV in Australian *P. monodon* and *P. merguiensis* which resembled PBV in *P. plebejus*, while Spann and Lester (1996) discovered another strain of MBV from *Metapenaeus bennettae*. This virus was morphologically analogous to MBV, but did not react with a DNA probe for MBV (Spann and Lester, 1996). Therefore, this virus was designated as Bennettae Baculovirus (BBV) or *Metapenaeus bennettae* SNPV (MbSNPV). Experimental infection shows that *P. monodon* and *P. japonicus* are refractory to this virus (Lightner, 1996). Wild caught *M. monoceros* and *M. elegans* from southwest coast of India also contained MBV-type virus (Subramaniyan *et al*., 2003).

2.3.3. PjNOB I or BMN-type viruses

BMN-type virus (BMNV) reportedly infect wild and cultured *Penaeus japonicus* in Japan and Korea, as well as *P. monodon* in east and southeast Asia and and *P. plebejus* in Australia (Lightner, 1996). An experimental infection of BMNV in *P. chinensis* and *P. semisulcatus* has been succesfully conducted and whilst results were inconsistent in *Metapenaeus ensis* (Lightner, 1996).

2.3.4. WSSV-type viruses

WSSV is a generic name for a complex of white spot syndrome viruses (Table 2.1.). At least 5 (five) viruses with distinct morphometrics are included in the WSSV complex (Lightner, 1996), that is: HHNBV from China, RV-PJ #1 and RV-PJ #2 from Japan, China and Korea; SEMBV from Thailand; and WSBV group from Indonesia, Taiwan, Vietnam, Malaysia, India and Texas (U.S.). Now all these viruses are called WSSV.

Natural infections of WSSV occur in *P. monodon, P. japonicus, P. chinensis (=orientalis), P. indicus, P. merguiensis* and *P. setiferus* (Lightner, 1996). Lethal infections of SEMBV have been experimentally induced in *P. vannamei, P. stylirostris, P. aztecus, P. duorarum* and *P. setiferus*.

2.4. Importance of Viruses in Prawn Aquaculture, Environment and Human Health

2.4.1. Prawn viruses in aquaculture

Following the extensive application of electron microscopy in early 1960's, investigation into virulent viruses infecting marine invertebrates started to escalate (Johnson, 1984). In crustacean species, in particular, since the 1970s more than 30 viruses, or virus-like agents, have been discovered (Ahne, 1994). Amongst several virulent penaeid viruses, baculoviruses are the major pathogen threatening the existence of the prawn aquaculture industry (Lightner *et al.*, 1983; Lightner, 1996; Flegel & Pasharawipas, 1998).

Globally, between 1984 and 1995, the prawn farming sector grew 16% (Subasinghe *et al.*, 1998). In 1995, crustacean aquaculture contributed 4.1% of a total aquaculture production of 27.76 million mt, or 17.3% of a total value of US$ 42.32 billion (Rana, 1997). Moreover, by 1995, the world penaeid aquaculture accounted for 96.3% of cultured crustaceans in the world, with a total production of 668,000 mt (Subasinghe *et al.*, 1998). The production of *P. monodon* alone increased considerably from 31% (54,000 mt) in 1984 to 56% (503,000 mt) of the total world production in 1995 (Rana, 1997). With fish consumption of 13 kg/year/person on average, it is predicted that the global demand for aquaculture products will increase two-fold by the year 2010 (Subasinghe, 1996). However, in 1997, FAO reported that the global expansion of prawn aquaculture declined during 1990-1995 due to degrading environment, poor culture practices, and diseases (US National Research Council, 1992; Subasinghe *et al.*, 1998; Bachère, 2000).

Baculoviral diseases have reportedly caused significant damage to prawn aquaculture worldwide (Ahne, 1994; Lightner, 1996). For instance, Taiwan's US$ 600 million-a-year prawn farming industry suffered a sudden drop of *P. monodon* production from 95,000 mt in 1987 to 30,000 mt in 1988 associated with MBV attack (Rosenberry, 1988; Liao, 1989). With the emergence of more viruses, in 1995 the production of cultured prawns in Taiwan fell 40-70% from 1992 production levels (Peng *et al.*, 1998). Similar discouraging reports also come from other countries, such as the People Republic of

China, the Philippines, Thailand, Japan, Mexico, India and Indonesia, Spain, Netherlands, Bangladesh and Cambodia (Subasinghe, 1996; Rana, 1997).

Beside baculoviruses, WSSV is also another serious threat to prawn aquaculture since its outbreak in Japan and China in 1993 (Mohan *et al.*, 2002). Since then, large losses of cultured prawns due to WSSV have been reported throughout Asia (Wongteerasupaya *et al.*, 1995; Mohan *et al.*, 1998; Park *et al*, 1998) and recently from the Americas (Calderon *et al.*, 2000). In addition, Wang *et al.* (1999) reported an increasing prevalence of WSSV in *P. monodon* grow-out farms in Malaysia from 23% (in 1994) to 20-50% (in 1995) and to 80% (at the end of 1996). The situation has become more complicated with the emergence of other deadly viruses such as yellow-head virus (YHV) from Thailand in 1992 (Wongteerasupaya *et al.*, 1995) which reportedly co-exists with MBV and WSSV. Mohan *et al.* (1998) found dual infection of YHV and WSSV caused mass mortalities of *P. monodon* cultured along the east coast of India in 1994. Ramasamy *et al*. (2000) put an estimated loss of productivity between 10,000 to 12,000 mt (around US$6 – 8 billion) per year for shrimp aquaculture in India due to viral diseases alone. Primavera (1998) in Kautsky *et al.* (2000) calculated the economic losses due to combination of viral and bacterial diseases to be US$750 million in 1993 in China and US$210 million in 1995-1996 in India. The harvest losses in 1996 in Thailand due to WSSV which were about 70,000 mt or around over half a billion US dollars (Flegel and Alday-Sanz, 1998). Obviously, a total loss of several billion dollars was suffered by the whole of Asia due to prawn viruses.

Since penaeid aquaculture is an important source of revenue and employment the threat of viral diseases has prompted global attention and co-operation to improve disease control and health management in aquaculture, including health certification and standard quarantine policy (Liao, 1989; US National Research Council, 1992; Subasinghe, 1996; Subasinghe *et al.*, 1998). As a research priority, Rodríquez and Le Moullac (2000) argue that the study of immunology aimed to develop criteria for selection of SPF or SPR prawns, is the prime area of disease prevention for sustainable aquaculture. However, Bachère (2000) stresses the need for a network of simultaneous research in immunology, genetics, physiology, environment and pathology of the cultured prawns. There are

other research possibilities such as the efficacy of probiotics in terms of strains, dosages and routes of administration (Uma, 1999), or bioremediation of the environment and 'vaccines' for the viruses (Jory, 1998). Furthermore, the state of immunological studies of lower animals, including penaeids infected by baculoviruses, will be complex given the interdependence of ecological factors, and animal physiology and phylogeny (Cooper and Parrinello, 1996). Thus, understanding the virology of penaeid prawns is deemed necessary to produce control strategies and technologies. This creates opportunities for greater economic benefits, especially for highly intensified culture systems. For example, the increasing demand for SPF or SPR-strains of broodstock and larvae have a potential market in Western Hemisphere of an estimated value of US$13.5 million for nauplii, US$ 68.4 million for PLs, and US$ 3.0 million for broodstock. In Asia alone, over US$ 90 million worth of postlarvae are needed to supply the Philippines, Indonesia, Thailand and Taiwan (O'Sullivan, 1997). Reasonably, Australia has a competitive advantage over other western (developed) countries, if it develops technology of SPF or SPR strains, due to its close proximity to major countries in Asia that culture penaeids.

2.4.2. Prawn viruses in environment

Undoubtedly, penaeid aquaculture will expand relentlessly to meet the global demands for seafood (O'Sullivan, 1997; Rana, 1997). However, the challenges of viral diseases are mounting as the quality of the environment deteriorates due to self-pollution of prawn aquaculture (Lightner, 1985; Subasinghe and Barg, 1998; Kautsky *et. al*., 2000).

Worst still, prawn viruses from a farm can contaminate the coastal water. For example, WSSV infected adult *P. monodon*, *P. japonicus*, *P. semisulcatus* and *P. penicillatus* caught from southern coast of Taiwan, as well cultured *Metapepenaeus ensis* (sand shrimp), *Macrobrachium rosenbergii* (the giant fresh water prawn) and *Scylla serrata* (mud crab) (Lo *et al.*, 1996 a). Morever, the pest crab *Helice tridens*, the small pest Palaemonidae prawn, and pupae of an Ephydridae insect from prawn farms were found infected by WSSV. In Thailand, WSSV infected and killed the sand crab *Portunus pelagicus*, the mud crab *Scylla serrata*, and krill *Acetes* spp. (Supamattaya *et al.*, 1998). In the USA, penaeid viruses reportedly also spread to the natural environment and infect

wild shrimp (Iversen, 1997). It is therefore well established that prawn viruses can threaten the aquaculture industry and natural environment (Bachère, 2000).

On the other hand, wild arthropods can serve as reservoir of prawn virueses which are later transferred to cultured prawns. For examples, MBV was found in wild caught juveniles and broodstock of *P. monodon* along the east coast of India (Ramasamy *et al*., 2000). Wild caught *Metapenaeus bennettae* in Australia (Spann and Lester, 1996) and *M. monoceros* and *M. elegans* in India (Subramaniyan *et al*., 2003) also contained MBV-type viruses. Kanchanaphum *et al.* (1998) confirmed that crabs *Sesarma* spp., *Scylla serrata* and *Uca pugilator* in the vicinity of prawn farms can transfer WSSV to healthy *P. monodon*. Again, the prevention and control of viruses in prawn aquaculture need serious attention to sustain the industry and natural environment.

2.4.3. Prawn viruses in human health and scientific interests

It is well-known that arbovirus (arthropod-borne virus of vertebrates) can accidently infect humans through contact with animals such as birds or monkeys (Dimmock and Primrose, 1994). However, no concerns regarding the impacts of penaeid baculoviruses on human health were found in the literature. Nevertheless, research on penaeid viruses is important to screen and prevent penaeid viruses that may be dangerous for humans (Subasinghe *et al.*, 1998). As Cooper and Parrinello (1996) point out, studying marine organisms susceptible to pathogens (external threat) and mutated cells (internal threat) may be applicable to humans.

2.5. Diagnosis of Viral Diseases in Penaeid Prawns

The improvement of diagnostics for viral diseases in cultured prawn species has become one of the major objectives of prawn farming industry, government and scientists in Taiwan (Liao, 1989), the United States (US National Academy of Sciences, 1992) and other countries. Accordingly, the advance in diagnostics increases the accuracy of assessment of the health status of prawns and their environment. Thus, highly sensitive, standardized, inexpensive and simple methods should arise from research and development (Lightner *et al*., 1990).

With regard to MBV diagnostics to date, Lightner (1996) has compiled a reasonably complete list of methods. Therefore, the following methods are taken from Lightner's handbook, except where stated otherwise.

2.5.1. Classical methods

The conventional data gathering for disease diagnostics in prawn aquaculture includes data on the history of the culture facility, gross and clinical signs, direct examination and microscopy, isolation and culture of pathogens, histology and histochemistry, electron microscopy, and serological tests with immune sera (Lightner, 1996).

The history of the facility gives general ideas with regard to the management of the health of the cultured prawns. For routine and non-random monitoring of prawn health status, direct examination and microscopical study are needed to assess the gross and clinical signs of infections. Histopathology is commonly used to observe the lesions, if any, while electron microscopy and serological tests assist the detection of viral materials and immunological components in the animal tissues.

For baculoviral diseases, the conventional diagnostics with reasonable sensitivity include: (1) direct bright field, phase, or darkfield light microscopy of tissue impression smears, wet mounts and stained whole mounts, and (2) histopathology. Enhancement of the infection by stressing infected animals followed by histology of the infected tissues has also been used succesfully for MBV and BP diseases. Other methods available are bioassay (the best for WSSV detection), transmission electron microscopy (TEM) which has been applied for all baculoviruses and scanning electron microscopy (SEM) for BP diagnostics (Lightner, 1996).

2.5.2. Molecular methods

Advanced molecular technologies and sciences make it possible to diagnose viral diseases in penaeids using serological tests with monoclonal antibodies and gene probes. However, their application for detecting baculoviruses in penaeid prawns is still limited.

A fluorescent antibody (FA) method has been used for detecting BMN (with reasonable sensitivity) and BP. Enzyme-linked immunosorbant assays (ELISA) using monoclonal and polyclonal antibodies have been applied for BP diagnostics. The development of DNA probes makes it possible to detect MBV and BP, both with reasonable sensitivity. The use of polymerase chain reaction (PCR) has also improved the diagnosis of BP, MBV, WSSV and other viruses (Lightner, 1996).

2.5.3. Diagnostic procedures for MBV disease

The diagnosis of MBV, in particular, can be categorized broadly into two situations : (a) disease situation, and (b) asymptomatic infections.

(a) Disease situation

The initial step for MBV diagnosis (presumptive diagnosis) is to examine the clinical signs as described in Section 2.6.2. This data can be combined with the history of the facility, region or species cultured.

Presumptive diagnosis in disease situation can use the following methods:

1. Direct microscopy/wet-mount method

Squash preparations of hepatopancreas, midgut, or faeces can be stained with 0.05% aqueous malachite green, and then examined under phase or bright field microscopy. Spherical occlusion bodies (stained green) indicate the presence of MBV.

2. Histological method

Histology of hepatopancreas and midgut tissues stained with hematoxylin and eosin (H&E) stains may reveal the presence of MBV occlusion bodies and cellular changes as described in Section 2.6.2.

Confirmative diagnosis with radioactive and non-radioactive DNA probes has been developed for MBV. Commercial Dixogenin-11-dUTP-labelled DNA probes for *in situ* tests are available from DiagXotics, Inc. (US). With DIG-labelled probes, the presence of MBV in fixed tissue sections can be detected with high sensitivity and specificity (Lightner, 1996).

Alternative diagnostic procedures are also available (Lightner, 1996), such as:

1. Transmission electron microscopy (TEM) method

Thin sections of infected cells examined using TEM will show enveloped virions of MBV in nucleus and occlusion bodies.

2. Phloxine/epifluorescence method

Smears of tissues or faeces containing MBV occlusion bodies are stained with 0.001% aqueous phloxine, or with 0.005% phloxine in the eosin of H&E stains. The occlusion bodies will fluoresce a bright yellow green against a pale green background under an epifluorescent microscope.

3. Acridine orange/epifluorescence method

Dried hepatopancreatic squash stained with 0.025% solution of acridine orange in 2% acetic acid can be used to detect MBV DNA which fluoresces bright green under an epifluorescent microscope.

4. Antibody test

An immunological method to detect MBV may be possible since the protein of the MBV occlusion bodies can cross-react with antibodies to proteins of BPV or BP occlusion bodies (Lightner, 1996). Obviously, purification of the protein of MBV OB is needed.

(b) Asymptomatic infections

Prawns suspected of carrying MBV can be subjected to crowding stress for 10 to 60 days, which will allow the infection to proliferate. Routine histological or wet mount methods can then be used for diagnosis.

Either in symptomatic or asymptomatic conditions, the basic conventional diagnostic methods commonly practiced include wet mount, routine histological staining and light microscopy. These techniques have low sensitivity to detect chronic infection in a prawn population with a low prevalence rate (Lightner *et al*., 1990). However, they are low-cost methods for experts and technicians in third world countries. Quantitative approaches

may amplify the power of these methods for research, educational and diagnostic purposes.

2.6. Quantitative Approaches in Prawn Virology

2.6.1. Quantitative approaches in viral characterization and diagnosis

(a) Nucleocapsids and enveloped virions

The quantitative dimension of viral ultrastructure is of interest in the diagnostics of the penaeid baculovirueses. In diagonal cross-section, the virions of MBV-type viruses are rod-shaped having a nucleoprotein core, capsid and single envelope (Lightner *et al.*, 1983; Doubrovsky *et al.*, 1988; Johnson & Lightner, 1988; Lu *et al.*, 1993; Spann & Lester, 1996). In longitudinal cross-section, enveloped virions appear pencil-shaped with apical extensions, supported by a reflexed filament. The surface of the envelope has spikes (Mari *et al.*, 1993). The envelope measures 16.8 ± 4.1 nm in thickness with a range of 16.5 to 17.7 nm (Lightner *et al.*, 1983), and separates from the virion along its length (Doubrovsky *et al.*, 1988). However, slight morphometric differences of nucleocapsids exist between and within diverse types of penaeid baculoviruses (Table 2.1.).

(b) Sizes and numbers of the occlusion bodies

Occluded viruses, like BP-type and MBV-type viruses, produce intranuclear occlusion bodies (OBs) that can be distinguished easily by their forms and sizes. Thus they can provide useful quantitative information on the viruses. The occlusion body (OB) provides protection for the viruses against the natural environment (Summers, 1977). The OBs from penaeid baculoviruses are formed by a crystalline protein matrix (Lightner *et al.*, 1983), refered to as polyhedrin by Summers (1977). An ultrastructural study by Bonami *et al.* (1997) revealed that the polyhedrin of OBs from penaeid baculoviruses contained large icosahedral nucleoprotein subunits (SuOBs) with diameter of 17 to 23 nm and peak absorption of UV light at around 280 nm wavelength. The major polyhedrin

protein bands of both OBs were 52 kDa (BP type from *P. vannamei*) and 58 kDa (MBV type from *P. monodon*).

The BP viruses generally have triangular or, more rarely quadrangular, unenveloped OBs (Bonami *et al.*, 1997), with size ranging from less than 0.1 μm to nearly 20 μm along the pyramidal height, and vertical length of 8-10 μm (Lightner, 1996). The MBV-type OBs are also unenveloped, but spherical, with diameter from less than 0.1 μm to nearly 20 μm (Lightner, 1985; Lester *et al.*, 1987; Lightner, 1996; Bonami *et al.*, 1997). These differences in OB morphometry are useful for diagnostic purposes using histology and microscopy for baculoviruses.

The variation of the number of OB/cell in MBV-type viruses may give a quantitative tool for diagnosis and differentiation of MBV based on prawn species, geography, culture system and severity of infection. MBV-type viruses can be found as single to multiple OBs in nuclei of infected cells (Lightner, 1996). In *Metapenaeus bennettae* from Australia, Spann and Lester (1996) found 1 to 8 OBs/ cell. Ramasamy *et al*. (2000) found 1 to 12 OBs/cell present in infected hepatocytes of *P. monodon* in India.

(c) Other quantitative features of infected cells

Some features of the cells change quantitatively during viral infections. For example, in MBV infection, an infected cell will undergo a reduced cytoplasmic volume and an increased number of vacuoles (Lightner and Redman, 1981; Lightner *et al*., 1983; Couch, 1989; Vogt, 1992; Lu *et al*., 1993, 1996; Ramasamy *et al*., 2000).

2.6.2. Quantitative approaches in prawn pathology

(a) Cellular and histological lesions

The mechanism and effects of MBV infection in penaeid cells have been reported quite extensively. Lightner *et al.* (1983) suggest that the uptake of baculovirus by prawns may be through ingestion of free virions or occlusion bodies, or cannibalism. Unfortunately there are no reports on the initial sequences of baculoviral entry into susceptible cells of

penaeid prawns. However, some scientists have attempted to study the stages of virogenesis of penaeid baculoviruses inside the cells. For example, Lu *et al*. (1996) has described four stages of MBV development in an infected cell, that is: (1) the appearance of presumed virogenic stroma (an electron dense structure, or a chromatin-like network, associated with nucleocapsid production), (2) the appearance of virions, (3) the formation of occlusion bodies, and (4) the release of virions. Using insect cell-lines infected with a polyhedral baculovirus from *P. orientalis*, Lee and Kim (1994) estimated that all the stages could be completed within 84 hr post-injection.

Doubrovsky *et al.* (1988), whilst studying the development of MBV in *P. monodon* and *P. merguiensis* from Australia, observed the formation of membranous labyrinths which were a highly organized cellular membrane structure in cytoplasm. In MBV infection, membranous labyrinths are formed slightly earlier than the occlusion bodies and before viral maturation (Lu *et al*., 1996). This is similar to observations in BP-infected penaeid shrimp cells (Couch, 1989).

Doubrovsky *et al.* (1988) reported that the labyrinths originated from mitochondria, vacuoles and the nuclear membrane, while Lu *et al*. (1996) noticed that the nuclear outer membrane, endoplasmic reticulum (ER) and Golgi apparatus were the sources of the labyrinth. Nevertheless, it is agreed that the morphogenesis of the membranous labyrinth is associated with the viral production or, the process of viral reproduction (Lightner *et al.*, 1983; Lester *et al.*, 1987; Doubrovsky *et al.*, 1988; Couch, 1989; Lu *et al*., 1996). However, no membranous labyrinth was reported in BBV infection in *Metapenaeus bennettae* (Spann and Lester, 1996).

In spite of cellular changes due to MBV infection as described above, apoptosis (generalized programmed cell death), necrosis or lysis of the host's cells may not necessarily occur (Fegan *et al.*, 1991; Spann and Lester, 1996; Flegel and Pasharawipas, 1998; Ramasamy, 2000). Flegel and Pasharawipas (1998) call this condition viral accommodation, in which under normal conditions, penaeid prawns can survive with baculoviruses present in their cells. They speculate that penaeid prawns may accomodate the viral genomes throughout their life after being exposed during larval stages. When a

baculovirus initially infects the prawn, the prawn will 'learn' to recognize the virus, such that in subsequent viral infections, the prawn cells will not display apoptosis or necrosis. Hence, the prawns become tolerant rather than resistant to the viruses (Flegel and Pasharawipas, 1998). This phenomenon is thought to relate more to the genetics rather than the immunology of the prawns since baculoviruses isolated from insects contain anti-apoptotic genes called p35 which inhibits the proteolytic activity of cysteine proteases (Clem *et al.*, 1996). However, the roles of penaeid immunology in that phenomenon should be treated cautiously since research on penaeid immune reactions against baculoviruses is scarce. This problem arises from the lack of special interest in the immunology of cultured penaeids (Bachère, 1998; van de Braak *et al*., 2002).

To date, there is no report on penaeid immune reactions against penaeid baculoviruses. Johnson (1984, 1988) described rod-shaped and non-occluded viruses that infected the haemocytes of crabs *Carcinus maenas, C. mediterraneus* and *Callinectes sapidus*. However, Johnson (1988) did not explain the haemocyte response against the invading viruses, other than the appearance of milky and less densed hemolymph with high content of granules. Van de Braak *et al*. (2002) confirmed the presence of haemocytic granules around *P. monodon* tissues infected by WSSV, based on staining with monoclonal antibody WSH 8. However, previous studies showed that penaeid baculoviruses do not elicit haemocitic infiltrations into the chronically or acutely infected tissues (Lightner *et al.*, 1983; Duobrovsky *et al.*, 1988; Lightner, 1996; Flegel & Pasharawipas, 1998). Under this condition, the role of circulating cellular and humoral factors might not be important in prawn defence against baculoviruses. Van de Braak *et al*. (2002) argued that although not as massive as in bacterial infection, the granular haemocytes did migrate from circulation to tissues infected by penaeid viruses. Moreover, haemocytes produce lectins which are thought to be related to non-self recognition mechanisms in penaeids because they can bind to specific sugar derivatives on various cell surfaces (Marques and Barracco, 2000). It was found that the sugar derivative, *N*-acetylglucosamine can inhibit the lytic activity in the crayfish haemolymph (Johansson *et al*., 2000). Thus it is possible that the granular haemocytes binding to MBV-infected hepatopancreacytes can release such antihaemolytic substances to avoid the cytolysis of the infected cells. Obviously,

studies on cell-cell communication in prawn immune system could clarify the roles of prawn immune system in viral accommodation.

Either through genetic mechanisms or immune responses, or both pathways, viral accomodation provides a basis for quantitative approach. The proportion of abnormal cells due to nuclear hypertropy, cytoplasmic granulation, apoptosis, necrosis or cytolysis in any internal organ of a prawn may be useful to determine the general magnitude of the effects of baculoviruses on cells and tissues. Combine this data with total haemocyte count and disease prevalence along with survival and mortality rates, one might get a robust assessment of the effectiveness of prevention and control of viral diseases being applied. This approach can be coupled with a grading system of infection severity.

(b) Grading the severity of diseases in prawns

In the development of MBV infection in *P. monodon*, Lightner *et al.* (1983) observed that the assembly of complete virions was synchronised with the formation of OBs. Hence, complete virions are usually found abundantly in and outside of the OBs with an advanced infection. Therefore, the stages of OB formation are suitable for a quantitative index of MBV effect on the prawn organs or, population.

Lightner (1996) has proposed a generalized grading system of severity due to infectious or non-infectious diseases in prawns (Table 2.2.). This grading system transforms a qualitative data into a semi-quantitative data. When combined with other factors, such as target tissue or organ, stages of viral development and mortality or survival rate, this grading system can generate useful information for the understanding of a viral outbreak in farm or an other environment.

Table 2.2. A generalized grading system of severity of diseases in prawns (Lightner, 1996).

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(c) Severity of viral infection and organ tropism

Every particular prawn virus has a tendency to infect certain tissues or organs. For example, MBV and BP usually infect the midgut and hepatopancreas, while WSSV infects the cuticular epithelial cells and connective tissue cells (Lightner, 1996). Thus it is meaningful to correlate the severity of a viral infection to the organ tropism. Of all tissues or organs, hepatopancreas, anterior midgut and lymphoid organ usually change the most with viral infections.

1. Hepatopancreas and anterior midgut

Cellular changes associated with viral development in these organs can be quantified to describe the severity of the infection. For example, MBV development in the epithelia of hepatopancreas and anterior midgut of *P. monodon* can be described in three stages : early, developing and final (Lightner *et al.*, 1983; Doubrovsky *et al.*, 1988; Ramasamy *et al*., 2000). These stages can be indexed as 1, 2 and 3, respectively.

Stage 1 (the early stage) : The cytoplasm and organelles appear morphologically normal. Golgi aparatus and membranes of smooth endoplasmic reticulum proliferate with the formation of membranous labyrinths, and the numbers of secretory granules, vacuoles

and lipid droplets decrease. The nucleus is slightly swollen as the nucleolus migrates to the periphery, or disintegrates into a region of virogenic stroma, containing densely marginated chromatin, virogenic patches, and materials of viral envelopes and, sometimes a few complete virions. The cytoplasm is granular and basophilic, and looks like a woman's engagement or signet ring.

Stage 2 (the developing stage) : In the cytoplasm, the membranous labyrinths become larger and more copious, and the number of autopaghocytic vesicles and free ribosomes increases, while the granular endoplasmic reticulum decreases. A paracrystalline substance accumulates in the cytoplasm, but without virions. Early in the developing stage, the nucleus has enlarged further, and contains one or more weakly eosinophilic occlusion bodies. Free complete virions in the karyoplasm aggregate adjacent to the nuclear membrane or the virogenic stroma. Viral envelopes are produced in the central area of the karyoplasm and in the periphery of the virogenic stroma.

Stage 3 (the final stage) : The cytoplasm diminishes in size and becomes very dense with free ribosomes, large membranous labyrinths, large mitochondria and microfilaments. The nucleus is grossly hypertrophied due to the presence of numerous, large, deeply eosinophilic, spherical, single or multiple, occlusion bodies. Numerous complete virions exist in the virogenic stroma, and in between two layers of the nuclear membrane. Finally, the inner membrane ruptures followed by the outer membrane. The cell then undergoes necrosis and/or cytolysis releasing the occlusion bodies and virions. No budding of virions occurs through the nuclear or cellular membrane.

By counting the proportion of nuclei with each stage of the viral development in each susceptible organ of an indvidual prawn, a comparison of viral development can be made between organs and individuals from a batch or different batches, or from different species of prawns, or culture systems.

2. Lymphoid organ

Lymphoid organ (LO) of penaeid prawns is generally regarded as the major organ for antiviral defense and undergoes major changes during viral infection (Hasson *et al*., 1999 a, b; Anggraeni and Owens, 2000). Along with haematopoietic and ovary tissues, LO is considered to possess high generative capacity for tissue culture (Colorni, 1989). Normal lymphoid cells (known as stromal matrix cells) surround a central vessel, while abnormal cells clump into a spherical body without a central vessel (later known as spheroid cells) (Lightner *et al*., 1987; Bell and Lightner, 1988; Spann *et al*., 1995).

Owens (in Anggraeni and Owens, 2000) classified changes in lymphoid organ spheroid (LOS) into three phases: tumorous-like phase, fully encapsulated spheroid phase, and vacuolative degeneration of spheroids. Moreover, Hasson *et al.* (1999 b) introduced three different morphotypes of LOS as seen in the development of LOS in chronic phase of infection of Taura syndrome virus (TSV) in *P. vannamei*. The earliest LOS arises from LO tubule phagocytes called type A. The succeeding type B contains necrotic cells with positive TSV by *in situ* hybridization after an acute phase infection. The terminal type C LOS has cells with condensed basophilic nuclei, reduced size and progressive atrophy as shown by apoptotic cells.

Anggraeni and Owens (2000) compared the area occupied by LOS in lymphoid organ of farmed and laboratory-held *P. monodon*. The authors found that farmed prawns had a smaller area and smaller proportion of spheroids compared with the laboratory prawns. A comparison between indviduals, batches or species of prawns with or without baculoviral presence can also be done by comparing the proportion of each LOS morphotype in LO, or by counting the total number of LOS in lymphoid organ.

(d) Severity of viral infection and growth parameters of the prawn

Besides its relation to the organs, the severity of viral infection can be related to the growth parameters of the prawn. For example, Flegel *et al.* (1999) related the stunted growth of grow-out *P. monodon* to hepatopancreatic parvo-like virus (HPV), or a dual

infection by HPV and MBV. Among other things, the authors showed that either MBV-infected and uninfected prawns, or HPV-infected and dually-infected prawns, had similar length distributions. However, the severity of HPV infection had a negative correlation with the prawn length, while that of MBV had no relation to the prawn length. These data gave rise to a hyphothesis that HPV has a casual role in stunting, and warrants further investigation. This quantitative approach can be useful to understand the magnitude and management of infection by a cocktail of viruses in a farm, or a natural environment.

2.6.3. Quantitative approaches using epidemiology

Depending on the information needed by researchers, epidemiology is often an inter-disciplinary science involving statisticians, microbiologists, virologists, pathologists, serologists, clinicians, and so on (Campbell, 1983). Combined field and laboratory studies can reveal the possible causes and natural history of unknown diseases, followed by development and evaluation of strategies and resource allocation in the prevention, diagnosis and management of the disease (Campbell, 1983; Dawson-Saunders & Trapp, 1994; Christie *et al.*, 1997). For the study design, epidemiology of prawn diseases follows the theories and practices in human health and veterinary sciences as the origins of this study.

Veterinary epidemiology encompasses qualitative and quantitative assessment of a disease (Fig. 2.1.) (Thrusfield, 1986). The components of quantitative evaluation of veterinary epidemiology consist of modelling, studies (observational and experimental) and surveys (cross-sectional, monitoring and surveillance). They assist in the assessment of the etiology, mechanisms and economic impact of the disease in question.

Modeling simulates disease dynamics and the effects of various control strategies using mathematical formulas (Thrusfield, 1986). This approach has been used intensively in the field of human and animal epidemiology. Yet, no literature on epidemiology modeling was found for penaeid viruses.

Epidemiological studies are aimed at comparing groups of animals, while surveys deal only with description a group of animals (Thrusfield, 1986). Study designs in epidemiology are cross-sectional, case control, cohort and experimental studies. The first three are observational, i.e. without intervention or manipulation of the animals (Dawson-Saunders and Trapp, 1994). According to Thrusfield (1986), cross-sectional study focuses on the relationship between a disease and hypothesized causal factors. Case-control study deals with the outcomes of exposure to hypothesized (possible) causal factors or risk factors upon disease and healthy animals. Cohort study compares the disease development in animals exposed and not exposed to hypothesized causal factors. Sometimes these studies can take place at the same time. For example the report by Lightner *et al*. (1983) can be seen as cross-sectional study because it described the relationship of pathology and baculoviral infection in *P. monodon*. The same report also followed the outcomes (case-control study) and development (cohort study) of MBV in disease and healthy *P. monodon* postlarvae from Taiwan, Philippines and Tahiti. Of six independent populations observed, only two did not develop signs of MBV infection. The cellular pathology and ultrastructure of MBV were described. In the populations of MBV-infected prawns, the prevalence and severity of MBV and mortality rate increased with crowding, gill disease and bacterial infections, but decreased with age. Antibacterial and formalin treatment that reduced bacterial and fouling organisms also decreased the mortality rate due to MBV. This treatment falls into experimental (intervention) study as defined by (Thrusfield, 1986; Dawson-Saunders and Trapp, 1994). Here, human intervention changed the potential or actual development of MBV disease together with other pathogenic diseases. Therefore, it is possible to run observational and experimental studies at once by using some quantitative approaches. Experimentation confirms the results of logical quantitative analysis of observations (Peller, 1967).

Surveys usually use cross-sectional design, although some can also use case-control and cohort designs (Dawson-Saunders and Trapp, 1994). Lightner (1996) has compiled the results of surveys of penaeid viruses from around the world, including the species of susceptible penaeids, diagnostic methods, geographic distribution, characters of the viruses, environment, disease management and rearing systems. One type of survey is

diagnostic screening in which a quick test separates healthy from disease individuals (Thrusfield, 1986). Diseased individuals are then subjects for further examination. For example, Ramasamy *et al*. (2000) used routine histology to screen for and describe MBV from cultured larvae and wild caught broodstocks of *P. monodon* from the east coast of India.

Monitoring and surveillance deal with routine recording and transmission of data on health status, productivity and environmental conditions, although the latter is more intensive in data recording (Thrusfield, 1986). In prawn hatcheries and, semi-intensive and intensive grow out ponds, these activities are part of daily management and operation (Apud, 1988; Licop, 1988). Since international movement of penaeid larvae and broodstock increases with global development of penaeid aquaculture, the need for global joint venture in monitoring and surveillance becomes paramount to prevent and control penaeid viruses. Failure to include many farmers and technicians in this strategy can continue to lose billions of dollars to viruses. Again, standardized, applicable and user-friendly quantitative approaches may contribute to such an important strategy.

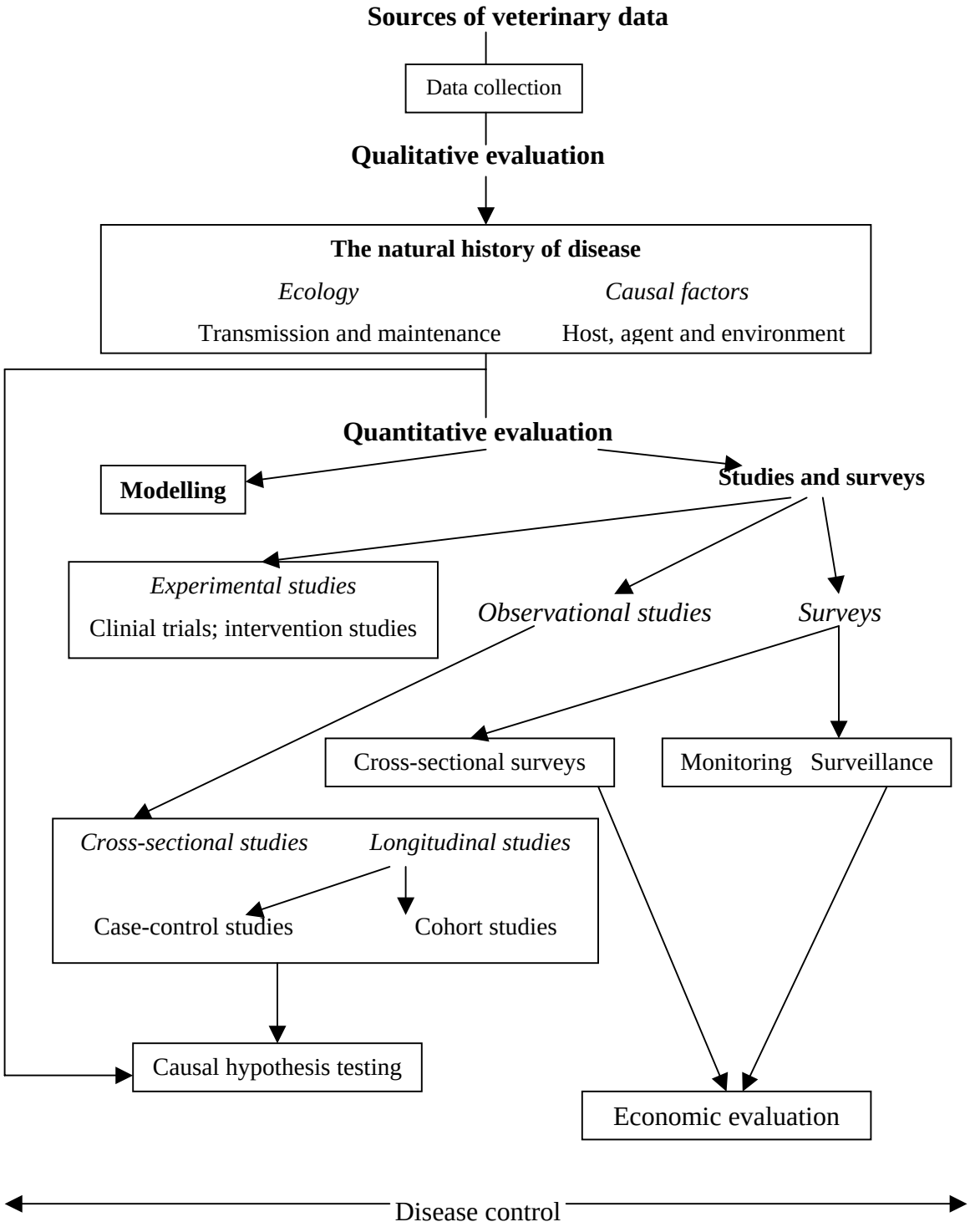


Figure 2.1. Components of veterinary epidemiology (adapted from Thrusfield (1985) in Thrusfield, 1986).

2.7. Discussion

Scientific studies into penaeid viruses have produced data on the strains, diseases, diagnosis and control of viral diseases in aquaculture, such as documented by Johnson (1984), Johnson and Lightner (1988), Ahne (1994), Bower *et al*. (1994) and Lightner (1996). Yet, some information needs to be clarified, such as geographical distribution of strains and potential hosts of viruses (Spann and Lester, 1996), stress and nutritional links to the prevalence of the viral diseases (Stuck *et al*., 1996), prevention of the diseases through the application of immunostimulants and probiotics (Rengpipat *et al*., 1998; Uma, 1999), and good husbandry practices in general (Subasinghe, 1995) as well as efficiency and cost-benefit analysis of methods for diagnosis and control of the diseases (e.g. Dagger and Jory, 1999) and cost of the disease. These are potential opportunities for research and development.

The proposal of active viral accomodation by Flegel and Pasharawipas (1998) is quite novel in adding a new perspective in understanding the baculovirus-penaeid relationship. However, new viral diseases keep emerging and the list of diseases of cultured penaeids is increasing. Penaeid viruses have also infected wild prawns from aquaculture facilities, such as in US (Iversen, 1997) and Thailand (Supamattaya *et al.*, 1998). With the expansion of penaeid aquaculture worldwide, it is reasonable to expect that such phenomena is also taking place around the world. In particular, in developing countries in Asia, where aquaculture health management is not always applied (Subasinghe and Barg, 1998), transmission of viral diseases from cultured to wild penaeids might be widespread. This kind of potential threat justifies the need for more education, research and extension services in the field of penaeid virology.

Bachère (2000) stresses the need for a network of simultaneous research in immunology, genetics, physiology, environment and pathology of the cultured prawns. In prawn virology, Lightner *et al*. (1990) propose for the development of diagnostic methods and procedures which are highly sensitive, easily standardized, inexpensive and technically simple. Indeed, it has been more than two decades since the application of advanced

molecular techniques in the diagnostics and research of prawn viral diseases (Vaughan, 1996). These technologies increase the diagnostic sensitivity, accuracy and speed (Benzie, 1998). However, many farmers and technicians in third world countries may not take part in this exciting drive for excellence. They lack training (Liao, 1989; Fulks and Main, 1992) and, perhaps, basic education in prawn virology and diagnostic methods. To bridge the gap, quantitative approaches as outlined above might be useful in integrated actions to prevent and control prawn viruses around the world.

Quantitative approaches may also play an important role in research and the decision making process of penaeid virology. They can operate with basic histology, light microscopy, database maintainance and statistical analysis. As more accurate diagnostic methods are available, combining all features of viral morphology, cellular and histological pathology and epidemiology appears a powerful tool for speedy decision making process in the prevention and control of viral diseases in penaeid aquaculture.

CHAPTER 3
GENERAL METHODOLOGY

3.1. Prawn Fixation

Routine histology was used to produce tissue sections for analyses of viral occlusion bodies, tissue tropism, pathological severity and, heart and lymphoid organ histopathological changes in prawns. The prawns were preserved in Davidson's fixative (Bell and Lightner, 1988) as per Appendix 3. For juvenile prawns, the fixative (0.5 mL in volume) was directly injected into the hepatopancreas and adjacent areas of cephalothorax, whereas the larvae were directly immersed in the fixative. The ratio of tissue to fixative was 1:10 for 24-72 hours, after which the samples were transferred to 50% ethanol for an indefinite time.

For embedding preparation, the cephalothorax was cut in half longitudinally, placed in a histocassette, transferred to 70% ethanol and then processed for routine histological examination using standard paraffin embedded procedures (Bell and Lightner, 1988). The larvae were also embedded using the same procedure, except they were pressed between two square sponges inside the histocassette.

3.2. Tissue Embedding

Tissues were dehydrated in graded ethanol (70%, 80%, 90%, 95%, 95% and 100%), cleared in xylene and stored in paraffin wax (Paraplast). This took place in a Shandon-Elliot processor. A Tissue-Tek II embedding center was used to embed the tissues in paraffin wax. Sections were cut at 5 μm using a microtome and dried in an oven at 60 ^{o}C overnight.

3.3. Hematoxylin and Eosin Staining

The following procedure was applied in the histology laboratory of the School of Biomedical Sciences, JCU. Dried sections were processed for haematoxylin and eosin

(H&E) stain as follows: rehydrated through 2 x two-minutes in xylene, 1 x two-minutes in absolute ethanol, 2 x one-minute in absolute ethanol, 1 x one-minute in tap water, stained in Mayer's haemotoxylin (Appendix 3.2.) for 8 minutes, immersed in tap water for 30 seconds, and in Scott's tap water substitute (Appendix 3.3.) for 30 seconds, counterstained in Young's eosin (Appendix 3.4.) for 4 minutes, differentiated in tap water for 1 minute, dehydrated in 2 x ten-dips in absolute ethanol, cleared in 1 x two-minutes in xylene, 1 x one-minute in xylene, and mounted in a DPX (dibutylphthalate-polystyrene-xylene). The slides were dried in an incubator at 37 ^{o}C for at least one hour.

3.4. Microscopy and Micrometry

Tissue sections were examined microscopically using a Labourlux K light binocular microscope (Leitz®, Germany). The sizes of viral occlusion bodies, cells or nuclei were measured using a 10X objective micrometer (Olympus®, Tokyo, Japan).

3.5. Microphotography

Microphotography was carried out using a photomicroscope BH2 Olympus® (Japan) connected to an Olympus® video digital camera. The results were transferred to Microsoft® Photo Editor 3.0 for editing and printing purposes.

3.6. Computer Software for Statistical Analyses

All statistical analyses were done using computer software Statistical Program for Social Sciences (SPSS) version 11.0 and Microsoft® Excel 2002 for Windows 2000 Professional.

3.7. Data Screening

Data used for statistical analysis were screened to remove suspected extreme outliers by using the stem-and-leaf and box plot analyses. These analyses identified extreme data

points that are anomalous and likely to be erroneous, such as a very high oxygen concentration in high temperature and high salinity conditions.

3.8. Measures of Variability, Frequency Distribution and Confidence Limits

The dispersions of quantitative data were generally presented as mean and standard error (SE) of the mean. In certain cases, the frequency distribution and confidence interval limits of the mean were presented.

3.9. Analysis of Variance and Post-Hoc Test

All quantitative data for one-way analysis of variance (ANOVA) were subjected to normality test (Q-Q plots) to determine their suitability for Univariate ANOVA with $p < 0.05$ as the significant level for all statistical tests. For $p < 0.01$, the notation is $p << 0.05$, whereas for $p > 0.10$ the notation is $p >> 0.05$. Non-normal data were transformed to natural logarithmic or another suitable transformation, and tested for normality (Q-Q test) again until a normal distribution was found. The Least Square Difference (LSD) test was run when the ANOVA returned significant differences.

3.10. Analysis of the Strength of Associations

Pearson's product moment correlation test (Pearson's test) was used through out the study to describe the probability of correlation between two different variables. The description of the relationship was shown with a scattergram produced by Microsoft® Excel. The strength of correlation between the variables was judged according to the product moment or Pearson's correlation coefficient (*r*).

Simple or multiple regression analysis was also carried out to find the adjusted coefficient of determination (R^2), slope and intercept of the correlation model between two or more variables. The value of adjusted R^2 determines the percentage of variation represented in a straight line relationship between two variables (Fry, 1993). In this study, the

magnitude of the correlation (based on R) was determined according to Morton *et al*. (1990), i.e. strong (R = 0.8 – 1.0), moderate (R = 0.5 – 0.8), weak (R = 0.2 – 0.5) and negligible (R = 0 – 0.2). The trendline and regression equation for all regression models were produced by Microsoft® Excel and analyzed for statistical significance with SPPS program.

Odds ratio (φ) assessment was used to measure the degree of association between diseases or abnormalities in a prawn population with pathogens or environmental stressors based on a 2 x 2 contingency table (Thrusfield, 1986).

$$\textbf{Odds ratio } (\varphi) = (a \times d) / (b \times c)$$

in which, a = the number of samples containing diseases or abnormalities with the presence of pathogens or environmental stressors; b = the number of samples not containing diseases or abnormalities with the presence of pathogens or environmental stressors; c = the number of samples containing diseases or abnormalities with the absence of pathogens or environmental stressors; d = the number of samples not containing diseases or abnormalities with the absence of pathogens or environmental stressors.

Using the same 2 x 2 contingency table, an attributable risk (δ) was calculated to measure the extent of risks of diseases or abnormalities associated with pathogens or environmental stressors (Thrusfield, 1986).

$$\textbf{Attributable risk } (\delta) = 1 - [[c/(c+d)] \div [(a+c)/(a+b+c+d)]]$$

3.11. Prevalence

The prevalence of diseases or abnormalities was measured as “time-point prevalence”, i.e. the prevalence of a disease in a designated point of time, assuming that a disease was in progress when a prawn was sampled.

3.12. Grading System of the Severity of Infection

A grading system of severity of diseases in prawns (Table 2.2.) was adopted from Lightner (1996) and simplified as follows: grade 0 (G0) = no lesion or infection seen, grade 1 (G1) = lesions or infection present in < 25% area of an organ or tissue section, grade 2 (G2) = lesions or infection present in > 25 - 50% area of an organ or tissue section, grade 3 (G3) = lesions or infection present in > 50 – 75% area of an organ or tissue section, and grade 4 (G4) = lesions or infection present in > 75% area of an organ or tissue section.

CHAPTER 4

MORPHOLOGY AND HISTOPATHOLOGY OF MONODON BACULOVIRUS (MBV) FROM AUSTRALIAN *P. monodon* AND *P. merguiensis*

4.1. Introduction

Monodon baculovirus (MBV) is typically prevalent in the postlarvae (PLs) of susceptible penaeids (Doubrovsky *et al*., 1988; Baticados *et al*., 1991; Chen *et al*., 1992; Natividad and Lightner, 1992; Lightner, 1996; Ramasamy *et al*., 2000). This virus infects the hepatopancreas (HP) and midgut (MG) epithelia of penaeids (Lightner and Redman, 1981; Vogt, 1992). Therefore, PL stages and both anatomical sites are suitable for MBV diagnosis. Histologically, spherical eosinophilic occlusion bodies (OBs) are present in MBV-infected cells (Lightner, 1996). Yet, in favourable environment, heavily infected penaeids can well accommodate the virus (Flegel and Pasharawipas, 1998). This viral accomodation is useful for quantitative analysis of the morphology and cytopathology of MBV in penaeids by measuring the diameters and numbers of MBV OBs, numbers of infected and healty nuclei, size of infected and healthy cells, and so on.

Doubrovsky *et al*. (1988) have described the similarities in the ultrastructure and cytopathology of MBV in *P. monodon* and in *P. merguiensis* from Australia. However, these MBV-type viruses may be distinct viruses or a single virus displaying differently in various hosts (Lightner, 1996). The multiple hosts and variants of MBV provide models for the development of simple quantitative analysis on phenotype and cytopathology of MBV in different penaeids. By using a histological archive of prawn samples, this analysis may also establish the occurrence of prawn diseases in the past through a longitudinally retrospective study as defined by Thrusfield (1986).

4.2. The Objectives of the Study

In prior observations, MBV in *P. monodon* was seen exhibiting distinct morphometrics and tissue tropism from MBV in *P. merguiensis*. Thus, this study was aimed at

applying quantitative analyses to characterize and differentiate the features of MBV and its histopathology in *P. monodon* and *P. merguiensis*

A reference of quantitative features of MBV in Australian *P. monodon* and *P. merguiensis* may be useful for comparison with serological and genomic studies of MBV from both prawn species. Information of the origins of prawn samples may illustrate limited history of viral diseases in Australian and Indonesian penaeids.

4.3. Materials and Methods

A longitudinally retrospective study was carried out to characterize MBV using quantitative methods. The studied features included MBV prevalence, cytopathology, severity of MBV infection, OB morphometrics, number of OB(s) in infected nuclei and tissue tropism in both penaeid species. Also noted were the origins of the prawns and time of submission. Microscopic examination used a Labourlux K light microscope (Leitz®, Germany). All quantitative data were analysed statistically using Microsoft Excel 7.0 and SPSS 10.0 for Windows 97.

4.3.1. Prawn samples

The archive of the Discipline of Microbiology and Immunology JCU, preserved 174 blocks and 201 H&E-stained histological slides of prawns that were useful for this study. The samples were submitted to the Discipline between 1988 – 2002 (Table 4.1. & Appendix 4). They were submitted by 6 farms in Australia, 2 farms in Indonesia, and 1 field sampling of wild penaeids from Indonesia. The life stages of the prawns varied from postlarvae (PLs) to adults (spawners). Nevertheless, only cultured *P. monodon* and *P. merguiensis* from Australia were of particular interest for the quantitative study.

Table 4.1. Histological archives of penaeids in JCU submitted from 1988-2002.

Year	No. of Entries	No. of Slides	Prawn Species and Origins
1988	3	20	Farmed *P. monodon* and unknown species from Australia

1989	2	27	Farmed *P. monodon* and unknown species from Australia
1990	8	97	Farmed *P. monodon* from Australia and Indonesia
1991	2	42	Farmed *P. monodon* from central Java (Indonesia)
1992	1	32	Farmed *P. monodon* from Australia
1999	2	6	Farmed *P. monodon* from Australia
		13	Farmed *P. merguiensis* from Australia
2000	2	22	Farmed *P. merguiensis* from Australia and wild caught *P. merguiensis* from Kupang Bay (Indonesia)
		13	Farmed *P. monodon* from Australia and wild caught *P. monodon* from Kupang Bay (Indonesia)
2001	2	54	Farmed *P. monodon* from Australia and used for bioassays
2002	3	51	Farmed *P. monodon* from Australia and used for bioassays, and farmed *P. monodon* and *P. vannamei* from Bali (Indonesia)

4.3.2. Prevalence study

The prevalence study was only for the PL stages because each histological block of PLs contained more than one individual animal. Thus, it was possible to compare MBV prevalence in PLs based on prawn species and anatomical sites. Here, each block was arbitrarily regarded as an independent population despite their origins. Only 28 blocks were properly labelled with the name of the prawn species, or withstood routine histological procedure. Therefore, only 22 slides with 282 *P. monodon* postlarvae and 6 slides with 56 *P. merguiensis* postlarvae were available for this prevalence study.

4.3.3. Anatomical sites and cytopathology of MBV infection

This study focused on histological blocks of MBV-infected PLs and juveniles to acquire as many as possible cells with MBV (Table 4.1 & Appendix 4).

Using a 40X light microscope, each slide was examined systematically to include all prawn samples in the slide. For each prawn, the numbers of normal and MBV-infected cells in each anatomical site (HP or MG) were quantified. In HP, the number of cells in each tubule was counted, and the results from all tubules were then totalled. Likewise, the total number of MG epithelial cells of the same prawn were counted. Only one field of observation was used on each anatomical site of a prawn due to its small size. Since

the number of cells in HP or MG varied from prawn to prawn, the number of infected cells for each anatomical site in each prawn was expressed as a percentage of the total number of cells. This allowed for quantitative comparasion of the data between prawn species or anatomical sites.

The pathological conditions of individual cells in each tissue were characterised as stages of nuclear change due to MBV infection (Fig. 4.1.) as described by Lightner *et al.* (1983) and Doubrovsky *et al.* (1988), namely:

(a) *Stage 1 (S1)*: slightly hypertrophied nucleus, nucleolus migrates to the periphery (dense accumulation of basophilic material) and chromatin margination.
(b) *Stage 2 (S2*): increased hypertrophied nucleus and developing one or more weakly eosinophilic occlusion bodies.
(c) *Stage 3 (S3*): grossly hypertrophied nucleus with well developed eosinophilic occlusions.

For quantitative analysis, each stage of infection was assigned a numerical value, i.e. 1 (one), 2 (two) and 3 (three) for S1, S2 and S3 stages, respectively. Then, the percentage of stage 1, 2, or 3-MBV nuclei within the total nuclei seen under an observation field of a 40X light microscope, was determined for each target tissue in each individual prawn.

Subsequently, a sum of multiplication between a ratio of infected nuclei and its corresponding infection stage produced a time-point severity index (SI) for a prawn assuming the prawns were sampled when the infection was in progress. For each anatomical site of individual prawns, SI was obtained using the following equation:

$$\mathbf{SI = \Sigma \{the\ numerical\ value\ of\ S_n\ X\ ratio\ of\ nuclei\ with\ S_n\}}$$

in which: S_n = the stage of MBV infection, where n = 1, 2 or 3. Ratio of nuclei with S_n is the number of nuclei with S_n, divided by the total number of cells seen.

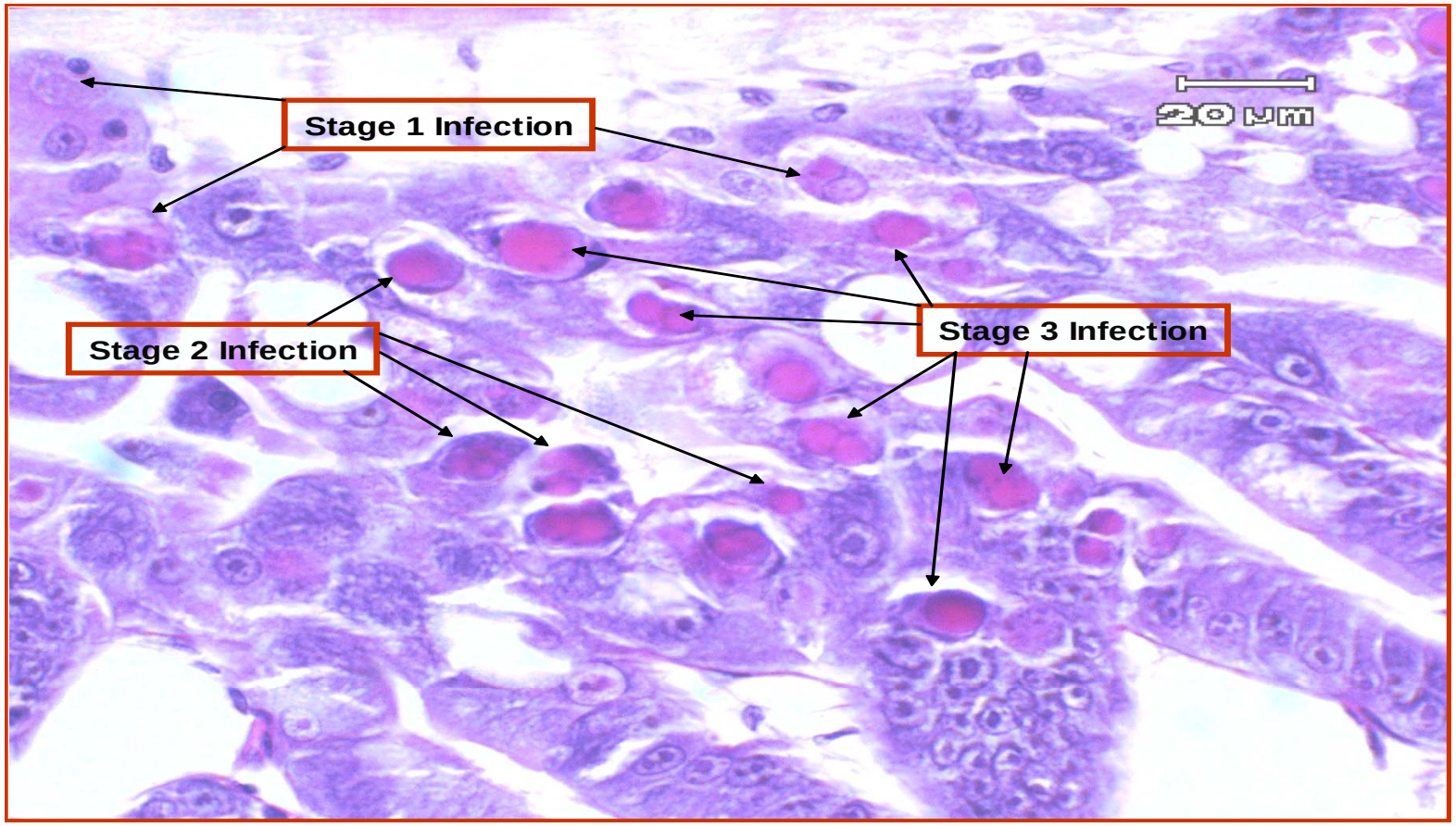


Figure 4.1. Stages of MBV infections based on occlusion body development in the nuclei. H&E stained.

Although cellular changes normally accompany viral development and replication in penaeid prawns, apoptosis (generalized programmed cell death), necrosis or lysis of the host's cells may not necessarily occur (Fegan *et al.*, 1991; Spann and Lester, 1996; Flegel and Pasharawipas, 1998). The cellular changes might be due to other diseases or natural causes. Here, the apoptotic, necrotic or lysed cells were regarded as 'abnormal' cells as opposed to healthy cells. Therefore, the ratio of abnormal cells in an organ can be calculated within the same field of observation as the severity analysis. The ratio of abnormal cells (including cells containing MBV) in the total number of cells was determined using the following formula:

$$\textbf{Ratio of Abnormal Cells } (R_{ac}) = (N_{ac}/N_t) \times 100\%$$

in which, N_{ac} = number of abnormal cells and N_t = total number of cells seen.

4.3.4. Morphometrics of MBV occlusion bodies

Two hundred and thirteen OBs from HP and MG of both prawn species were analyzed. The number of OBs in each infected nucleus of each anatomical site in each individual prawn was counted under a 40X light microscope. The diameter of each OB was measured using a 10X objective micrometer (Olympus®, Tokyo, Japan). The data were grouped based on prawn species, anatomical sites and number of OB in nuclei.

4.3.5. Statistical analyses

All quantitative data were subjected to normality test (Q-Q plots) to determine their suitability for parametric tests with $p < 0.05$ as the significant level. Non-normal data were transformed to natural logarithmic values. The variability of the means of OB diameters were analysed concurrently with a generalised linear model ANOVA across three independent variables, i.e.: (1) prawn species (*P. monodon* and *P. merguiensis*), (2) anatomical sites (HP and MG), and (3) number of OB per cell. In the latter case, Least Square Difference (LSD) was run when the ANOVA result was significant. The same

statistical procedures were used to analyse the prevalence of MBV based on prawn species and anatomical sites.

Pearson's correlation analysis or regression analysis was also conducted to determine the degree of correlation between R_{cc} (ratio of cytolytic cells) and SI (severity index) of MBV infection, and between the number of OBs/cell with the diameters of OBs.

4.4. Results

4.4.1. Histological archives of the prawns

Samples submitted from 1993 to 1998 were not included in this study (Table 4.2. and Appendix 4) because they were samples of diseases other than baculoviruses. However, samples from 2001 to 2002 were included although they did not have MBV because they were used for a bioassay related to the study in Chapter 5.

Samples between 1988 to 2000 (excluding 1993 – 1998) from 5 farms in Australia and 1 farm in Indonesia contained MBV (Table 4.2 & Appendix 4.). Even some samples submitted in 1988, 1990 and 1992 from Australia contained dual infection of MBV and a non-occluded virus. However, after the year 2000, these viruses were not seen.

Table 4.2. Baculoviral occurrence in penaeid histological archives of JCU (1988-2002)

Year	Prawn Species and Diagnosis
1988	*P. monodon* juveniles and unknown species; none to heavy MBV & a non-occluded virus in hepatopancreas (HP).
1989	*P. monodon* larvae and unknown prawn species; Very low MBV in HP.
1990	Mostly *P. monodon* larvae. None to heavy MBV in HP & a non-occluded virus
1991	*P. monodon* postlarvae and adults. None to heavy MBV.
1992	*P. monodon* juveniles; MBV and a non-occluded virus.
1999	*P. monodon* PLs; None to moderate MBV.
	P. merguiensis PLs; None to heavy MBV.
2000	*P. merguiensis* larvae and juveniles; None to heavy MBV.
	P. monodon larvae and juveniles; None to heavy MBV.
2001	*P. monodon* juveniles; no MBV; used for bioassays.
2002	*P. monodon* and *P. vannamei* juveniles; no MBV; used for bioassay.

4.4.2. MBV prevalence based on species and anatomical sites

The prevalence of MBV was distributed non-normally, so it was normalized using a transformation power of 1.46. The prevalence varied with species and anatomical sites (Table 4.3.). The range of MBV prevalence in *P. monodon* (0 to 100%) was wider than that in *P. merguiensis* (20 to 100%). However, *P. merguiensis* had a significantly higher mean of MBV prevalence (80 ± 7%) than that in *P. monodon* (30 ± 5%) (*t (2-tailed) = -4.8; df = 54; p << 0.05*).

The mean values of MBV prevalence based merely on the anatomical sites, when data from both prawn species were combined, were not significantly different (*F = 0.01, df = 1, 52; p >> 0.05*). This was because the mean values of MBV prevalence in MG of both prawn species were spread widely from 11 ± 4% in *P. monodon* to 91 ± 6% in *P. merguiensis* (Fig. 4.2). These values intersected with those of the HP of both prawn species, i.e., from 50 ± 8% in *P. monodon* to 68 ± 11% in *P. merguiensis* (Fig. 4.2.). However, the mean values of prevalence were significantly different based on the joint effects of anatomical sites and prawn species (*F = 14.7; df = 1, 52; p << 0.05*). The anatomical site factor alone had non-significant effects on MBV prevalence.

Table 4.3. Prevalence of MBV infection among infected batches of *P. monodon* and *P. merguiensis* and anatomical sites of the prawns.

Species	Anatomical Sites	N (slide)	Prevalence (%)				
			Min	Max	Mean	SE	SD
Penaeus monodon	Hepatopancreas	22	0	100	50	8	37
	Midgut	22	0	50	11	4	17
	Pooled	44	0	100	30	5	35
Penaeus merguiensis	Hepatopancreas	6	20	100	68	11	27
	Midgut	6	67	100	91	6	14
	Pooled	12	20	100	80	7	24

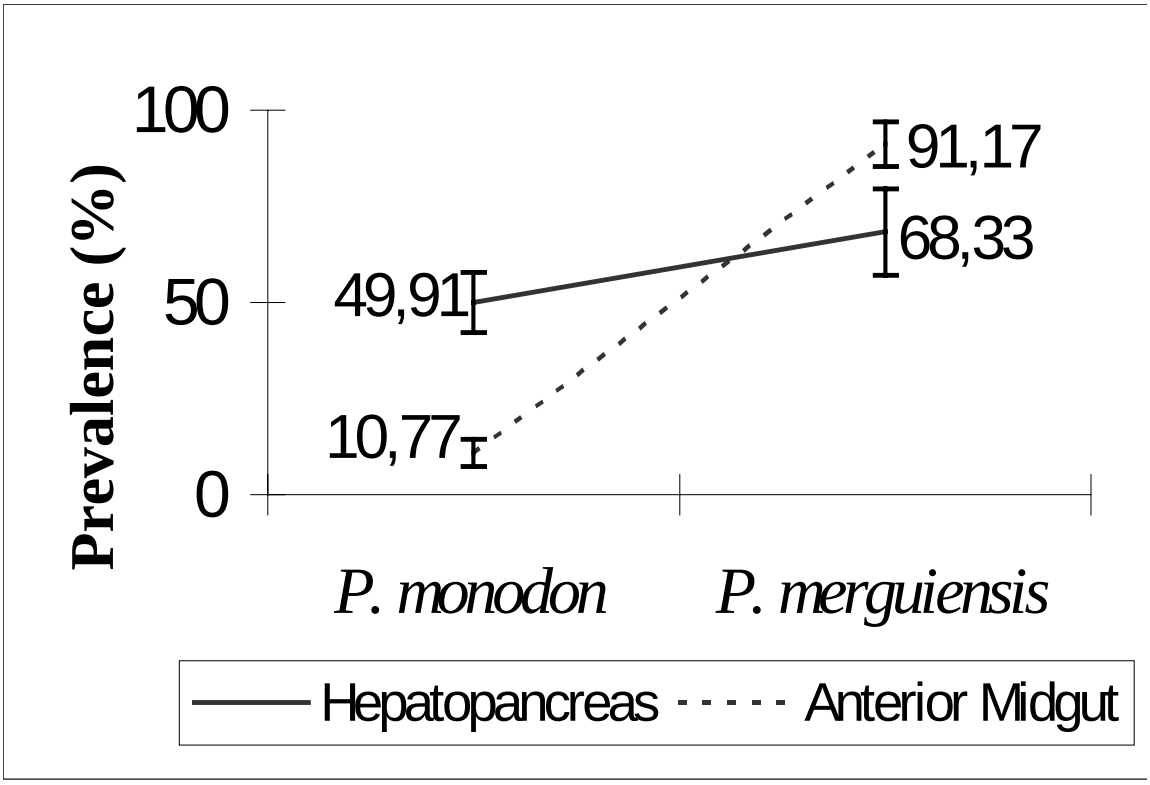
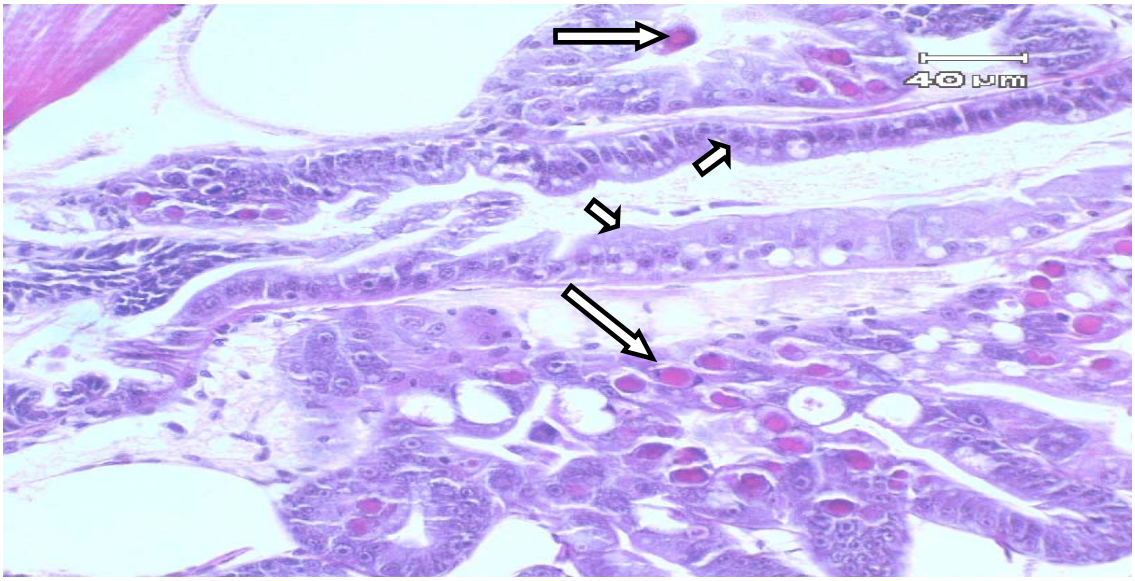


Figure 4.2. The mean prevalence of MBV infection based on species and tissues. The bars represent standard error measurement (SE).

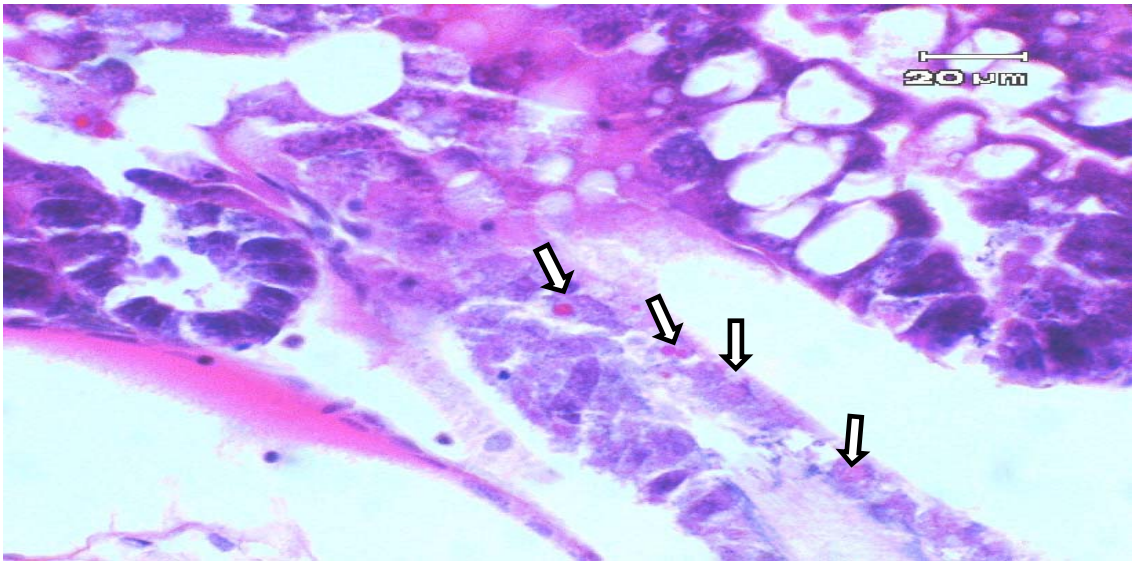
Tissue tropism of MBV in both penaeid species was also apparent in histology (Fig. 4.3.). In *P. monodon*, MBV was found more abundant in the HP than in the MG. The opposite occurs in *P. merguiensis* where MBV was seen along the MG epithelial cells.

4.4.3. Stages of nuclear infection and ratio of infected nuclei (R_{in})

Since some slides contained HP or MG only, the sample size for this particular study varied for each anatomical site in each prawn species (Table 4.4.). The occurrences of MBV in the HP and MG of *P. monodon* were 78% and 85%, respectively. The occurrence of MBV for *P. merguiensis* was 74% in HP and 100% in MG. In non-infected prawns, two samples of MG in *P.monodon* and three samples of HP in *P. merguiensis* were undergoing necrosis.



(a) *Penaeus monodon*



(b) *Penaeus merguiensis*

Figure 4.3. Tissue tropism of MBV. (a) *P. monodon*: MBV occlusion bodies in the hepatopancreatic tubules (long arrows) and healthy epithelia of midgut (short arrows). (b) *P. merguiensis*: MBV occlusion bodies along the epithelial cells of midgut (arrows) and healthy hepatopanceatic tubules. H&E stained.

Table 4.4. The sample sizes of each anatomical site in each prawn species with absence or presence of MBV infection.

Species	Anatomical Site	MBV Absent		MBV Present		Total	
P. monodon	Hepatopancreas	26	22%	92	78%	118	100%
	Mid-gut	14	15%	80	85%	94	100%
P. merguiensis	Hepatopancreas	9	26%	26	74%	35	100%
	Mid-gut	0	0%	27	100%	27	100%

In MBV-infected samples, the mean proportion of infected nuclei (R_{in}) for *P. monodon* (64±3%) and that for *P. merguiensis* (46±3%) (Table 4.5.) differed significantly (*t (2-tailed) = - 3.9; df = 260; p << 0.05*). However, the mean R_{in} in HP (61 ± 3%) and that in MG (53 ± 3%) (Table 4.5.) did not differ significantly (*t (2-tailed) = 1.6; df = 260; p >> 0.05*). The stages of infection caused significant differences in the mean R_{in} (*F = 10.1; df = 2, 250; p << 0.05*). Hence, the pooled means of nuclear proportions for S1 (66 ± 3%) and S3 (61 ± 4%) did not differ significantly, but they were significantly higher than that of S2 nuclei (35 ± 4%) (Table 4.5.).

Table 4.5. The mean percentage ± SE of the proportion of nuclei of HP and MG of *P. monodon* and *P. merguiensis* with different stages of MBV. N = the number of animals with a certain stage of MBV infection. *Total number of animals with MBV present (Table 4.4.). Different characters denote a significant difference (p > 0.05).

Species	Sites	Parameter	S1	S2	S3	Pooled
P. merguiensis	HP	N	15	9	13	26*
		Mean±SE	$56 \pm 7^{a,c}$	28 ± 6^{b}	46 ± 8^{a}	$46 \pm 5^{\#}$
	MG	N	16	15	22	27*
		Mean±SE	$34 \pm 7^{b,e}$	30 ± 5^{b}	$64 \pm 5^{c,d}$	$45 \pm 4^{\#}$
	Total	Mean±SE	$45 \pm 5^{(1)}$	$29 \pm 4^{(2)}$	$58 \pm 5^{(3)}$	$46 \pm 3^{\Diamond}$
P. monodon	HP	N	65	22	31	92*
		Mean±SE	74 ± 5^{d}	$35 \pm 8^{b,e}$	$69 \pm 7^{c,d}$	$65 \pm 4^{\$}$
	MG	N	29	14	11	80*
		Mean±SE	73 ± 7^{d}	$43 \pm 10^{a,e}$	$53 \pm 12^{a,c,e}$	$61 \pm 5^{\$}$
	Total	Mean±SE	$74 \pm 4^{(4)}$	$38 \pm 6^{(1,2)}$	$65 \pm 6^{(4)}$	$64 \pm 3^{\Diamond}$

There was no significant effects on the proportion of infected nuclei with stages of infection and prawn species (*F = 2.6; df = 2, 250; p >> 0.05*), the stages of infection and anatomical sites (*F = 1.2; df = 2, 250; p >> 0.05*), or the prawn species and anatomical sites (*F = 0.04; df = 1, 250; p >> 0.05*). However, the interaction between prawn

species, stages of infection and anatomical sites caused significant differences on the mean proportion of infected nuclei (*F = 3.3; df = 2, 250; p < 0.05*). For example, the mean proportion of S1 nuclei in *P. monodon* HP (74 ± 5%) was significantly higher than that in *P. merguiensis* HP (56 ± 7%) (Table 4.5.).

In *P. monodon*, the mean proportion of infected nuclei for HP (65 ± 4 %) and that for MG (61 ± 5%) (Table 4.5.) did not significantly differ (*t (2-tailed) = 0.61; df = 170; p >> 0.05*). Furthermore, the proportion of HP nuclei with a particular infection stage of MBV did not differ significantly with that of MG nuclei containing the same infection stage (*F = 0.87, df = 2, 172, p >> 0.05*). For example, the mean proportion of S3 in *P. monodon* HP (69 ± 7%) did not differ significantly from that in its MG (53 ± 12%) (Table 4.5.) However, they differed significantly based on the stage of infection (*F = 10.31, df = 2, 172; p << 0.05*). Hence, the mean proportion of S2 nuclei (38 ± 6%) in *P. monodon* was significantly lower than those of S1 (74 ± 4%) and S3 nuclei (65 ± 6%), while those of S1 and S3 nuclei did not differ significantly (Table 4.5.).

In *P. merguiensis*, the mean proportion of infected nuclei in HP (46±5%) was not significantly different from that in MG (45±4%) (Table 4.5.), (*t (2-tailed) = 0.11; df = 88; p >> 0.05*). However, they were significantly different with the interaction between the stages of infection and anatomical sites (*F = 5.2; df = 2, 89; p < 0.05*). Hence, the S1 nuclei (56±7 %) were dominant in HP like the S3 nuclei (64±5 %) were in MG (Table 4.5.).

Furthermore, in *P. merguiensis*, the mean proportion of infected nuclei was significantly different based on the infection stage (*F = 7.2, df = 2, 89; p < 0.05*). Here, the proportion of nuclei with S3 infection was significantly higher (58 ± 5%) than S1 (45 ± 5%) and S2 (29 ± 4%) (Table 4.5.). However, the proportion of S1 nuclei was significantly higher than that of S2 nuclei ($p << 0.05$).

4.4.4. Severity index (SI) and ratio of abnormal cells (R_{ac})

The mean SI based on the number of the infected cells for *P. monodon* (1.6 ± 0.1) was significantly smaller than that for *P. merguiensis* (2.1 ± 0.1) (Table 4.6.), (*t (2-tailed) = - 4.2; df = 213; p << 0.05*). Likewise, the mean of total R_{ac} of *P. monodon* (34 ± 3%) was significantly lower than that of *P. merguiensis* (76 ± 5%) (Table 4.6.), (*t (2-tailed) = - 6.7 (df = 213; p << 0.05*).

Table 4.6. Cell-based severity index (SI) and ratio of abnormal cells (R_{ac}) in different anatomical sites of different prawn species. n = the number of anatomical organ.

Prawn Species	**Anatomical Sites**				**Total**	
	Hepatopancreas		**Mid-Gut**			
	SI	R_{ac} (%)	SI	R_{ac} (%)	SI	R_{ac} (%)
P. monodon	1.6±0.1 (n=127)	28±3 (n=127)	1.5±0.1 (n=44)	56±8 (n=44)	1.6±0.1	34±3
P. merguiensis	1.9±0.1 (n=19)	49±7 (n=19)	2.4±0.1 (n=25)	91±3 (n=25)	2.1±0.1	76±5

The means of SI also differed significantly based on anatomical sites (*F = 8.1, df = 3, 214, p << 0.05*). Here, the mean SI for *P. merguiensis* MG (2.4 ± 0.1) was significantly higher than those for *P. merguiensis* HP (1.9 ± 0.1), and *P. monodon* HP (1.6 ± 0.1) and MG (1.5 ± 0.1) (Tables 4.6. and 4.7.). However, the mean SI of the last three sites did not differ significantly from one another (Table 4.7).

Table 4.7. The levels of significant difference of SI and R_{cc} means by LSD test based on anatomical sites of both prawn species.

		P. monodon		***P. merguiensis***	
		HP	**MG**	**HP**	**MG**
P. monodon	**HP**		0.2 (SI) << 0.05 (R_{ac})	0.3 (SI) << 0.05 (R_{ac})	<< 0.05 (SI) << 0.05 (R_{ac})
	MG			0.1 (SI) 0.5 (R_{ac})	<< 0.05 (SI) << 0.05 (R_{ac})
P. merguiensis	**HP**				< 0.05 (SI) << 0.05 (R_{ac})

All means of R_{ac} for different anatomical sites of both species differed significantly from each other at $p << 0.05$, except between those of *P. monodon* MG (56 ± 8%) and *P. merguiensis* HP (49 ± 7%) (Table 4.7).

The simple linear relationship between SI and R_{ac} varied in terms of the models, R^2, R and strength (Table 4.8.). Based on the values of R^2, only 1 – 34% of the variability was represented in the straight line. The strength of the relationship appeared moderate in the HP of both prawn species, but negligible and weak in the MG of *P. monodon* and *P. merguiensis*, respectively.

Table 4.8. The model, R^2, R and strength of correlation between SI (x) and R_{ac} (y) for different anatomical sites in both prawn species.

Species	Sites	Models	R^2	R	Strength
P. monodon	Hepatopancreas	y = 25.9x – 14.6	0.34	0.58	Moderate
	Midgut	y = 6.9x + 39.1	0.01	0.10	Negligible
P. merguiensis	Hepatopancreas	y = 28.4x + 3.0	0.26	0.51	Moderate
	Midgut	y = - 10.2x + 115.5	0.10	0.32	Weak

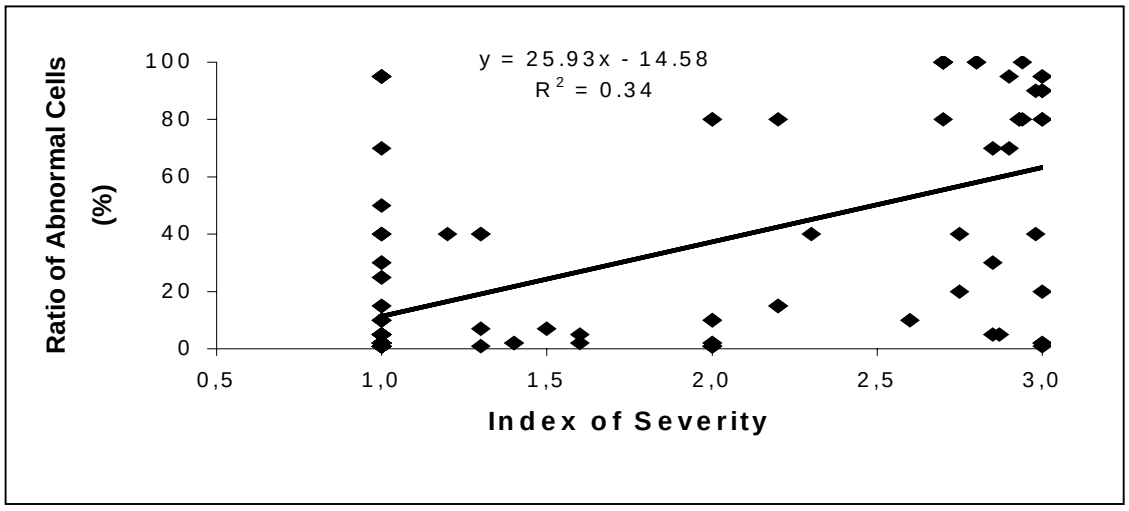


Figure 4.4. Relationship between index of severity (SI) and ratio of abnormal cells (R_{cc}) in the hepatopancreas of *P. monodon* infected by MBV.

Except for *P. merguiensis* MG, all modes of correlation between SI and R_{ac} were positive, such as represented by *P. monodon* HP in Fig. 4.4. However, the correlation between SI and R_{ac} for *P. monodon* ($R^2 = 0.19$) and for *P. merguiensis* ($R^2 = 0.16$) appeared weak.

4.4.5. Size and number of the occlusion bodies

The diameters of OBs were not normally distributed. Transformation of the raw data to a log natural data produced an adequately normal distribution of the data. Hence, the mean diameter of OBs in *P. monodon* (5.4 ± 0.3 µm*)* was significantly larger than that in *P. merguiensis* (3.5 ± 0.4 µm) (Table 4.9.), (*t (2-tailed) =- 7.0; df = 211, p << 0.05*). Likewise, the OB sizes in the HP of both prawn species (5.2 ± 0.4 µm) were significantly larger than those from the MG (3.5 ± 0.3 µm) (*t (2-tailed) = 5.7; df = 211; p << 0.05*).

The interaction between prawn species and anatomical sites also indicated a significant difference in OB sizes (*F = 8.9; df = 1, 209; p << 0.05*). The sizes of OBs from *P. monodon* HP and MG were 6.6 ± 0.4 µm and 4.3 ± 0.3 µm, respectively (Table 4.9.). They were bigger than those of *P. merguiensis*, i.e. 4.1 ± 0.6 µm in HP and 2.9 ± 0.4µm in MG (Table 4.9.).

Table 4.9. The diameters of OBs (in µm) based on prawn species and anatomical sites.

Prawn species	Anatomical sites	N	Min.	Max.	Mean ± SEM
P. monodon	Hepatopancreas	55	2.7	18.8	6.6 ± 0.4
	Midgut	55	1.3	11.8	4.3 ± 0.3
	Pooled	110	1.3	18.8	5.4 ± 0.3
P. merguiensis	Hepatopancreas	32	2.7	6.7	4.1 ± 0.6
	Midgut	71	1.3	8.0	2.9 ± 0.4
	Pooled	103	1.3	8.0	3.5 ± 0.1

Of the two hundred and thirteen OBs observed, 107 occurred as single, 73 as a couple, 29 as a trio, and 4 as a quartet per cell (in *P. merguiensis* only) (Table 4.10.). The diameters of OB significantly differed relative to the number of OB/cell (*F = 18.6; df = 3, 209; p << 0.05*). Accordingly, the mean size of a single OB/cell (6.1 ± 0.2 µm) appeared

significantly larger than that of the others' categories (Table 4.11.). The mean sizes of OBs appeared as a couple per cell (4.5 ± 0.3 µm) significantly differed from those appeared as a trio (3.0 ± 0.4 µm) and a quartet (3.3 ± 1.3 µm) per cell. However, the latter categories did not significantly differ from each other.

Table 4.10. OB size (in µm) based on prawn species, anatomical sites and number of OB/cell. HP = hepatopancreas, MG = midgut.

Prawn species	Sites	OB/cell	Mean	SE	N	%N
P. monodon	HP	1	10.0	0.4	30	55
		2	6.0	0.5	21	38
		3	3.7	1.1	4	7
	MG	1	6.2	0.4	28	51
		2	4.4	0.5	19	35
		3	2.3	0.8	8	14
P. merguiensis	HP	1	3.7	0.6	12	38
		2	4.4	0.8	8	25
		3	3.6	0.7	11	34
		4	4.7	2.2	1	3
	MG	1	4.4	0.4	37	52
		2	3.1	0.4	25	35
		3	2.2	0.9	6	9
		4	2.0	1.3	3	4

Table 4.11. The results of LSD test on OB sizes based on OB number/cell. md = the mean difference between the means of OB diameters from two categories being compared. se = standard error measurement. p = probability is significant at $p < 0.05$.

Categories	One OB/cell	Two OBs/cell	Three OBs/cell	Four OBs/cell
One OB/cell	-	md = 0.3 se = 0.1 p << 0.05	md = 0.7 se = 0.1 p << 0.05	md = 0.9 se = 0.3 p << 0.05
Two OBs/cell	-	-	md = 0.4 se = 0.1 p << 0.05	md = 0.5 se = 0.3 p < 0.05
Three OBs/cell	-	-	-	md = 0.2 se = 0.3 p = 0.5

The interaction between prawn species, anatomical sites and categories of the number of OB/cell demonstrated a significant difference of OB diameters (*$F = 3.9$; $df = 2, 209$; $p < 0.05$*). Indeed, the sizes of OBs declined with the increase of the number of OB/cell,

except those found in the HP of *P. merguiensis* that had relatively similar sizes across all categories (Table 4.10. and Fig. 4.5.).

Furthermore, the largest mean diameter of OB that was found as a single OB in the HP of *P. monodon* was 10.0 ± 0.4 µm. In contrast, the largest mean diameter of single OBs in *P. merguiensis* was 4.4 ± 0.4 µm (in the MG). Remarkably, in one *P. merguiensis* HP, there was a cell containing four OBs with a mean diameter of 4.7 ± 2.2 µm.

The correlation between the number of OBs/cell and OB diameters in both prawn species appears significantly negative (Fig. 4.5.). The regression model for *P. monodon* was ln y = - 0.49 x + 2.5 (*F = 55.9, df = 1, 108; p << 0.05*), in where y = OB diameter and x = the number of OBs/cell. For *P. merguiensis*, the regression model was ln y = - 0.16x + 1.52 (*F = 13.1, df = 1, 101; p << 0.05*). The correlation between both parameters seems moderate for *P. monodon* (R^2 = 0.34) and weak for *P. merguiensis* (R^2 = 0.12) (Fig. 4.5.).

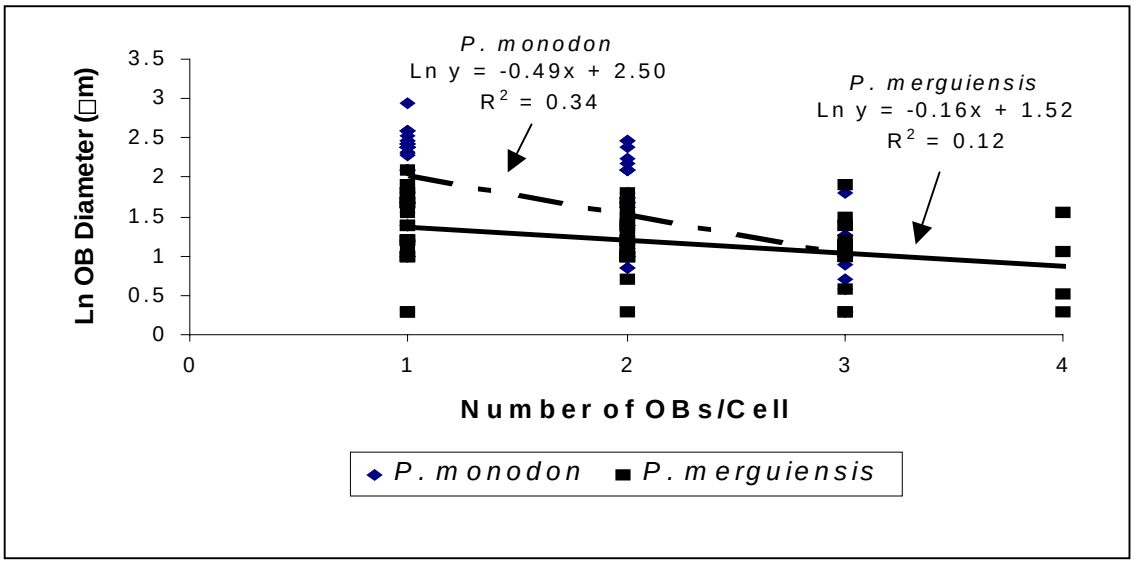


Figure 4.5. Regression between the number of OBs/cell and the OB diameter of MBV from *P. monodon* and *P. merguiensis*.

Seven quantitative features differentiate MBV from *P. monodon* and *P. merguiensis*, i.e. prevalence, ratio of infected nuclei, common infective stages, ratio of abnormal cells, severity index, the number of OB in nuclei and OB diameters (Table 4.12.).

Table 4.12. Summary of quantitative differences of MBV from *P. monodon* and *P. merguiensis* from Australia. Single numbers are the average values with $p << 0.05$.

Parameters	*P. monodon*	*P. merguiensis*	Note
1. Prevalence for PLs only	30%	80%	Studies (2) and (3) also included few *P. monodon* juveniles.
2. Ratio of abnormal cells	34%	76%	
3. Severity index	1.6	2.1	
4. Ratio of infected nuclei	64%	46%	Only used prawns infected by MBV
5. Common infective stage	S1 and S3	S1 (HP); S3 (MG)	
6. Range of OB in nuclei	1 to 3; 53% single	1 to 4; 48% single	
7. Diameter of OB	5.4 µm	3.5 µm	

4.5. Discussion

The cases of MBV infection for this study were limited in number. Indeed, between 1993 and 1998 and after year 2000, no MBV case was submitted. However, the archive might well indicate the changes of viral diseases in farmed penaeids in Australia and Indonesia between 1988 and 2002, but this seems unlikely (see below). The cultured prawns were from northern Queensland (northeastern Australia) and Java island (western Indonesia). These are the core regions of penaeid aquaculture in Australia and Indonesia. In 1990, northern Queensland housed most of approximately 30 hatcheries in Australia (Lester and Paynter, 1990). In fact, since the first studies of penaeid diseases in *Metapenaeus macleayi* from New South Wales began in 1984 (Lester and Paynter, 1990), economically important viruses have been found in northern Queensland. Hepatopancreatic parvo-like virus (HPV) was found in *P. esculentus* (Glazebrook *et al*., 1985; Lester and Paynter, 1990); PBV in *P. plebejus* (Lester *et al.*, 1987); MBV in *P. monodon* and *P. merguiensis* (Doubrovsky *et al.*, 1988); lympoidal parvo-like virus (LPV) in *P. monodon*, *P. merguiensis* and *P. esculentus* (Owens *et al*., 1991); lymphoid organ virus (LOV) in *P. monodon* (Spann *et al*., 1995); infectious hypodermal and haematopoietic necrosis virus (IHHNV) in *P. monodon* and *P. esculentus* (Owens, 1997); spawner-isolated mortality virus (SMV) in *P. monodon* and *P. merguiensis* (Fraser and Owens, 1996; Owens *et al*., 2003); and, BBV in *Metapenaeus bennettae* (Spann and Lester, 1996). Owens (1997)

and Munday and Owens (1998) also listed other viruses associated with wild-caught prawns, wild broodstock and prawn aquaculture in northern Queensland.

Recently, gill-associated virus (GAV) in *P. monodon* has plagued penaeid aquaculture in Australia (Spann and Lester, 1997; Spann *et al*., 1997; Liu and Walker, 2001; Tang *et al*., 2002; Spann *et al*., 2003). Meanwhile, Indonesia has been plagued by WSSV epidemics since mid-1994 (Winarno, 1995).

Thus, the lack of MBV cases in JCU archive from 1993 to 1998, and after year 2000, seems to reflect the rapid changes to studying more important viral diseases in cultured penaeids in Australia and Indonesia. However, prawn samples submitted in 1999 (case number 99/125) and 2000 (case number 00/28) from Seafarm contained larvae of *P. merguiensis* and *P. monodon* mostly with moderate to heavy MBV infection (Appendix 4.). Thus, it is most likely that MBV is still prevalent in cultured penaeids in Australia at low endemic levels, although MBV is well under control by good farming practices (Owens, 1997), or well tolerated by its' hosts (Flegel and Pasharawipas, 1998; Karunasagar *et al*., 1998). Monitoring, surveillance and risk analysis of MBV using simple and low-cost approaches can test those possibilities in the future.

To date, only Doubrovsky *et al*. (1988) have compared the ultrastructure and histopathology of MBV from Australian *P. monodon* and *P. merguiensis*. Vickers *et al*. (1992, 2000) and Belcher and Young (1998) only reported the ultrastructure and genetics of MBV from *P. monodon*. Thus, comparisons of genomes and serology of both penaeids from Australia as suggested by Doubrovsky *et al*. (1988) are lacking. Probably, this is because MBV from various areas and hosts are considered homologous (Lightner, 1996). Quantitative analyses in this study offer a way to compare MBV-type viruses in penaeids.

Using healthy and diseased prawns, this study has demonstrated some quantitative differences between MBV in *P. monodon* and *P. merguiensis* from Australia and its hosts' responses to infection (Table 4.12.). Clearly, MBV affected *P. merguiensis* PLs

more heavily compared to its effects on *P. monodon* PLs relative to the prevalence, population of abnormal cells and severity of infection. Here, a high MBV prevalence in both prawns parallels a high proportion of necrotic and apoptotic cells and high severity of infection. However, the prevalence trend might mislead since the sample size of *P. merguiensis* (56 individuals) was around 4 times smaller than that of *P. monodon* (228 individuals). Also these samples came from only 5 farms. Thus, it is very likely that the samples did not represent a random sub-set of hatcheries and farms in Australia, or their respective populations of prawns. Besides, around 91% of *P. merguiensis* samples were infected by MBV as seen from MBV prevalence in their MG epithelia (Table 4.3.). These samples mostly came from one farm with a suspected MBV outbreak in 1999 - 2000 (Dr. L. Owens, Discipline of Microbiology and Immunology JCU, *pers. comm*.). Thus, the prevalence of MBV in this study might be biased in many ways.

To omit the calculation bias, thus increasing the accuracy of the analyses, a subsequent assessment was done solely on MBV-infected prawns (Table 4.4.). Surprisingly, *P. monodon* had a significantly higher proportion of infected nuclei (64%) than *P. merguiensis* (46%) did (Tables 4.5. and 4.12.). Moreover, the infected nuclei of HP or MG of *P. monodon* were predominantly the early (S1) and final (S3) infection stages. In contrast, the HP of *P. merguiensis* was dominated by the S1 nuclei, while the MG was dominated by the S3 nuclei. This suggests that MBV from Australia is more likely to prefer *P. monodon* than *P. merguiensis*.

This finding conforms to earlier reports that MBV could infect *P. monodon* as young as PL_3 stage in hatcheries in the Philippines, with prevalence from 20% to 100% (Baticados *et al*., 1991). Most of those samples had developed stage 2 and 3 nuclei without specific gross signs of MBV infection. Ramasamy *et al.* (2000) found that *P. monodon* PLs from hatcheries in India, with an MBV prevalence of 84%, had a survival rate of 45% over a 48-h period. Thus, it seems correct that MBV usually infect PLs of penaeids, but they may appear clinically healthy (Lightner and Redman, 1981; Lightner *et al*., 1983; Nash *et al*., 1988; Baticados *et al*., 1991; Fegan *et al*., 1991; Vijayan *et al*., 1995; Lightner, 1996;

Ramasamy *et al*., 2000). Experimental trials might clarify the host preference of MBV from Australian *P. monodon* and *P. merguiensis*.

In the present study, around half of the samples had single OBs and few with 2 to 4 OBs in infected nuclei (Tables 4.10 and 4.12.). Lester *et al*. (1987) found that more than half of PBV-infected PLs and juveniles of *P. plebejus* contained single OBs, few with more than 3, and only one cell with 9 OBs. Lester *et al*. (1987) also found a slightly higher multiple OBs in the PLs than juveniles of PBV-infected *P. plebejus*. However, the causes of mortality among the infected *P. plebejus* were unknown. In *Metapenaeus bennettae*, BBV generally appeared in clusters of 2 to 8 per nuclei of infected hepatopancreas (Spann and Lester, 1996). In the HP nuclei of *P. monodon* PLs from hatcheries in India, Ramasamy *et al.* (2000) found 1 to 12 OBs of MBV per nuclei.

However, the differences in the dominance of particular infection stage and number of OBs in nuclei can also indicate latent and chronic MBV infection in *P. monodon* and *P. merguiensis*. Hence, MBV might not develop well in the HP of *P. merguiensis* and, was shed into the gut and developed there to the stage 3 of infection. In contrast, MBV developed well in the HP and MG of *P. monodon*. Even so, the high prevalence of S3 nuclei might not necessarily cause the mortality of the infected hosts. Karunasagar *et al*. (1998) report a high prevalence of MBV in *P. monodon* larvae in India without serious problems in grow-out ponds under favorable environmental conditions.

Moreover, in the present study, the OB diameters of MBV in both prawn species differ from each other. Here, the mean diameter of OBs in *P. monodon* (5.4 µm) was significantly bigger than that in *P. merguiensis* (3.5 µm). This difference is not attributable to the number of OB in nuclei. In fact, in each prawn species, the diameters of OBs decrease significantly with the increasing number of OB per cell (Tables 4. 10, 4.11. and Fig. 4.3). The difference is that the diameters of OB in *P. monodon* were declining much faster than that in *P. merguiensis*, as judged from the slopes of their regression lines (Fig. 4.3.). In addition, the strength of that correlation (as defined by Morton *et al*., 1990) was moderate for *P. monodon* ($R^2 = 0.34$) and weak for *P.*

merguiensis (R^2 = 0.12). Thus, it is likely that MBV development in *P. merguiensis* was not controlled by the virus. In contrast, the host and the virus affected MBV development in *P. monodon*. The mechanisms involved in these circumstances are beyond the aims of this study. However, again, this result supports the hypothesis that there might be two different MBV types, or different responses by the hosts to the same type of MBV.

This study also reveals different tissue tropism by MBV that has not been previously reported from Australian *P. monodon* and *P. merguiensis*. In *P. merguiensis* MBV was mostly found in the MG epithelia with a prevalence of 91% (Table 4.3) causing high severity (SI of 2.4) and high number of abnormal (necrotic and apoptotic) cells (R_{ac} of 91%) (Table 4.6.). In contrast, MG epithelia of *P. monodon* had the lowest MBV prevalence (11%) and SI (1.5) with 56% R_{ac}. The values of MBV prevalence and severity for the HP of both prawn species did not significantly different from each other. In addition, they are also significantly lower than those for *P. merguiensis* MG. However, the HP of *P. monodon* had the lowest R_{ac} (28%) with the biggest mean diameter of OBs (6.6 µm). These values are significantly different from those for the HP of *P. merguiensis* (R_{ac} = 49% and OB diameter = 4.1 µm). However, the cytological changes in both tissues of both penaeids conform to those reported by Lightner *et al*. (1983), Doubrovsky *et al*. (1988), Vogt (1992) and Lightner (1996). Thus, overall, this study showed MBV in *P. merguiensis* is hostile to MG epithelia, while MBV in *P. monodon* is genial to its HP and AMG.

These facts indicated that: (1) necrotic and apoptotic cells were intimately related to MBV presence as previously reported by Doubrovsky *et al*. (1988), Vogt (1992), Lightner (1996), Ramasamy *et al*. (2000) and so on, and (2) *P. monodon* might accommodate to the virus as theorized by Flegel & Pasharawipas (1998).

In the present study, the biggest OB diameters of MBV found in HP and MG of *P. monodon* were 18.8 µm and 11.8 µm, respectively (Table 4.9.). These diameters are bigger than those reported by Lightner *et al*. (1983) from *P. monodon* (up to 8 µm), Doubrovsky *et al*. (1988) from *P. monodon* and *P. merguiensis* of Australia (up to 10

µm), Johnson and Lightner (1988) from penaeids (around 4.8 µm), Baticados *et al*. (1991) from *P. monodon* of the Philippines (up to 7.7 µm), Vijayan *et al*. (1995) from *P. monodon* and *P. indicus* of India (up to 8.4 µm) and Spann and Lester (1996) from *Metapenaeus bennettae* (up to 9 µm). Nevertheless, the biggest diameter of OB found in *P. merguiensis* in the present study was 8.0 µm (Table 4.9.).

The variability of OB diameters found in this study, and previous studies, are not peculiar since the diameter of MBV OBs is in a range of 0.1 µm to around 20 µm (Lightner *et al*., 1992). However, the present study shows that the sizes of OBs in the hepatopancreas of *P. merguiensis* were relatively similar regardless their numbers in the nuclei (Table 4.10). It suggests that *P. merguiensis* might suppress the size of the OB in its' hepatopancreas, yet be unable to do so in its' midgut. This suppressive ability might explain the relatively low severity index and proportion of necrotic and apoptotic cells seen in the hepatopancreas of *P. merguiensis* (Table 4.6.). Again, the likely explanations may be related to the presence of another MBV strain in *P. merguiensis*, and an attempt by the prawn to accommodate the viral genome.

Thus, tissue tropism and OB diameter might indicate different host responses to a single MBV strain, or the existence of different MBV strains in these two prawn species. The results support the latter hypothesis based on the differences in the proportion of infected nuclei, tissue tropism, level of severity and OB diameters. The mechanisms behind these differences are not known. Nevertheless, these phenomena might be associated with the presence of a novel MBV strain, or another nucleopolyhedrovirus in *P. merguiensis*. Indeed, MBV strains found in Australian penaeids (*P. monodon, P. merguiensis* and *P. plebejus*) differ ultrastructurally from those found in Asian and American penaeids (Doubrovsky *et al*., 1988; Lightner, 1996). Although, Doubrovsky *et al*. (1988) did not report any ultrastructural difference between MBV found in Australian *P. monodon* and *P. merguiensis*, it is possible that host-specific strains of MBV may exist as indicated by the present study. Possibly, like *P. monodon, P. merguiensis* may be in the process of accommodating the foreign viral genome.

Finally, the present study has established the power and usefulness of combining histopathology and statistics to differentiate the morphology and histopathology of MBV in Australian *P. monodon* and *P. merguiensis*. Its limitations with regard to the sample bias and lack of study design for epidemiology can be easily rectified by using appropriate sample size and proper sampling methods. The results can, then, be compared to serological and genomic studies relative to prawn health performance and farm production to understand the relevance and reliability of this quantitative analysis in practical application. Moreover, in places lacking facilities for molecular and serological diagnosis like Indonesia or other third world countries, the combination of histopathology, or even gross pathology, and statistics such as in this study will provide a powerful analytical tool to comprehend viruses and other pathogens in various aquatic animals living in controlled or wild environment.

CHAPTER 5

A NOVEL QUANTITATIVE METHOD FOR ANALYSIS OF LYMPHOIDAL ANTI-VIRAL ACTIVITIES IN *Penaeus monodon* AGAINST A BACULOVIRUS

5.1. Introduction

Extensive studies have been done on the effects of MBV on the digestive system of the penaeids (Lightner and Redman, 1981; Lightner *et al*., 1983; Johnson and Lightner, 1988; Vogt, 1992). However, reports on penaeid immune responses to MBV infection are scarce due to the lack of experimental studies and cell lines for MBV culture. Therefore, in this study, the immune response of *P. monodon* against baculoviral infection was assessed using *Autographa californica* nuclear polyhedrosis virus (AcNPV), an insect baculovirus, as the substitute for MBV. AcNPV was considered appropriate for this study because AcNPV has been widely used as the basis for the development of gene probes and polymerase chain reaction (PCR) for MBV (Vickers *et al*., 1992; Belcher and Young, 1998; Hsu *et al*., 2000). The assessment was done on the lymphoid organ (LO) of the prawns since LO is regarded as the major organ for antiviral defense and undergoes considerable histological changes during viral infections (Owens and Hall-Mendelin, 1990; Hasson *et al*., 1999 b; Anggraeni and Owens, 2000; Spann *et al*., 2003).

Lymphoid organ is anterior to the hepatopancreas and ventral to the stomach (Lightner *et al*., 1987). The nomal LO cells (stromal matrix cells) surround a central lumen, while abnormal cells clump inside a spherical body (lymphoidal spheroid) without a central lumen (Lightner *et al*., 1987; Owens and Hall-Mendelin, 1990; Spann *et al*., 1995). Thus, it is relatively easy to distinguish diseased tissue from a normal tissue in the organ using light microscopy and conventional histology.

The spheroid was once suggested as an inflammatory response with no association to any pathogen as it is found in healthy and diseased prawns (Lightner *et al*., 1987). However, since Owens and Hall-Mendelin (1990) first described LO spheroids (LOS) in *P.*

merguiensis spawners from Australia, several virions have been found associated with the LOS of various penaeids. Among other things, there are lymphoidal organ vacuolization virus (LOVV), lymphoidal parvo-like virus (LPV), lymphoid organ virus (LOV) (Owens *et al*., 1991; Bonami *et al*., 1992; Spann *et al*., 1995; Owens, 1997), Taura syndrome virus (TSV) (Hasson *et al*., 1995, 1999 a, b), spawner-isolated mortality virus (SMV) (Fraser and Owens, 1996; Anggraeni and Owens, 2000), white spot syndrome virus (WSSV) (Wang *et al*., 1999), gill-associated virus (GAV) (Tang *et al*., 2002; Spann *et al*., 2003), and MBV (Vijayan *et al*., 1995). Since the lymphoid organ is connected to the heart through the epigastric artery (Bell and Lightner, 1988), Anggraeni and Owens (2000) suggest that LOS originates from haemocytes that phagocytose and subsequently remove viral materials from penaeid bodies by migrating through the haemal sinuses of the organ.

Hasson *et al.* (1999 b) described three different morphotypes of LOS in *P. vannamei* infected by Taura syndrome virus (TSV). The earliest LOS arises from activated LO tubule phagocytes called type A. The succeeding type B contains necrotic cells with positive TSV by *in situ* hybridization after an acute phase infection. The terminal type C LOS has cells with condensed basophilic nuclei, reduced size and progressive atrophy as shown by apoptotic cells. Hence, Hasson's classification required a TSV gene probe. However, Owens' classification simplifies the histological development of LOS into three phases: tumorous-like phase without fibrocytes encapsulating the spheroids, fully encapsulated spheroid phase, and vacuolative degeneration of spheroids (Anggraeni and Owens, 2000). Thus, Owens' classfication was preferable for the current study.

In studying the development of LOS, Anggraeni and Owens (2000) analysed the area occupied by LOS in the lymphoid organ using a video camera, a computer and image analytical softwares. To make the analyses low-cost, simple and speedy, the present study addressed the prospect of a quantitative assessment of the histopathological conditions of LO using a "transect technique". This quantitative approach was tested on penaeid prawns from grow-out ponds and quarantine holding facility.

5.2. The Objectives of the Study

The present study was carried out to:

1. Develop a transect method to quantify histopathological changes in the lymphoid organ, and
2. Assess the reliability and sensitivity of the transect method in detection of histopathological changes in a time-series manner.

5.3. Materials and Methods

5.3.1. Bioassays

Prior to use, all quarantine bins, filter systems and experimental aquaria were sterilized with liquid chlorine (100 g/l) at 30 ppm overnight with aeration. The following day, these bins and aquaria were scrubbed and washed twice with fresh water. All equipment such as nets, heaters and corner filters were chlorinated (30 ppm) and dried prior to use.

One hundred and fifty sub-adult tiger prawns, *Penaeus monodon*, from a commercial farm in northern Queensland were transferred to the Microbiology and Immunology (JCU) aquatic quarantine room on December 12th 2001. In the quarantine room, the animals were immediately acclimated in two 1000-L circular bins (75 prawns/bin) containing continuously aerated saltwater of 33 ppm salinity and 27 ^{o}C temperature and equipped with a large biological trickle filter system. Four bottle-holding plastic boxes were put inside each bin to provide hiding places for the prawns. The quarantine room was lighting between 8 am to 5 pm each day to stimulate natural light. The animals were fed with prawn pellets of 10% total body weight divided into 3 rations a day. The water was exchanged to 50% volume each week, in conjunction with the removal of waste on the inside of the tank by scrubbing, swabbing and siphoning.

Upon arrival, a sample of 30 prawns was taken randomly, anesthetized in icy saltwater from the pond and measured for their weight and length. Each prawn was blotted dry

with paper towel before its weight was measured using a portable scale (A & D EK-120A) with measurement accuracy of 0.01 g. The length of the prawn was measured from the tip of its rostrum to the telson using a ruler with an accuracy of 1 mm. The means ± SD of their weights and lengths were 11.7 ± 0.9 g and 11.7 ± 0.4 cm, respectively.

Subsequently, sixty prawns were used in two time-series experiments (chronic and acute infections) in which they were injected with *Autographa californica* nuclear polyhedrosis virus (AcNPV). The insect baculovirus recombinant of $7x10^7$ pfu/ml was obtained from Invitogen® (USA) (Kit No. 45-0421 and 50-0048) and stored at 4 ^oC prior to use.

Each bioassay was conducted in two 200-L aquaria in a closed aquatic research room. Each aquarium was equipped with one air-powered corner biological filter and an air stone. The tops of the aquaria were covered with hard plastic boards to prevent the prawns from jumping out. Each aquarium had a large black insect net submerged in the surface of the water in aquaria. This net became the hiding place for the prawns. The room temperature was stabilized at 28 ^oC using a thermostat. The water salinity and temperature fluctuated between 33-35 ppt and 26-28 ^oC, respectively, prior to and during the experiment. The prawns were acclimated in both aquaria for 4 days prior to each experiment. Control groups for each experiment consisted of 2 prawns randomly taken from the aquaria immediately before the start of each experiment. The food was given each day, except when the prawns seemed to not eating their food from the previous day. Sampling of the prawns was done using a hand-held aquarium net. The procedures and methods of sample fixation, routine histology and H&E staining were described in Chapter 3.

For the 1st experiment (chronic infection), 15 prawns were transferred from the quarantine bin to each aquarium on December 13th 2001. After acclimated for 4 days, each prawn in the aquaria was injected with 50 µl of $7x10^7$ pfu/ml of AcNPV into the heart using a sterile 27Gx1/2" Terumo® needle on a sterile, non-pyrogenic Terumo® syringe (one-ml capacity with 10-µL accuracy). Two infected prawns (one per aquarium)

were sampled randomly at zero hour post-injection (h p.i.) and processed for routine histology and H&E staining. The subsequence sampling was carried out in a logarithmic fashion from 2 hour-post injection (h p.i.) to 45 day-post injection (d.p.i.).

In the 2nd experiment (acute infection) on April 22nd 2002, the similar protocol as per the 1st experiment was carried out. This experiment lasted for 16 hours with a sampling frequency of every two hours.

5.3.2. Severity of LO infection

The severity of LO infection was graded as described in section 3.12. with a slight adjustment as per Hasson *et al*. (1999 b), i.e.: grade 0 (G0 = no LOS), grade 1 (G1= LOS area is < 25% of the organ), grade 2 (G2 = LOS area is between 25 - 50% of the organ), grade 3 (G3 = LOS area is between 50-75% of the organ) and grade 4 (G4 = LOS area is > 75% of the organ).

5.3.3. Transect analysis of lymphoid organs

This simple technique quantified histopathological changes in the lymphoid organ based on the abundance and length of LOS morphotypes using a light microscope and a micrometer (section 3.4. in Chapter 3). Here, the scale line on the micrometer measures the length of normal tubules and LOS along its track. Only parts of LO that came into contact with the bands in the either side of the scale line were measured. The primary measurement was done along the longest plane of the LO (Fig. 5.1.). When the LO section in an individual prawn was large, a secondary or tertiary measurement was made along the diagonal section to the longest track. Care was exercised in order to keep the measurement on the right track and direction.

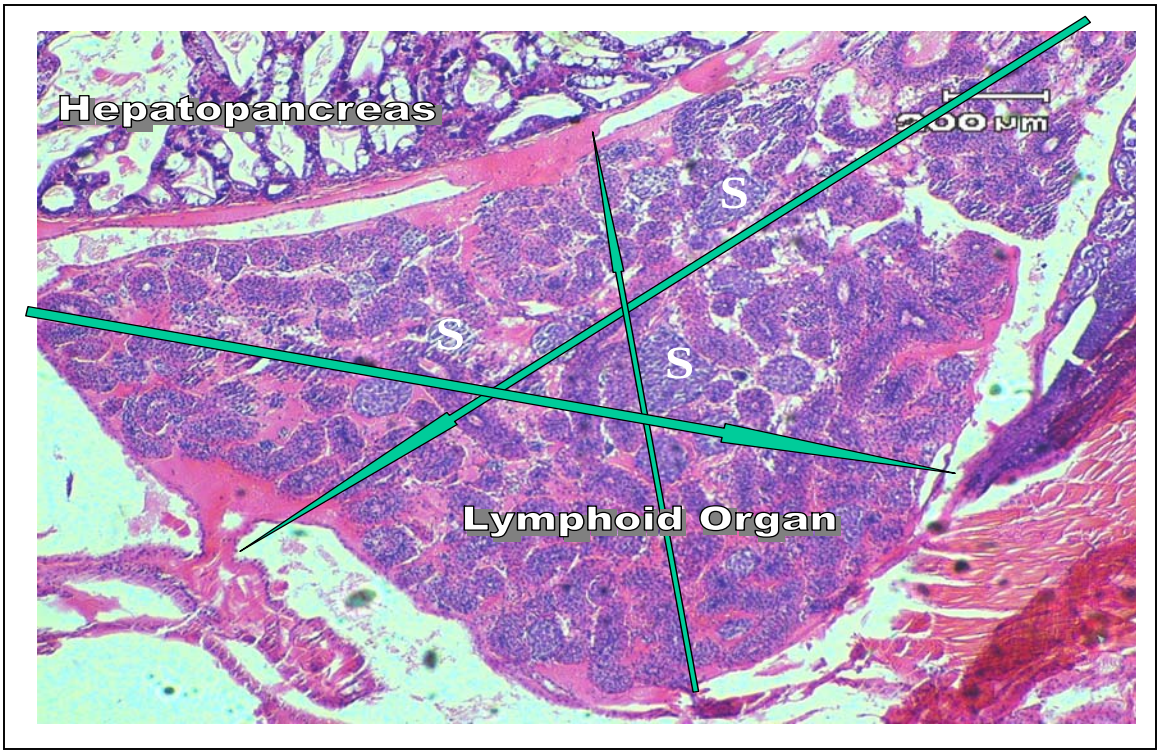


Figure 5.1. Three transect planes for measurement of the length of spheroids (S) and tubules in the lymphoid organ. The severity grade = 2. H&E stained.

Since the sections of the three dimensional lymphoid organ were cut at random, it is very likely that the two dimensional slide section chosen for the measurement of spheroid length was no identical in each prawn. Therefore, a ratio for the spheroid length was used to circumvent the bias. Hence, the total lengths of normal tubules and LOS from all transect lanes were used to create the spheroid-total length (STL) ratio, i.e. the total length of the spheroids against the total length of the transect track (total length of spheroids and normal tubules):

STL Ratio = [(LOS total length) ÷ (transect total length)] x 100%

The morphotypes of LOS in this study were differentiated based on Owens' classification (Anggraeni and Owens, 2000) with a slight modification, i.e. phase one or type (t_A), intermediary between type A and type B (t_{AB}), phase two or type B (t_B), intermediary between type B and C (t_{BC}), and phase three or type C (t_C) (Fig. 5.2.).

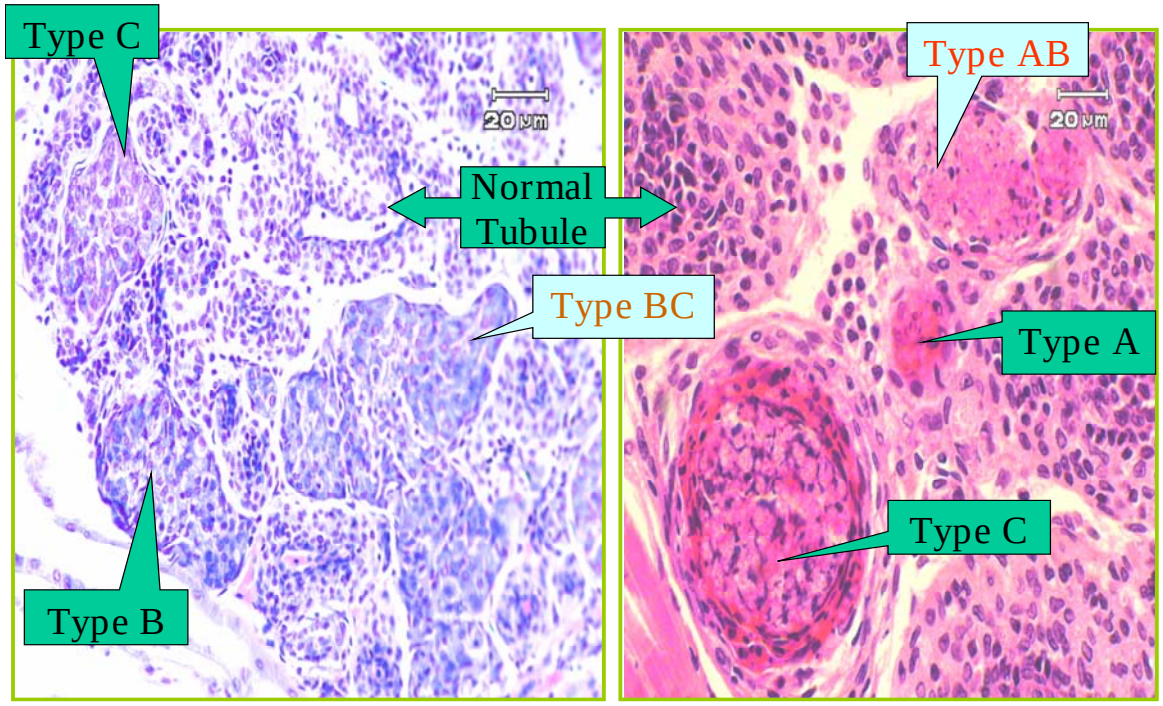


Figure 5.2. Morphotypes of lymphoid organ spheroids (LOS): type A (early), B (developing) and C (final), AB (intermediate between A and B), BC (intermediate between B and C). H&E stained.

To determine the dominant LOS type, each LOS morphotype was assigned a numerical value, i.e. 1 (t_A), 1.5 (t_{AB}), 2 (t_B), 2.5 (t_{BC}) and 3 (t_C). These values generate a morphotype score (MT score) calculated by the following formula:

$$\textbf{MT Score} = [\Sigma (\text{numerical value of } t_x \times \text{the number of } t_x)]/\text{total } N_{tx}$$

in which: t_x = LOS morphotype with *x* as A, AB, B, BC or C. N_{tx} = the sum of the numbers of all LOS morphotypes counted along the transect trail.

Therefore, the range of MT scores is from 1.0 (the numerical value for LOS morphotype A) to 3.0 (the numerical value for LOS morphotype C).

5.3.4. Statistical analyses

Pearson's correlation analyses (section 3.10.) were carried out to determine the strength of relationship between the severity of LO infection (based on LOS area in Hasson *et al*.,

1999 b) with the spheroid-total length (STL) ratio. Patterns of changes in morphotype (MT) score were analysed for each LOS monotype. Comparison of the means of STL ratio and MT score was done for the acute bioassay only.

5.4. Results

5.4.1. Reliability of the transect method

The combination of data from the chronic and acute bioassays showed that the natural logarithm of the total length of spheroids across the transect line correlated significantly to the number (abundance) of the spheroids (*Pearson's r = 0.79; p << 0.05; N = 36*). Likewise, the number of the spheroids significantly correlated to the natural logarithm of the STL ratio (*Pearson's r = 0.65; p << 0.05; N = 36*). For the chronic and acute bioassays, the number of spheroids can be predicted using the equations in Table 5.1. Here, the number of spheroids positively and significantly correlated to an increase in the total length of the spheroids, or the STL ratio.

Table 5.1. Predictive equations for the number of spheroids (SN) based on the spheroid total length (STL) and the ratio of spheroid length to transect total length (STL Ratio).

Infection	Equation	R	R^2	F	df	p
Chronic	SN = -17.31 + 4.47 ln STL	0.73	0.54	22.1	1, 19	<< 0.05
	SN = 0.42 + 3.02 ln STL Ratio	0.48	0.23	5.7	1, 19	< 0.05
Acute	SN = -10.72 + 2.78 ln STL	0.88	0.77	42.2	1, 13	<< 0.05
	SN = 0.80 + 1.75 ln STL Ratio	0.62	0.39	8.2	1, 13	< 0.05

5.4.2. Qualitative histopathology of prawns in the chronic bioassay

The lymphoid organ (LO) appeared to have various observable histological conditions. For example, in a prawn without LOS sampled at 8 h p.i., approximately 90% of the LO lumens were filled with strongly basophilic cells. The LO of this particular prawn also had deeply-stained eosinophilic material within the hemal sinuses. The hemal sinuses contained few pyknotic cells, debris and round granular cells.

In two prawns with a heavy haemocytic infiltration in the LO, haemocytes filled the hemal sinuses without the eosinophilic materials. One of these prawn (sampled at day 3) had around 5% of the LO area filled with spheroids (severity grade = 1) while around 85% of the LO lumens were packed with strongly-stained basophilic cells. The other prawn (sampled at day 20) had around 85% LOS area (severity grade = 4) with most of the LO tubules were empty.

In four prawns, the hemal sinuses were quite prominent and had different sizes depending on the dimension of empty spaces between the healthy tubules (intertubular spaces) (Fig. 5.3.a.). In a prawn sampled at day 10, hemal sinuses were found adjacent to the terminal phase of LOS (type C LOS) (Fig. 5.3.b.). Thus, the hemal sinuses seem to be intimately connected (no clear boundaries) to the type C LOS and clearly not connected to a healthy tubule close by since there were spaces containing fibrous connective tissues between the sinuses and healthy tubules.

A prawn sampled at day 25 contained a mass of spheroids surrounding two lymphoidal tubules (with empty lumens) in which some spheroids were inside the stromal matrix of cells around the tubules (Fig. 5.4.a, b.). Few spheroids were right in the endothelial layers of the lumens. Two prawns sampled at days 15 and 25 contained spheroids in the spongy connective tissue of hematopoietic tissue (Fig. 5.4.c.) and necrotic columnar cuticular epithelium of the stomach lumen (Fig. 5.4.d.), respectively. A spheroid-like body was present in the lumen of a hepatopancreatic tubule adjacent to the LO in a prawn sampled at day 35.

Two prawns sampled at days 25 and 45 with approximately 5% and 25% LOS areas, respectively, had lost defined LO tubules without any apparent eosinophilic material present within the hemal sinuses. A prawn sampled at day 30 contained LOS like melanized nodules of a bacterial infection. However, no Gram stains were done to ascertain the presence of bacteria.

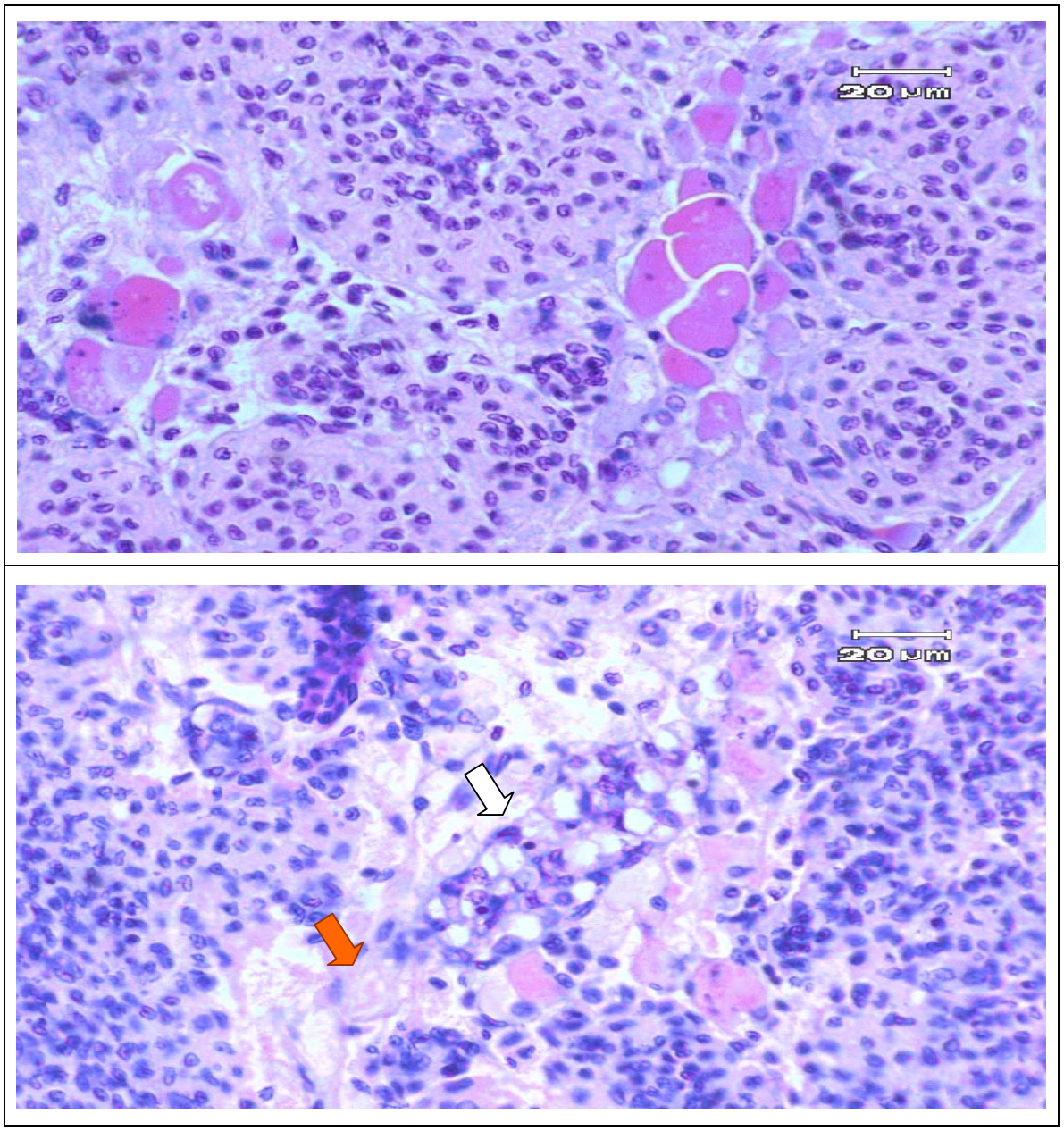


Figure 5.3. Lymphoid organ: (a) Eosinophilic material within the hemal sinuses in intertubular spaces lacking defined lymphoidal tubules. (b) A vacuolated or type C spheroid (white arrow) surrounded by an array of fibrous connective tissues (red arrow) and hemal sinuses containing eosinophilic materials. H&E stained.

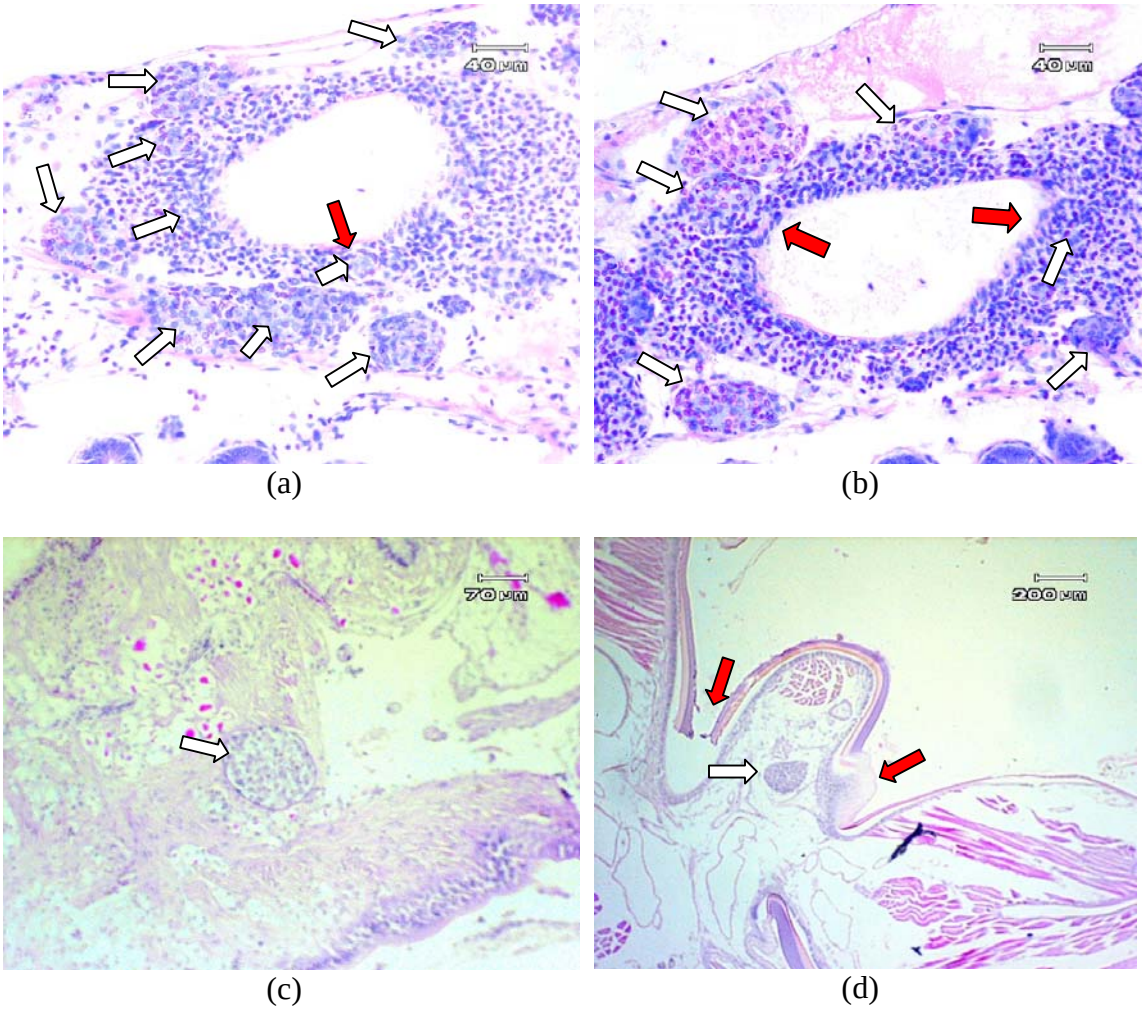


Figure 5.4. Lymphoidal spheroids (white arrows): (a, b) surrounding haemal sinuses; few spheroids thrusting the endothelial layers (red arrows) into the lumens. (c) the spongy connective tissue sheath of hematopoietic tissue. (d) adjacent to necrotic columnar cuticular epithelia of the stomach lumen (red arrows). H&E stained.

5.4.3. Quantitative histopathology of prawns in the chronic bioassay

From 32 prawns sampled during the chronic bioassay, only 24 were useful for this study since 2 samples were not properly fixed and 6 samples contained scattered LO sections. Of the 24 prawns, the prevalence of LOS was 87.5% (21/24).

In lymphoid organs with LOS, the mean STL ratio for each LOS morphotype during the 45-day chronic infection ranged from 1 to 42%, in which type C LOS had the highest

mean of STL ratio, i.e. 12 ± 4% (Table 5.2.). The means of STL ratio of types B and BC LOS were not significantly different ($p >> 0.05$) from one another and from those of types A and AB, but significantly different ($p < 0.05$) from that of type C. The means of STL ratio of types A and AB did not significantly differ from that of type C ($p >> 0.05$).

Table 5.2. The mean STL ratio (%) of each LOS monotype in the lymphoid organ of *P. monodon* during the chronic infection with AcNPV based on the sampling time. N = the number of sampling time when LOS was seen.

LOS Monotypes	N	Min	Max	Pooled Mean	SE
Type A	15	1	24	8	2
Type AB	7	1	18	6	2
Type B	10	1	20	5	2
Type BC	5	1	6	3	1
Type C	11	1	42	12	4
TOTAL	15	1	66	26	5

Among the prawns with LOS, type A LOS were seen at all sampling points (15/15) followed by types C (11/15), B (10/15), AB (7/15) and BC (5/15) (Table 5.2. and Fig. 5.5.). Overall, the pooled mean of STL ratio for all morphotypes of LOS in the chronic assay was 26 ± 5% with a range from 1% (at 0 and 8 h p.i.) to 66% (at 4 h p.i.) (Table 5.2. and Fig. 5.5.). The latter was from an individual prawn only.

During the chronic infection, the severity of infection based on the sampling time ranged from 0 (no LOS) to 4 (LOS in > 75% area of the organ) with a mean ± SE of 1.9 ± 0.3. Based on the sampling time, the severity of infection was significantly and positively correlated to the natural logarithm of the means of STL ratio (*Pearson's $r = 0.59$; $p < 0.05$*).

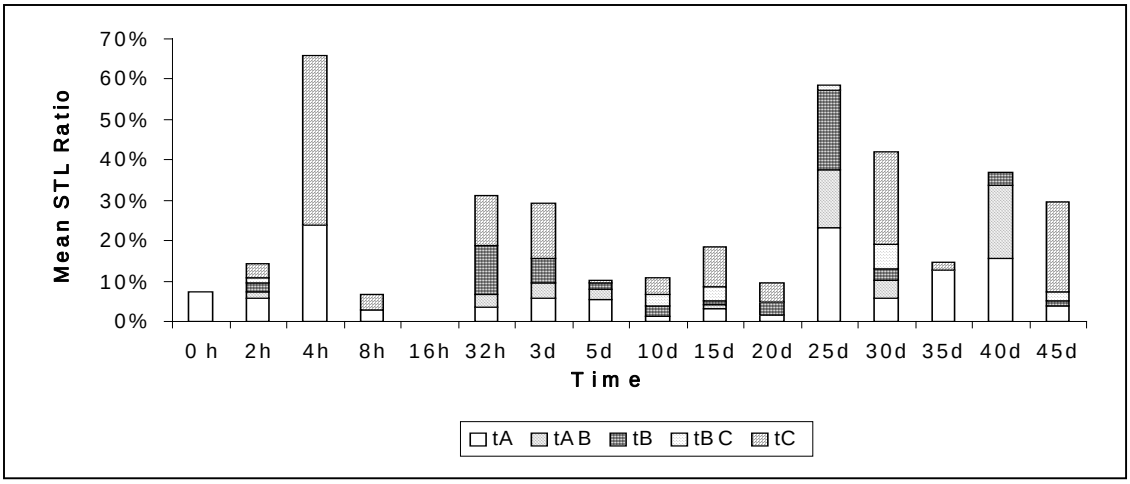


Figure 5.5. Distribution of the spheroid-total length (STL) ratio from various samples during a chronic infection of AcNPV based on the morphotypes of the spheroids. tA = type A. tAB = intermediary from type A to B. tB = type B. tBC = intermediary from type B to C. tC = type C.

5.4.4. Quantitative histopathology of prawns in the acute bioassay

In the 16-h acute bioassay, 18 prawns were sampled, but one prawn was poorly fixed. Of the 17 prawns available, the prevalence of LOS was 82.5% (14/17) since three prawns taken at 4, 6 and 12 h p.i., respectively, did not contain LOS. Nevertheless, all prawns with LOS had grade 1 severity of infection (LOS in < 25% area of the organ).

No clear trend was obvious. If a possible trend is to be found, perhaps it is as follows. Two levels of total STL ratios may occur during the acute infection. The first one was from 0 to 6 h p.i. when the total means of STL ratio were relatively low (1.3 – 8.8%) (Fig. 5.6.). The second one was from 8 to 14 h p.i. when the total means of STL ratio were persistently high (9.8 – 15.4%) (Fig. 5.6.). The successive changes of the morphotypes from type A to C possibly seems more structured from 8 – 14 h p.i. than from 0 – 6 h p.i. (Fig. 5.6.).

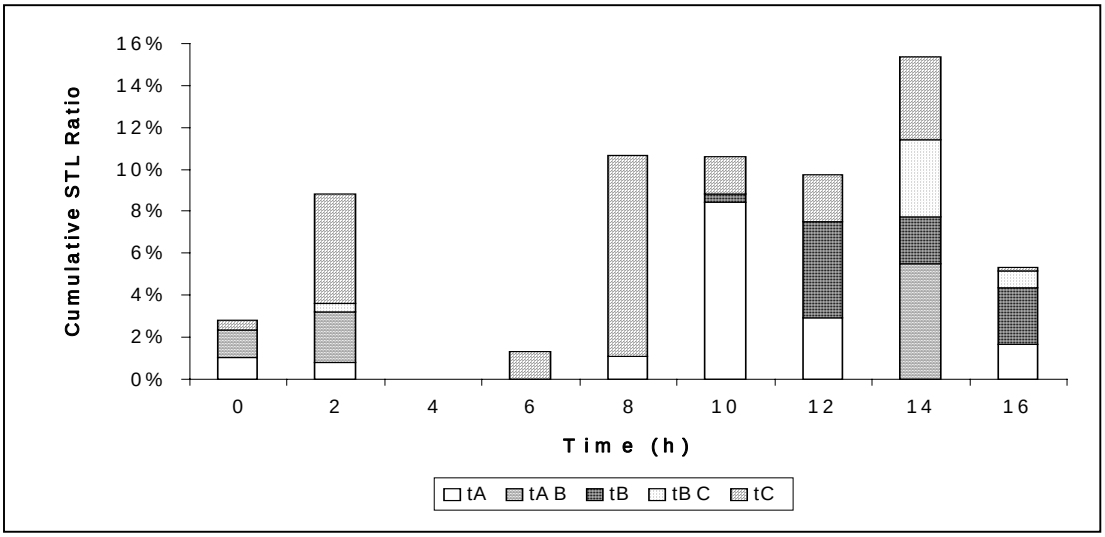


Figure 5.6. The distribution of the spheroid-total length (STL) ratio from various samples during an acute infection by AcNPV based on the morphotypes of the spheroids. tA = type A. tAB = intermediary from type A to B. tB = type B. tBC = intermediary from type B to C. tC = type C.

Apparently, the average MT score for each LOS morphotype also fluctuated considerably overtime (Fig. 5.7.). However, there was no linear correlation between the MT score and STL ratio (*Pearson's $r = 0.35$; $p >> 0.05$*).

During the period of low STL ratios (from 0 to 6 h p.i.), the MT scores ranged from 1.8 (close to type B LOS) to 3 (type C LOS) (Fig. 5.7.). In contrast, during the period of high STL ratios (from 8 to 14 h p.i.), the MT scores ranged from 1.3 (close to type AB LOS) to 2.6 (close to type C LOS) (Fig. 5.7.). The MT scores also demonstrated a successive shift of morphotypes from type C LOS to type AB from 6 to 10 h p.i., and a successive production of LOS morphotypes from type AB to type BC from 10 to 14 h p.i. during the period of high STL ratios (Fig. 5.7.).

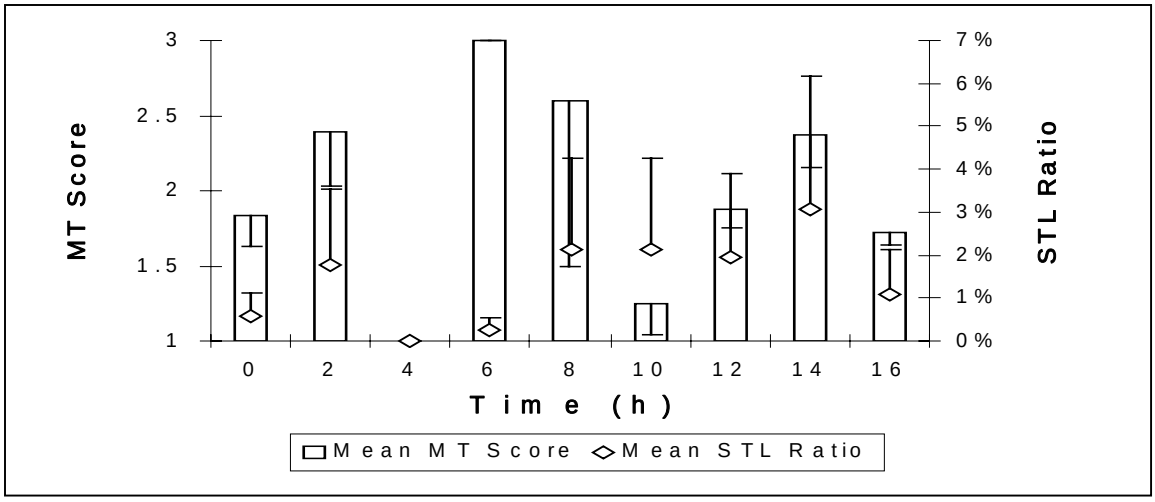


Figure 5.7. The distribution of the mean monotype (MT) score and mean spheroid-total length (STL) ratio during the acute infection by AcNPV. Bars are the standard error of the means for both factors.

Finally, the mean STL ratio for prawns from the first 16 h of the chronic trial (23.6 ± 14.2%) was higher than that for prawns from the 16-h acute trial (8.1 ± 1.6%). However, they were not significantly different from each other (*t (2-tailed) = 1.5; df = 10; p >> 0.05*). This might be due to a large time-related variation of STL ratios in the first 16-h of the chronic assay. However, the overall mean of STL ratio during the 45-day chronic assay (25.7 ± 4.8%) was significantly higher than that of the 16-h acute trial (8.1 ± 1.6%) (*t (2-tailed) = 3.4; df = 21; p << 0.05*).

5.5. Discussion

The internal organs of aquatic animals have long been used as indicators of diseases and environmental changes associated with the diseases. The studies of internal organs also assist in detecting the susceptibility of various anatomical sites to pathogens and the spread of the pathogens within various anatomical sites. This knowledge will help in verifying the route(s) of horizontal or vertical transmission of diseases.

The characteristics of internal organs that are suitable for biological indicators include easily recognisable cytological and ultrastructural changes due to stress and other related

causes (Manisseri and Menon, 1995). Lymphoid organ of the penaeids fits into this category since the lymphoidal changes occur in healthy and virus-infected prawns with different severity (Lightner *et al*., 1987; Owens *et al*., 1991; Hasson *et al*., 1999 b; Anggraeni and Owens, 2000; Cowley *et al*., 2000; Spann *et al*., 2003). Moreover, the lymphoid organ is considered as a defence organ against viruses in penaeids (Anggraeni and Owens, 2000).

In this study, it is rather difficult to establish a correlation between the changes in the lymphoid organ to AcNPV infection, although the presence of high STL ratios and successive morphogenesis of LOS morphotypes in the acute assay occurred under 8 h p.i. In this stage, it can be hypothesized that baculoviruses, introduced systemically, may elicit non-specific immune responses in the prawns under 8 h post infection. This argument supports the finding by Paynter *et al*. (1992) that a two hour exposure time was sufficient to allow MBV infection by oral route. Lightner *et al*. (1992) reported that the incubation period of MBV and other penaeid baculoviruses, such as baculoviral midgut gland necrosis virus (BMNV) and baculovirus penaei (BP), based on oral transmission from prawn to prawn was about 24 h. Nevertheless, Paynter *et al*. (1992) and Lightner *et al*. (1992) only reported the changes in the epithelial cells of hepatopancreas, not the immune responses of the prawns. Possibly, because of the route of infection, to date, there is no report on penaeid immune reactions against penaeid baculoviruses. Although Johnson (1984, 1988) described the haemocyte-infecting rod-shaped and non-occluded viruses in crabs *Carcinus maenas*, *C. mediterraneus* and *Callinectes sapidus*, Johnson (1988) did not explain the haemocyte response against the invading viruses. Perhaps lymphoid organ may provide a general picture of the immune response by the penaeids against systemic baculoviruses.

Although using a much simpler technique than those used by other investigators, the present study showed that significant and positive correlations occurred between the length and number (abundance) of the LOS (Table 5.1.). Likewise, the spheroid-total length ratio correlated positively and significantly with the grades of LO severity of infection based on the LOS area in the organ (Hasson *et al*., 1999 b). Thus the reliability

of the transect technique appears to be well established. Nevertheless, larger sample size was needed to validate the quantitative models developed in this particular study.

The transect technique also demonstrated that the total mean of STL ratio in the first 16 h of the chronic bioassay (23%) was higher (though not significantly) than that in the 16-h acute bioassay (8%). However, the overall means of STL ratio for chronic assay (26%) was significantly different from the latter. These findings show that this technique was sensitive because the prawns in both trials came from the same pond in the same farm, but their laboratory rearing period differed by more than 4 mo. The prawns for the chronic trial was injected with AcNPV only 4 days after their arrival in the laboratory. Thus their lymphoidal changes in the first 16 h of the trial might be affected largely by natural factors from the grow-out pond and stress-related factors in the aquaria. A separate observation in the farm shows that the penaeid postlarvae experienced a latent and chronic Vibriosis as indicated by their bent tails (Coco Soetrisno, Microbiology and Immunology JCU, *pers. comm.*). Moreover, Owens (1997) and Munday and Owens (1998) confirm that viruses are a normal facet of farmed penaeids in Australia, while Spann *et al*. (2003) argue that most of farmed *P. monodon* in Australia contain GAV. In contrast, the prawns used in the acute trial had been reared in the quarantine bins for more than 4 months, i.e. more than 4 mo older than the prawns in the chronic trial. The relatively clean bins, controlled water quality and room temperature, closed quarantine room, high sanitary condition and low density during the 4-mo quarantine period might have removed these prawns from natural stressors and pathogens. In fact, no mortality and viral infections occurred during the quarantine, although few prawns had typical bacteria-induced melanization in their hepatopancreai. Therefore, it was likely the prawns in the acute trial (laboratory held) had relatively lower pre-existing background activities in their lymphoid organs than those in the chronic trial. Again, the transect technique was sufficiently sensitive to detect and quantify this difference.

Anggraeni and Owens (2000) found LOS area in prawns held for 1 month in laboratory was significantly higher than that of prawns from ponds. However, the current study shows that after 1 month (30 days) in the aquaria, the prawns in the chronic trial appeared

to display a high STL ratio, i.e. 40% (Fig. 5.1.), while the highest mean of STL ratio for the laboratory prawns was 15% (Fig. 5.2.). Moreover, the prawns in the acute assay only suffered grade 1 (G1) severity of infection, i.e. LOS in < 25% of the lymphoid organ. In contrast, prawns used in the chronic trial mostly suffered from G2 severity of infection (LOS in 25 -50% of the lymphoid organ). Possibly, the laboratory prawns used by Anggraeni (1998) and Anggraeni and Owens (2000) had not completely adapted to the laboratory rearing condition, or the laboratory rearing condition at that time was unfavorable for the animals. Moreover, Anggraeni (1998) and Anggraeni and Owens (2000) compared prawns from two different commercial farms, although one population was reared for 1 month in laboratory. In the present study, the prawns were from the same farm with different quarantine period in the laboratory. Thus, the difference in LOS area (Anggraeni and Owens, 2000) or STL ratio (the present study) may reflect different rearing conditions and age of the prawns. Moreover, in GAV infection, Cowley *et al*. (2002) and Spann *et al*. (2003) argue that the age of the prawns may affect the ability of the prawns to overcome the infection and avoid disease. Therefore, at this stage, it is plausible to suggest that prawns from different origins and age can display different quantitative activities in their lymphoid organs. It implies that the transect technique on the lymphoid organ is a simple, economical and robust method for assessing the interaction between hosts (e.g. age and species of the prawns), pathogens (e.g. viruses) and environment (e.g. ponds, farms or coastal areas). Thus, the potential applications of this technique are extensive.

Anggraeni and Owens (2000) suggest that LOS as spent haemocytes might be shed during ecdysis in the moulting process. However, Anggraeni and Owens (2000) did not find correlation between LOS area and prawn growth in *P. monodon*. Therefore, moulting was not of particular interest in the present study. Moreover, in the present study, the highest area of LOS in the chronic trial was approximately 85% of the lymphoid organ section. Yet, the average grade of severity for the chronic trial was 2, i.e. 25 - 50% LOS in the organ. This finding agrees with Hasson *et al*. (1999 b) who found that *P. vannamei* infected by TSV mostly suffered grade 1 to 2 severity of infection. Moreover, in the present study, 12.5 and 17.5% of the prawn populations in the chronic

and acute trials, respectively, had no LOS. Spann *et al*. (2003) found around 50% of GAV-infected *P. monodon* from farms did not contained LOS, and only less than 10% of the lymphoid organ section of acutely GAV-infected *P. monodon* had LOS. These suggest a routine shedding of LOS occurs in penaeids, although the mechanism(s) is unknown at present.

Along with hematopoietic and ovary tissues, the lymphoid organ possess high generative capacity for tissue culture (Colorni, 1990), such as demonstrated by Lu *et al*. (1995) who used primary lymphoidal cells from *P. stylirotris* to examine cytopathogenic effect (CPE) and the $TCID_{50}$ titer of YHV. In the present study, the transect technique was able to show the successive and structured production of LOS types did occur. These findings corroborate Hasson *et al*. (1999 b) who found rapid increase in LOS number and successive LOS morphogenesis during a chronic infection of *P. vannamei* by TSV. However, the STL ratios in the present study did not originate from the same individual animals in a time-series manner. Rather, the mean value of STL ratio came from different animals sampled in different times. Therefore, the values were not valid to describe lymphoidal change, other than to describe the lymphoidal activities of indvidual prawns. This problem can be overcome by sampling the prawns after moulting in a time series manner given that the time of moulting is closely observed.

The presence of the eosinophilic materials in the hemal sinuses connected to type C LOS were also a common feature in the present study. These materials seemed to be connected to regenerative components of lymphoidal tubules. This phenomenon might be comparable to brown spot of melanin and haemolytic infiltration within foci of cuticular epithelia once containing TSV in *P. vannamei* (Hassons *et al*., 1999 a).

Hasson *et al*. (1999 a) found that after moulting, the survivors of acutely TSV-infected *P. vannamei* appeared to behave normally with numerous LOS in their lymphoid organs. Thus, it shows that moulting did not reduce LOS number in the lymphoid organ since a chronic phase of the infection generally follows the acute phase. However, since LOS was thought to originate from granular haemocytes with apoptotic, peroxidase and

phenoloxidase (PO) activities (Anggraeni and Owens, 2000), it is likely that the abundance of the virus(es) becomes lesser as they pass through the lymphoid organ. Based on TSV infection in *P. vannamei*, Hasson *et al*. (1999 a) proposed that in a chronic infection, the virus continues to replicate inside the type B LOS, some virions escape to the circulatory system, return to the lymphoid organ and elicit the formation of new spheroids. Spann *et al*. (2003) agreed with that model based on GAV chronic infection in *P. monodon*. The present study support this proposal since the prevalence of LOS in the prawns from the chronic trial was considerably high, i.e. 87.5 %. Likewise, the type A LOS was found at all sampling time during the chronic infection (Table 5.2.) suggesting that a cyclic infection occurred.

In the present study, LOS were seen in different tissues, such as connective tissue of the eosophagus and spongy tissue of the antennal gland (Fig. 5.4.). This agrees with Lightner (1987) and Hasson *et al*. (1999 b) who found LOS as ectopic foci in the gills, gonads, heart, antennal gland, muscle, hepatopancreas, nerve cord and tegmental glands of various penaeids. Owens *et al*. (1991) argue that the ectopic foci of LOS might show tissue tropism of virus or viruses connected to the foci since lymphoidal parvo-like virus (LPV), IHHNV and LOS appear in similar sites. However, Hasson *et al*. (1999 b) suggest that ectopic spheroids may develop outside the lymphoid organ without metastatic process as seen in the spheroidal formation in the tegmental glands of *P. vannamei* induced with TSV. Thus, Hasson *et al*. (1999 b) propose that LOS is a part of a cell-mediated inflamatory response in penaeids against small substances that cannot elicit melanization. Neverthless, Spann *et al*. (2003) did not find any LOS in connective tissues of hepatopancreas, midgut caeca and gills of *P. monodon* infected by GAV, although these tissues contained GAV DNA and LOS in the lymphoid organ were positively infected by GAV. The present study conforms to Hasson *et al*. (1999 b) who argue that LOS might migrate to tissues close to the lymphoid organ, but there was no proof of metastasis. Hasson *et al*. (1999 b) found the accumulation of LOS around the subgastric artery of the lymphoid organ in *P. vannamei* and *P. stylirostris*. In the present study, spheroids were found surrounding two lymphoidal tubules (Fig. 5.4.). Even few spheroids seem to thrust the endothelial layers of the lumens. This implies that LOS may

routinely migrate across the epithelial layers of other tissues bordering the lymphoid organ (Hasson *et al*., 1999 b), or be routinely discarded through hemal sinuses (Anggraeni and Owens, 2000). Again, these hypotheses are beyond the scope of the present study.

In summary, the present study has demonstrated that the lymphoid organ is appropriate for detecting changes in the general health performance or general immune status of the prawn relative to pathogens and environment. The present study has also demonstrated that the simple and cost-effective transect method could sufficiently and sensitively quantify various aspects of that interaction. Since this is a novel and preliminary development of quantitative models of lymphoidal changes, the technique needs validation with larger sample size and moulting as the starting point of reference. Nevertheless, STL ratio and MT score may be useful for monitoring purposes, yet they are not appropriate for a time-series experiment until biopsy of LO or other methods can be done to detect LOS without killing the animals. When farm records of mortality or survival rate, diseases and production are available, the validation of the transect technique and quantitative models of lymphoidal changes in this study can be related to management of prawn diseases in farm level. Likewise, the technique and models may be useful for ecological studies using the lymphoid organ as a bioindicator of pollution or other natural stressors in aquatic environment. The simplicity and cost effectiveness of this technique seem to warrant a wide range of potential applications.

CHAPTER 6

THE EPIDEMIOLOGY OF A POSSIBLE BACULOVIRAL DISEASE IN A PRAWN FARM IN NORTHERN QUEENSLAND, AUSTRALIA

6.1. Introduction

The studies in Chapters 4 and 5 only assessed the relationship between the hosts (i.e. penaeids) and pathogens (i.e. baculoviruses). In reality, infectious diseases occur through interplay between a susceptible host, pathogens and the environment (Weber and Rutala, 2001). Thus, the assessment of baculoviral diseases in previous chapters was void of the environmental factors.

The multi-factorial determinants or risk factors of a disease expression in human or animal population are of interest for epidemiologists (Campbell, 1983). There are two different modes in epidemiological investigations, i.e., dynamic and statistical (Lotz *et al*., 2001). Dynamic epidemiology concerns with cause-effect relationship and process of transmission of diseases. Statistical epidemiology focuses in the identification of risk factors of diseases.

In the present study, statistical epidemiology was used to analyze a possible MBV epidemic in a prawn farm in northern Queensland that cultured the tiger prawn (*Penaeus monodon*). Since 1991, the farm experienced a decline in productivity. Here, productivity was defined loosely as the biomass of prawns produced per crop, or annually. MBV infection was diagnosed by private diagnostic laboratories as one of determinants in the decline since the pattern of decline mimicked MBV-affected drop of prawn production in Taiwan in 1988 (Dr. Leigh Owens, Discipline of Microbiology and Immunology, *pers. comm*.). In actual fact, environmental and management factors also contributed to the collapse (Liao *et al*., 1992; Flegel, 1997). Nevertheless, the diagnosis of the present case has never been related to the environmental and farming management factors. Fortunately, the farm data from year 1991 to 1992, minus disease records, were handed over to Dr. Leigh Owens some years ago. The available data were used in this study to establish any potential risk factor in the decline in productivity. By including environmental and management factors in the analysis, this study would establish

whether MBV or other baculoviruses were really a risk in the decline of prawn production in the farm.

6.2. The Objectives of the Study

This statistical epidemiology study was aimed at:

1. Identifying any potential risk factor relative to the water quality (environment), hosts (biology), pathogens and farming management, and
2. Establishing any possible coherent link between the identified risk factors.

6.3. Materials and Methods

6.3.1. The source, content and size of the data

This non-concurrent longitudinal study utilizes data supplied by the farm to Dr. Leigh Owens of the Discipline of Microbiology and Immunology JCU. The data consisted of two sets of unrelated written information, and 35 histological blocks of juvenile *Penaeus monodon*. The prawn samples were taken in the end of May 1992 from the farm (Dr. L. Owens, *pers. comm.*). Therefore, pathogens and histopathology found in these samples were treated cautiously as a limited depiction of prawn diseases in the farm.

6.3.2. pH data

The values of pH were transferred from their measured values to their relevant $[H^+]$ values, which then were summed up and averaged to get a mean pooled pH for each pond, or each month, or the entire culture periods.

6.3.3. Prawn samples, microscopy and diagnosis of diseases

Thirty-five slides of the prawn histology were prepared from the histological blocks available in the archive of the Discipline of Microbiology and Immunology JCU. The prawn sections were stained with haematoxylin and eosin (HandE). Presumptive identification and diagnosis of disease were performed using a light microscope and based on Lightner (1996), Anggraeni (1998) and others. The severity grade for any viral infection was rated according to Lightner (1996).

6.3.4. Computational and statistical analyses

Data management and graphics were produced using Microsoft Excel 1997. The statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) 10.0 and 11.0 for Windows.

Summary of data included mean, variability and dispersion. Where necessary, a 95% confidence interval of the mean value was calculated. Each set of data was subjected to stem-and-leaf plot and box plot to identify and exclude outliners, before being tested for normality (Q-Q test). Abnormal data were transformed to natural log (x + 1) before any parametric tests because the standard deviations were proportional to the means and there was a strong positive skewness.

Comparison of the statistical means from relevant data used parametric tests, such as t-test, or univariate analysis of variance (ANOVA). Where possible, the strength of association among the environmental, management and/or biological variables was examined using Pearson's product moment analysis and stepwise regression analysis. Analyses of the strength of association between pathogens and anatomical lesions were focused on attributable risk (AR) and odds ratios (OR) (Thrusfield, 1986). Coherence and analogy were applied to establish the most possible correlations between the diseases, histopathology, feeding budget and prawn biomass. The level of $P<0.05$ was chosen for accepting statistical significance. $P<<0.05$ was used to show P approaching 0.01 but not reaching it. e.g. $P\sim0.017$.

6.4. Results

6.4.1. The data of environmental parameters and feed budget

The first set of written data reported herein contained raw (primary) data of the daily weather quality (Appendices 6.1. and 6.2.), water quality and feed quantity (Appendices 6.3. and 6.4.) during a culture period from 18 crops. However, only 15 crops generated useful information for the purpose of this study (Table 6.1.). The records extended from the 30th of August 1991 to the 30th of May 1992 covering a total of 265 working days. However, for convenience, all data from 30-31 August 1991 were included in September

1991. Some data of daily weather or water quality for some dates were missing, or not recorded, especially after January 1992.

All daily records contained the names of the responsible officers, sampling date, and comments on mechanical or water quality problems found in any pond. The supplementary comments indicated the use of paddlewheel aerators in the ponds.

Table 6.1. A reconstruction of culture periods for each crop. Shadowed boxes are the lengths of the culture period.

Pond No.	Year 1991				Year 1992				
	Sept	**Oct.**	**Nov.**	**Dec.**	**Jan.**	**Feb.**	**Mar**	**Apr**	**May**
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Although the quantity and quality of the environmental data were reasonable, nothing could be done to relate these data to the actual disease and mortality that occurred during the culture periods of each crop. Since no disease data recorded in this data set, the amount of feed was used as a surrogate measure of prawn health. It was assumed that the amount of feed might indirectly reflect the health condition of the prawns because under stressful or diseased condition penaeids usually become lethargic and lose their appetite (Lightner, 1996; Shariff *et al*., 2001). Thus, the amount of feed can become the proxy (indirect) measure of the prawn health and parallel the change of environmental stressors.

6.4.2. Stocking, culture period, ponds and prawn species

Some of the crop data lacked a record of stocking and harvesting dates. Yet, the available data helped to reconstruct culture periods for each crop (Table 6.1.). Some ponds were stocked twice, which apparently caused incomplete data for the whole culture periods for those ponds (Table 6.1.). Thus, most of the data only recorded the end of the 1st crop, or the start of the 2nd crop.

With the lack of stocking and harvesting dates, the length of culture period could not be determined for all 15 crops (Table 6.1.). Only 4 crops had the definite start and end of the culture period, i.e., crop #3 (6.5 months), crop #7 (5.5 months), crop #9 (3 months, in the first culture period), and crop #13 (5.5 months). Assuming one month contained 30 days, the mean (±SD) length of culture period was 154 ± 45 days with a 95% confidence interval of the mean between 111 – 197 days.

6.4.3. Weather condition

The weather parameters consisted of morning and afternoon rainfall, cloud cover, wind speed and temperature (Table 6.2 and Appendices 6.1. and 6.2.). On the average, the mean morning rainfall (7 ± 0.7 mm) was higher than that of the afternoon (6.5 ± 0.7) (Table 6.2.). In the morning, the air temperature ($23.7 \pm 6.8e^{-02}$ ^{o}C) was cooler than in the afternoon ($26.9 \pm 9.4e^{-02}$ ^{o}C) (Table 6.2.). The cloud cover in the morning (51.8 ± 1%) was lower than that in the afternoon (57.4 ± 1.1%). However, the wind speed in the morning (9.1 ± 0.2 knots) was higher than in the afternoon (8.3 ± 0.2 knots).

Table 6.2. Pooled data for weather condition from September 1991 to May 1992

Weather parameters	N	Min	Max	Mean	SE	SD
Morning rainfall (mm)	570	0	108	7.0	0.7	17.2
Afternoon rainfall (mm)	611	0	150	6.5	0.7	18.1
Morning temperature (^{o}C)	1086	16.8	33	23.7	0.07	2.2
Afternoon temperature (^{o}C)	875	21.4	35	26.9	0.09	2.8
Morning cloud cover (%)	1056	0	100	51.8	1.0	33.4
Afternoon cloud cover (%)	994	0	100	57.4	1.1	34.3
Morning wind speed (knot)	1145	0	30	9.1	0.2	7.3

Afternoon wind speed (knot)	1015	0	30	8.3	0.2	6.3

6.4.4. Screening of water quality data

The analyses of water quality were extensive since the prawns were directly in contact with the water. The water quality data included temperature, pH, dissolved oxygen (DO) level, transparency and salinity (Tables 6.3., 6.4. and Appendix 6.3.).

The raw data of water quality, especially the DO and transparency, were rather dubious. The DO levels of 12-18 ppm (higher than saturation), or the transparency of 140-150 cm followed by much smaller readings, might represent technical failures of the DO meter, or misreading of the Secchi disk depth. After the data were screened using the stem-and-leaf plot and box plot, and those questionable points were omitted, 93% of the total numbers of the data were useable (Table 6.3.). However, the useable data for morning DO and daily DO levels were only 72.64% and 74.73%, respectively (Table 6.3.).

Table 6.3. Changes of mean and standard deviation (SD) for pooled water quality from September 1991 to May 1992 (before and after the data screening).

Water Quality Parameters	**Unscreened data**			**Screened data**			**Useful N**
	N	Mean	SD	N	Mean	SD	
Morning temperature (^{o}C)	1641	26.0	2.5	1641	26.0	2.5	100 %
Noon temperature (^{o}C)	1133	27.9	2.4	1132	27.9	2.4	99.99%
Daily temperature (^{o}C)	1704	26.6	2.4	1695	26.7	2.3	99.47%
Morning DO level (ppm)	1513	7.1	2.8	1099	5.9	0.9	72.64%
Afternoon DO level (ppm)	951	8.2	2.3	869	7.6	1.2	91.38%
Midnight DO level (ppm)	722	7.0	1.4	660	6.7	0.8	91.41%
Daily DO level (ppm)	1591	7.7	2.7	1189	6.6	1.0	74.73%
Morning pH	1647	8.4	0.3	1589	8.4	0.2	96.48%
Afternoon pH	1083	8.6	0.3	1049	8.6	0.3	96.86%
Daily pH	1579	8.5	0.4	1534	8.5	0.2	97.15%
Transparency (cm)	889	57.7	23.8	883	56.7	22.3	99.33%

Salinity (ppt)	946	32.6	5.4	905	33.1	4.6	95.67%
						Total mean of useful data	92.93%

Table 6.4. Monthly water quality from September 1991 to May 1992. *A.M. = morning; N/A = no recorded data; **cannot be calculated for one datum.

Parameters		**Year 1991**				**Year 1992**				
		Sept.	**Oct.**	**Nov.**	**Dec.**	**Jan.**	**Feb.**	**Mar.**	**Apr.**	**May**
Tem-pera-ture (oC)	***A.M.****									
	Mean	23.75	24.08	26.20	28.82	29.15	27.58	27.38	24.75	24.29
	SD	1.63	1.43	1.59	1.02	1.24	1.99	1.38	0.85	0.97
	N	246	278	239	197	105	135	174	113	146
	Noon									
	Mean	25.77	26.77	28.32	30.66	30.86	28.53	29.96	N/A	26.14
	SD	1.52	1.56	1.54	1.31	1.37	1.85	1.06		1.38
	N	252	280	248	162	80	49	21		50
DO (ppm)	***A.M.***									
	Mean	6.02	6.10	5.69	5.33	5.79	5.81	6.46	6.25	6.90
	SD	0.76	0.98	0.87	0.64	1.21	0.74	1.05	1.42	0.92
	N	237	253	220	143	11	141	57	4	26
	Noon									
	Mean	7.56	8.06	7.45	6.51	10.08	8.88	5.79	N/A	8.91
	SD	0.96	1.18	1.32	0.74	0.81	0.82	0.80		1.38
	N	238	287	197	102	4	16	7		12
	Night									
	Mean	6.85	6.99	6.55	5.76	N/A	8.60	N/A	N/A	N/A
	SD	0.72	0.74	0.71	0.51		**			
	N	212	236	146	64		1			
pH	***A.M.***									
	Mean	8.45	8.48	8.39	8.40	8.64	8.31	8.31	8.28	8.35
	SD	0.22	0.22	0.23	0.24	0.21	0.19	0.29	0.26	0.30
	N	237	273	223	182	91	161	170	113	138
	Noon									
	Mean	8.45	8.48	8.59	8.48	8.85	8.51	8.59	N/A	8.68
	SD	0.22	0.22	0.24	0.23	0.17	0.19	0.25		0.25
	N	226	271	222	144	80	43	21		33
Trans pa-rency (cm)	Mean	54.89	53.83	63.10	71.01	66.37	N/A	50.88	35.49	34.13
	SD	23.67	20.70	17.77	18.50	17.61		21.51	20.73	12.84
	N	151	167	199	129	61		51	35	87
Sali-nity (ppt)	Mean	31.46	35.73	37.17	34.81	30.33	24.40	33.29	24.92	24.07
	SD	1.98	2.28	2.38	2.56	0.71	8.05	5.61	1.99	3.60
	N	212	176	202	131	9	5	23	38	144

A significant drop of pooled means after the data screening occurred in DO data, e.g., the mean of morning DO from 7.1 to 5.9 ppm, or daily DO from 7.7 to 6.6 ppm (Table 6.3.). Except the morning and afternoon temperature data, all screened data produced narrower standard deviations of the means than the unscreened ones. Further analysis in this report utilized the screened data only.

The analyses of Pearson's correlation among water quality parameters showed that on a daily basis all parameters correlated to one another, except acidity did not correlate to the transparency and salinity (Table 6.5.). However, detailed analysis of the correlations among the water quality parameters based on the time of sampling (i.e., morning, afternoon, night or daily) shows that 9 out of 65 paired parameters (14 %) did not correlate to one another (Appendix 6.4.). Among other things, DO level in the morning did not correlate to the transparency ($r = -0.05$ at $p >> 0.05$), whilst DO level in the afternoon did not correlate to the salinity ($r = 0.03$ at $p >> 0.05$) (Appendix 6.4.). Likewise, DO level at night did not correlate ($p >> 0.05$) to the morning pH ($r = 0.03$), daily pH ($r = 0.05$) and salinity ($r = -0.07$) (Appendix 6.4.). Also, transparency did not correlate ($p >> 0.05$) to the morning pH ($r = 0.01$) and daily pH ($r = -0.02$), while salinity did not correlate to the morning pH ($r = -0.02$ at $p >> 0.05$), afternoon pH ($r = 0.07$ at $p > 0.05$) and daily pH ($r = -0.03$ at $p >> 0.05$) (Appendix 6.4.).

Table 6.5. Pearson's correlation (2-tailed test) between daily water quality parameters. The bold numbers show significant correlation.

Parameters		**DO**	**pH**	**Transparency**	**Salinity**
Tempe-rature	Pearson's R Coefficient	**- 0.32**	**0.19**	**0.41**	**0.28**
	Probability	<< 0.05	<< 0.05	<< 0.05	<< 0.05
	N	1141	1484	841	876
Dissolved Oxygen (DO)	Pearson's R Coefficient		**0.07**	**- 0.18**	**- 0.10**
	Probability		<< 0.05	<< 0.05	<< 0.05
	N		1114	648	719
Acidity (pH)	Pearson's R Coefficient			- 0.02	- 0.03
	Probability			>> 0.05	>> 0.05
	N			780	815
Transpa-	Pearson's R Coefficient				**0.35**

rency	Probability				<< 0.05
	N				728

6.4.5. Water temperature

The pooled mean of daily water temperature was 26.7 ± 2.3 °C with the morning temperature (26.0 ± 2.5 °C) lower than that in the afternoon (27.9 ± 2.4 °C) (Table 6.3.). The monthly data for morning and afternoon temperatures also had a similar trend (Table 6.4.). However, the means of water temperature steadily increased over summer and decreased towards the middle of the year (Fig. 6.1.). Hence, the mean temperature increased from 24.70 ± 1.50 °C in September 1991, up to 29.86 ± 1.16 °C in January 1992, before declining to 24.75±0.85°C in April 1992, and remained in the same in May 1992 (Fig. 6.1.).

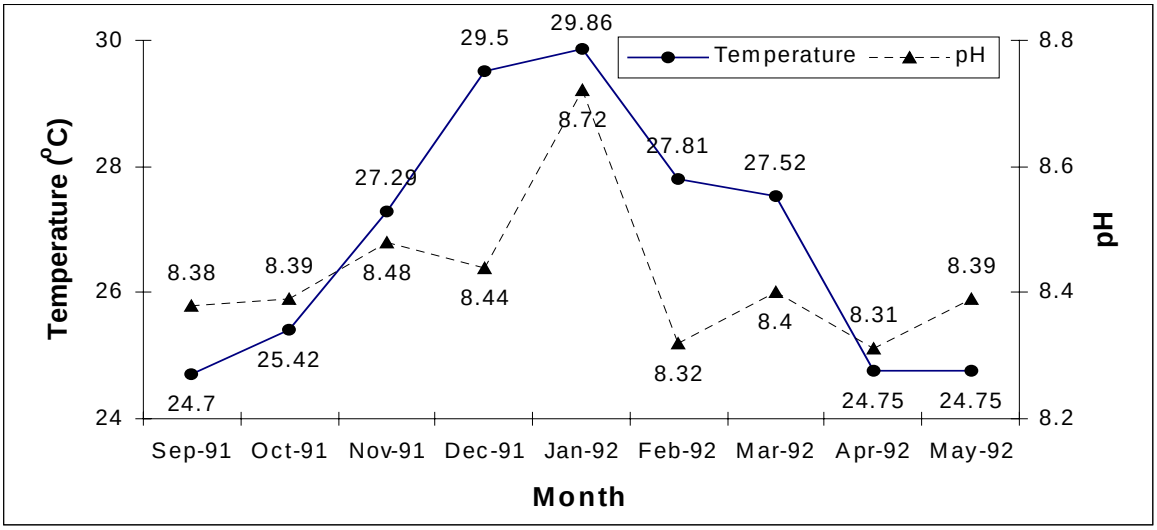


Figure 6.1. The monthly changes of the mean water temperature and pH.

On a monthly basis, the temperature changed in a relatively similar fashion as the pH (Fig. 6.1.). However, their correlation was not significantly linear (Pearson's 2-tailed test with $p > 0.05$, Pearson's coefficient = 0.65 and N =9).

On a daily basis, the temperature correlated negatively with daily DO level ($r = -0.32$ at $p << 0.05$), and positively with daily pH ($r = 0.19$ at $p << 0.05$), transparency ($r= 0.41$ at $p << 0.05$) and salinity ($r = 0.28$ at $p << 0.05$) (Table 6.5.). Daily pH and temperature had a

linear relationship (Fig. 6.2.) with an F = 56.2 (df = 1, 1482) at p << 0.05. Daily DO and temperature had a linear relationship (Fig. 6.3.) with an F = 133.67 (df = 1, 1139; p << 0.05). The daily temperature also was positively correlated with the salinity (Fig. 6.4.) with an F = 76.44 (df= 1, 874; p << 0.05).

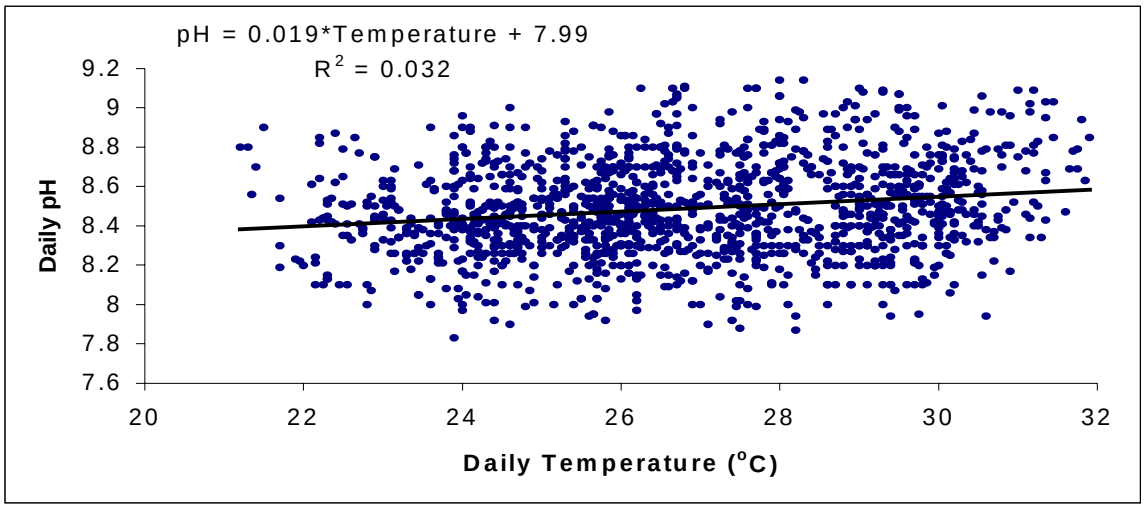


Figure 6.2. The regression between the daily water temperature and pH.

6.4.6. Dissolved oxygen (DO)

The pooled mean DO level in the afternoon (7.6 ± 1.2 ppm) was higher than the morning level (5.9 ± 0.9 ppm), the night level (6.7 ± 0.8 ppm), or the daily level (6.6 ± 1.0 ppm) (Table 6.3.). In 1991, the monthly DO in the morning was always lower than that at noon, or at midnight, while DO at noon was always the highest (Table 6.4.). In 1992, the highest DO occurred at noon, except in April where data was lacking (Table 6.4.).

The highest monthly DO occurred in October 1991 (7.06 ± 0.69 ppm) and May 1992 (7.10 ± 0.89 ppm), and the lowest level (5.68 ± 0.67 ppm) in December 1992 (Fig. 6.5.). Although the DO levels remained inside a 4-to-9 ppm boundary, nevertheless a rather substantial fluctuation occurred in January and April 1992 (Fig. 6.5.).

As well as being correlated with the temperature (Fig. 6.3.), the daily DO level also correlated with the daily pH (r = 0.07 at p < 0.05) (Table 6.5. and Fig. 6.6.), transparency (r = - 0.18 at p << 0.05) (Table 6.5. and Fig. 6.7.), and salinity (r = - 0.10 at p << 0.05)

(Table 6.5. and Fig. 6.8.). With the pH, the DO level correlated significantly at $p < 0.05$ ($F = 4.88$; df = 1; 1113).

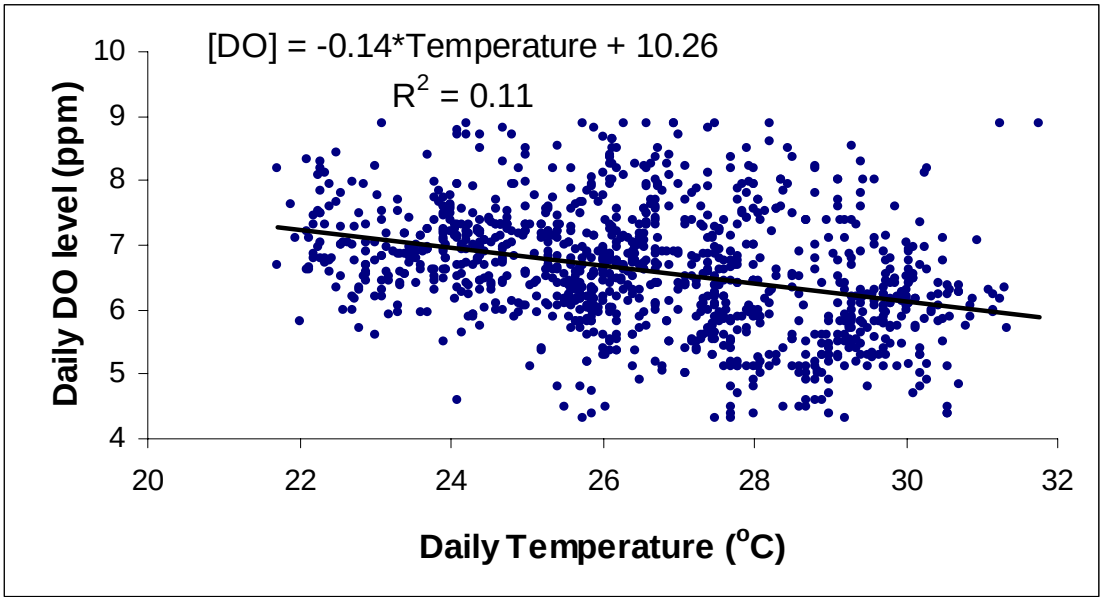


Figure 6.3. The regression between the daily water temperature and DO level.

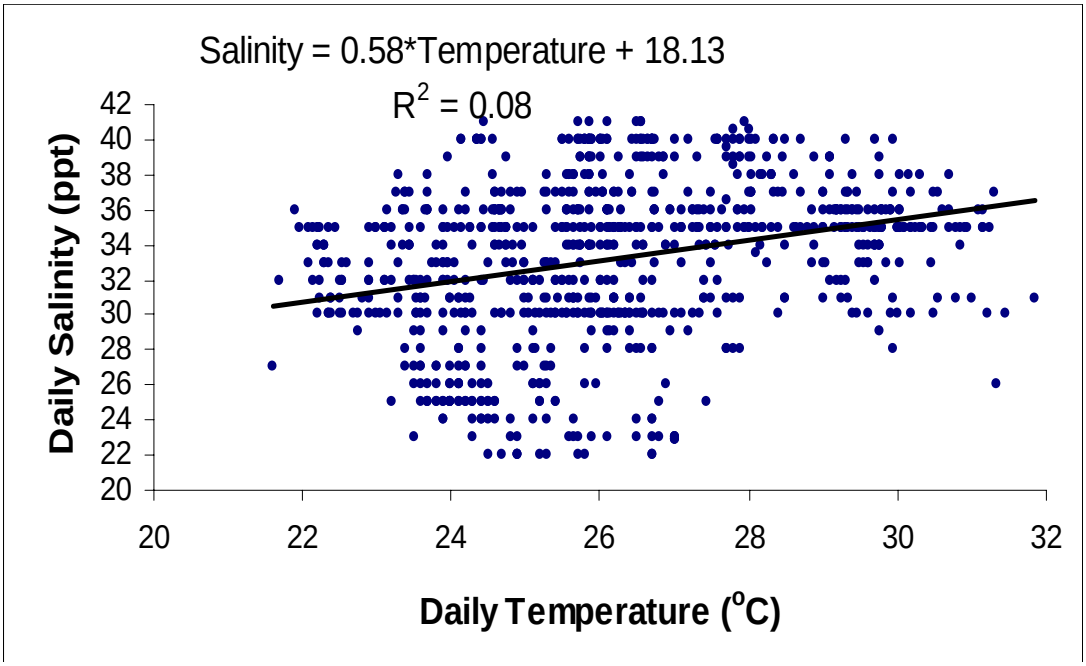


Figure 6.4. The regression between the daily water temperature and salinity.

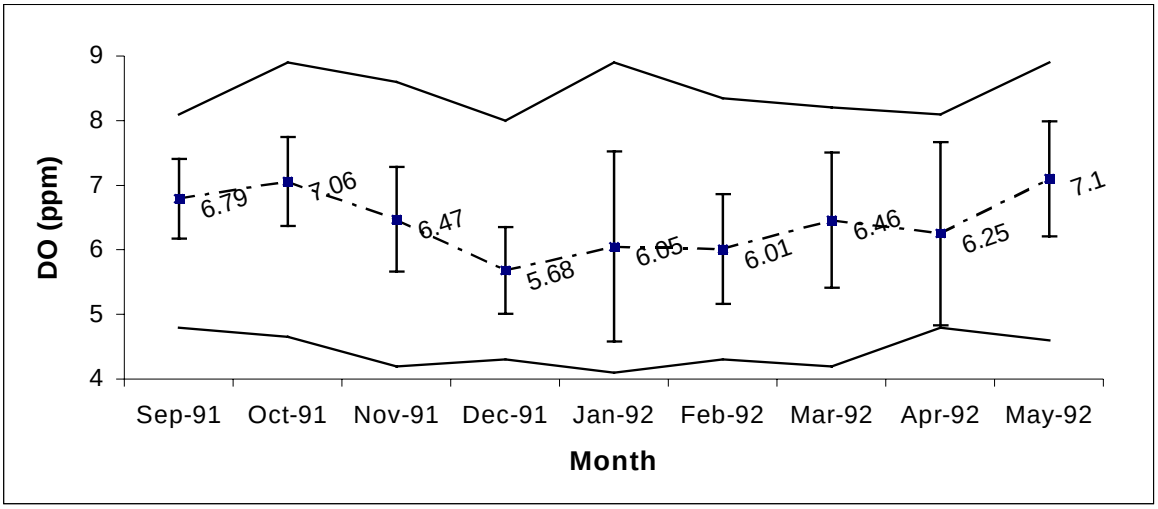


Figure 6.5. The monthly changes of the means of DO levels in the pond water. Bars are the standard deviations. The top line is the maximum boundary. The bottom line is the minimum boundary.

6.4.7. Water pH (acidity)

The pooled mean pH in the morning (8.4 ± 0.2) was lower than that in the afternoon (8.6 ± 0.3), or on a daily basis (8.5 ± 0.2) (Table 6.3.). On a monthly basis, the pooled means of pH in the morning was mostly lower than that in the afternoon (Table 6.4.). The peak of monthly pH was reached in January 1992 (8.7 ± 0.2 ppm), and the lowest was in February 1992 (8.3 ± 0.2) (Fig. 6.1.).

The daily pH had no linear correlation with the daily transparency ($p = 0.53$, Pearson's correlation = - 0.02), or salinity ($p = 0.46$, Pearson's coefficient = - 0.03) (Table 6.5.).

6.4.8. Transparency and salinity

The mean pooled transparency of the pond water was 56.7 ± 22.3 cm (Table 6.3.). However, the transparency increased from October 1991 (53.8 ± 20.7 cm) up to December 1991 (71.0 ± 18.5 cm) before decreasing from January 1992 onward to the lowest level (34.1 ± 12.8 cm) in May 1992 (Table 6.4.).

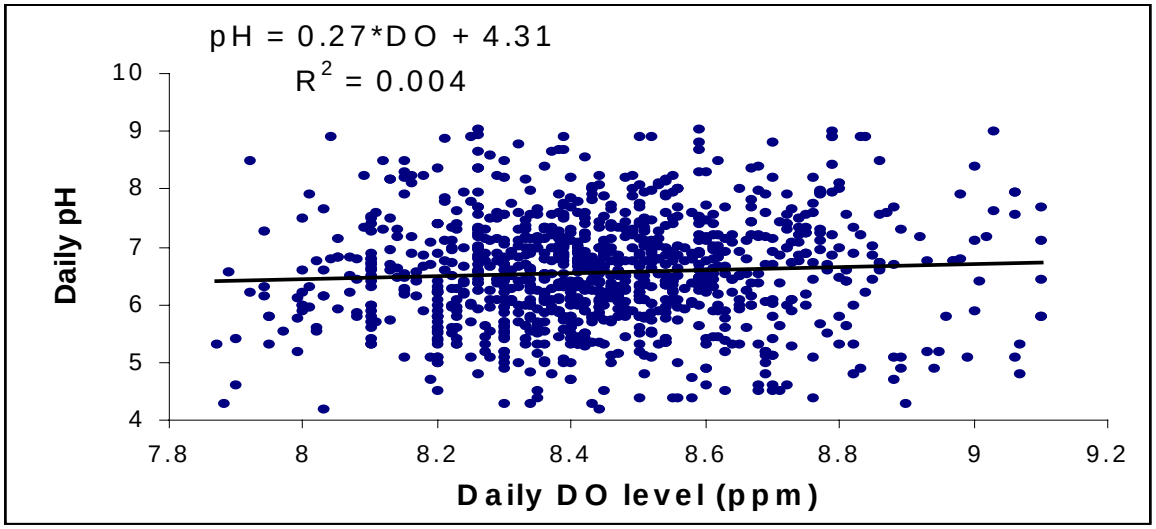


Figure 6.6. The regression between the daily water DO level and pH.

As well as being correlated significantly to daily temperature (section 6.4.5. and Fig. 6.7.) and DO level (section 6.4.6. and Fig. 6.8.), the daily transparency also correlated significantly to the salinity (Pearson's coefficient = 0.35, $p << 0.05$, N =728) (Table 6.5. and Fig. 6.10.).

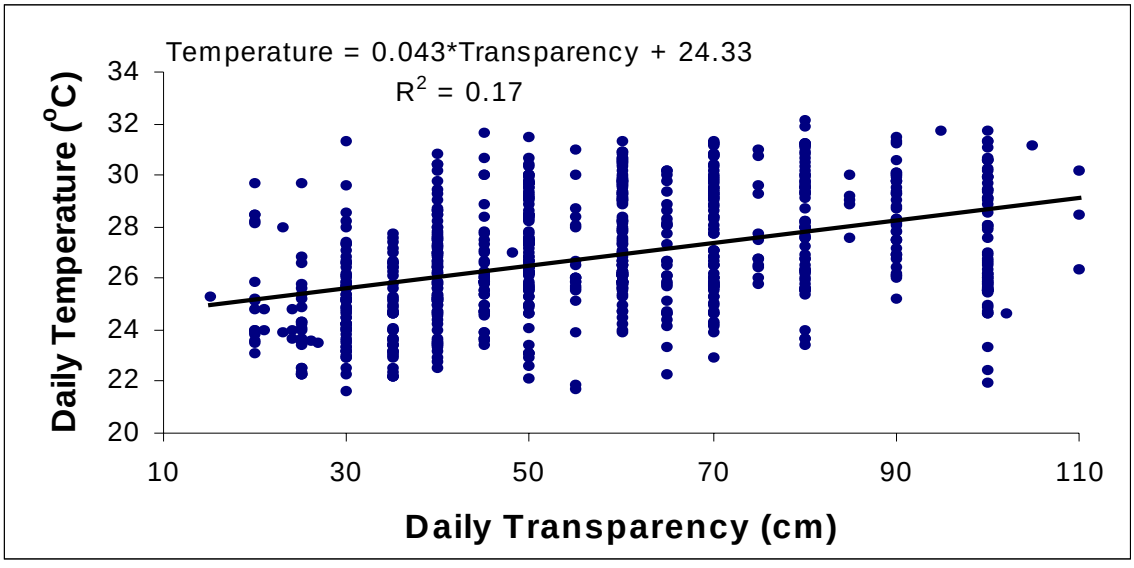


Figure 6.7. The regression between daily transparency and water temperature.

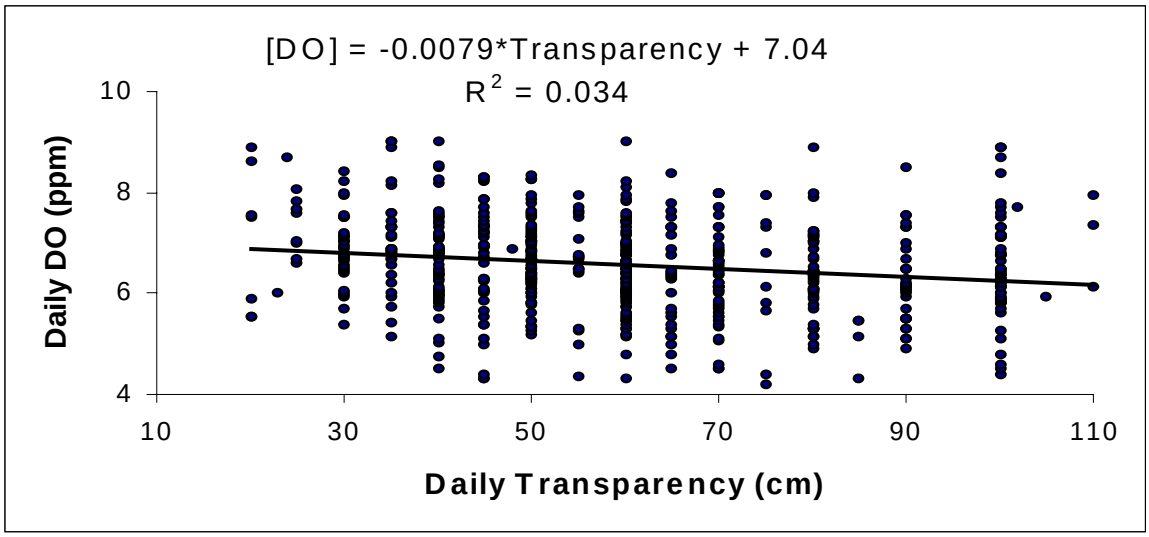


Figure 6.8. The regression of daily water transparency and DO level.

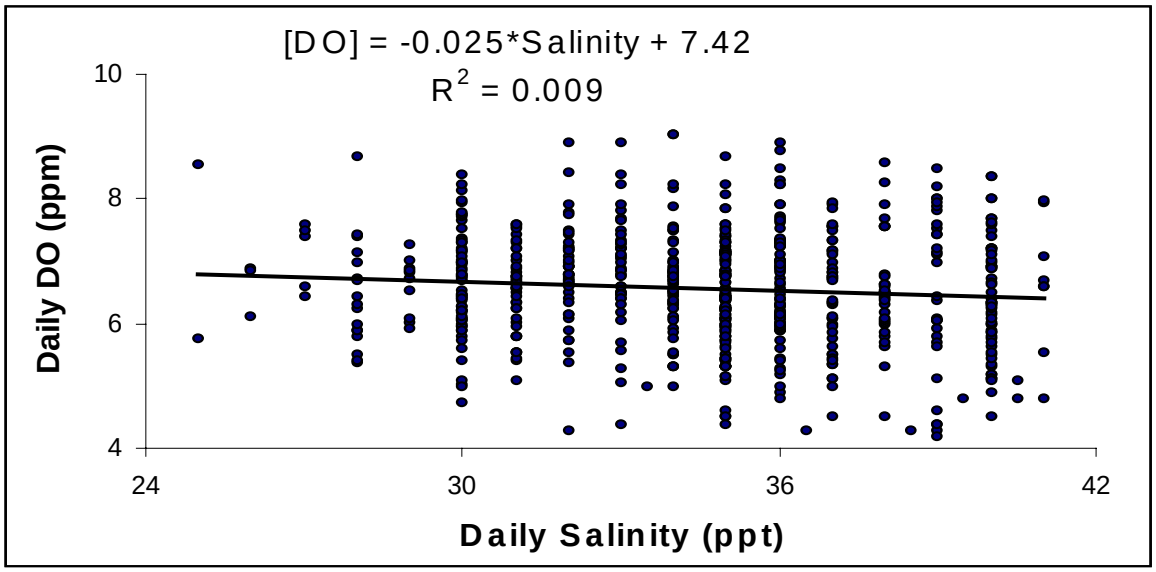


Figure 6.9. The regression between daily water salinity and DO level.

The monthly means of salinity also changed overtime in an increasing trend from September to December 1991, followed by a steady decline (except in March 1992) (Table 6.4.). The highest mean of salinity occurred in December 1991 (37.17 ± 2.38 ppt) and the lowest in May 1992 (24.07 ± 3.60 ppt) (Table 6.4.). The pooled mean of salinity was 30.96 ± 5.22 ppt (Table 6.3.).

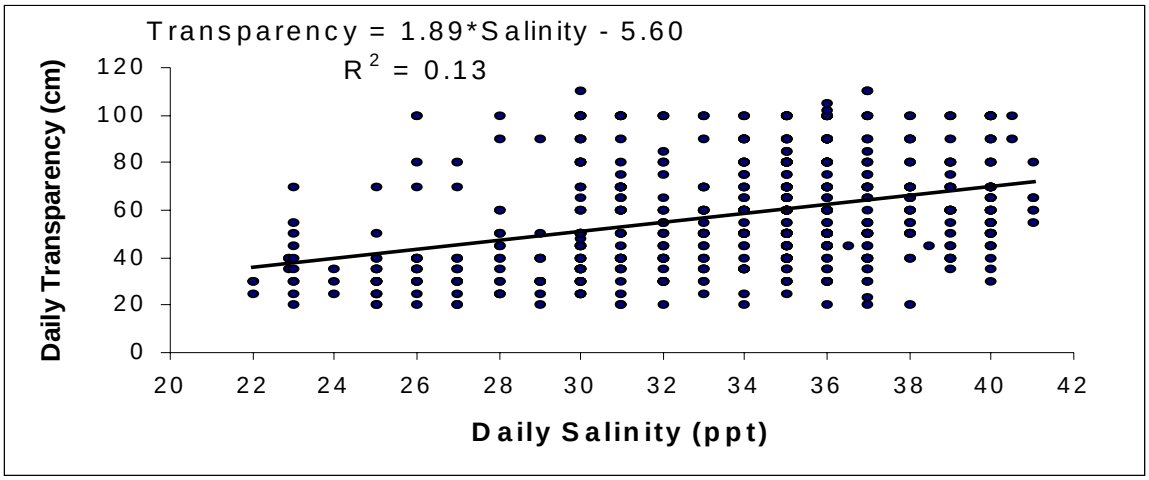


Figure 6.10. The relationship between daily water salinity and transparency.

6.4.9. Feeding activities

The prawns were fed 3 to 5 times a day with pellets with the amount fed being adjusted by the use of feeding trays. In some cases, the prawns were initially fed with a combination of *Artemia* and pellets, a couple of days after stocking in the grow-out ponds.

The amounts of feed given during a culture period in the ponds followed three phases of activity (Fig. 6.11. and Table 6.6.), namely:

(1) The initial phase, where very small amounts of pellets were given to the animals during the first days of stocking, when the animals were adjusting to the environment in the pond. *Artemia* was also fed to the prawns in this period.

(2) The increasing-feeding phase (supposedly when the animals ate intensely during their exponential growth).

(3) The feed-reduction phase (presumably the feed quantity was reduced prior to the harvesting time).

For example, in pond #14 (Fig. 6.11.), the initial phase of feeding activity lasted for 39 days, in which no feed was given during the first 15 days. Thus only 24 days were used to calculate the mean of the daily feed in the initial phase (Table 6.6.).

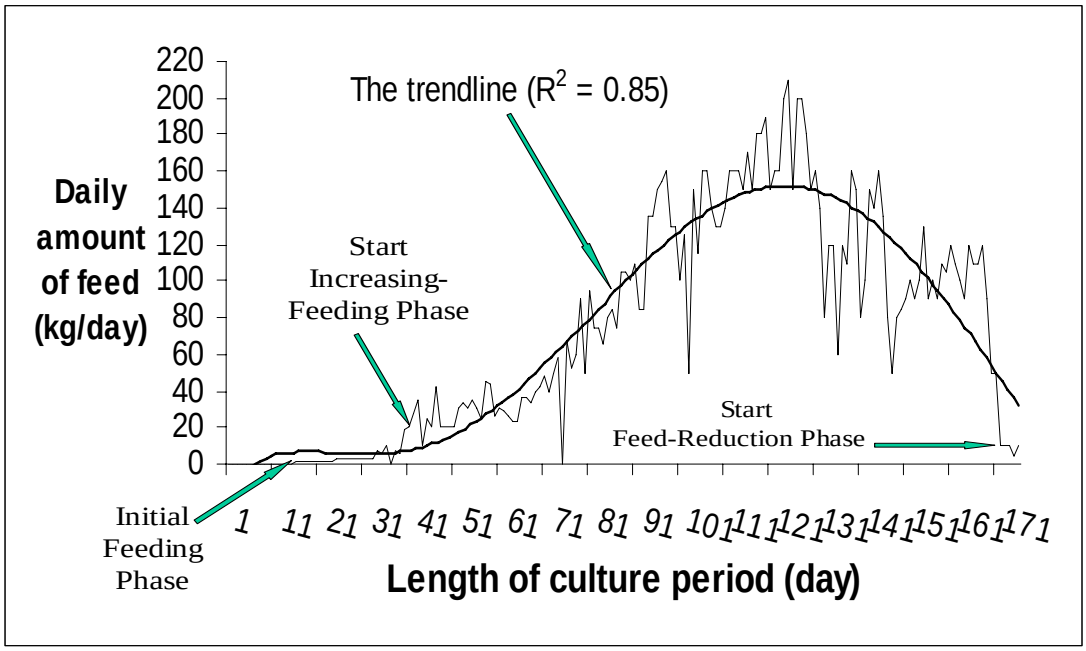


Figure 6.11. A typical feeding pattern in the grow-out ponds (data from pond #14), showing the actual starting points of the initial feeding phase, increasing-feeding phase and feed-reduction phase. The trend line clarifies the pattern.

The mean±SD of the daily amounts of fed were 3.3 ± 0.9 kg in the initial phase, 65.6 ± 30.8 kg in the increasing-feeding phase, and 9.1 ± 4.3 kg in the feed-reduction phase (Table 6.6.). On the average, the initial phase lasted for 26 ± 10 days, the increasing-feeding phase for 86 ± 41 days, and the feed-reduction phase for 3 ± 3 days (Table 6.6.). The pooled mean daily feed (59.2 ± 31.3 kg) was lower than that of the increasing-feeding phase (65.6 ± 30.8 kg) (Table 6.6.). Thus, to eliminate the effects of the initial phase and the feed-reduction phase, the analysis of feed amount only covered those given during the increasing-feeding phase.

6. 4. 10. Correlation between feed amount and water quality parameters

The correlation and degree of association between the amount of feed and water quality were conducted on a pond-to-pond basis (Tables 6.7., 6.8. and Appendix 6.5.). A comparison between the crops was not possible due to the lack of data on the stocking density, age, and mortality, or survival, rate of the prawns. Differences in the environmental conditions from pond to pond (Appendix 6.3.) also made the comparison between crops irrelevant.

Table 6.6. Daily feed in the grow-out ponds at different phases. A pond stocked twice was denoted A and B to distinguish two crops. Total feeding period and mean daily feed amount were calculated from the total amount of feed divided by the number of days when the prawns fed. Days without feeding were omitted from the calculation.

Pond No.	**Initial phase**		**Increasing-feeding phase**		**Feed-reduction phase**		**Total**	
	Period (day)	Mean (kg/day)	Period (day)	Mean (kg/day)	Period (day)	Mean (kg/day)	Period (day)	Mean (kg/day)
1			46	115.4	1	10.00	47	113.2
3			66	101.9	2	10.50	68	99.2
4	36	2.94	157	59.5			193	49
5			105	76.8			105	76.8
6			102	77.8	2	17.50	104	76.7
7			96	73.6			96	73.6
8	21	3.93	130	97.9	9	6.11	160	80.4
10A	42	1.78	42	15.6	1	5.00	85	8.7
10B	25	5.12	56	38			81	27.8
11A	29	3.90	58	24.4			87	17.6
11B	32	3.50	16	17.6			48	8.2
12A			103	74.6			103	74.6
12B	25	2.66	91	82.3			116	65.1
13A	17	2.47	87	49.9			104	42.1
13B			70	96.1	4	5.50	74	91.2
14	24	3.42	130	96.6	5	9.00	159	79.8
15A			33	39.9			33	39.6
15B	8	2.88	152	43.9			160	41.8

Mean	***26***	***3.3***	***86***	***65.6***	***3***	***9.1***	***101***	***59.2***
SD	***10***	***0.9***	***41***	***30.8***	***3***	***4.3***	***44***	***31.1***

Table 6.7. Significant correlation (marked with "x") between food amount given and water quality parameters for each pond during the high feeding phase. Mo= morning, Aft = afternoon, Nit = night, Av = daily mean, T = transparency, S = salinity.

Pond Number	**Temperature**			**Dissolved Oxygen**				**pH**			**T**	**S**
	Mo	Aft	Av	Mo	Aft	Nit	Av	Mo	Aft	Av		
1				X			X				X	X
3			X					X	X	X		X
4	X	X						X		X		X
5	X	X	X	X		X	X	X		X	X	X
6		X		X				X		X		
7	X	X	X	X			X				X	X
8	X	X	X					X	X	X	X	X
10A					X	X	X					
10B	X											
11A	X	X	X	X				X	X	X		X
12A	X	X	X	X			X				X	X
12B	X		X					X		X	X	
13A	X	X	X	X		X	X	X	X	X		
13B	X		X								X	
14	X	X	X	X				X	X	X	X	X
15A			X			X						
15B												X
Count	11	9	11	8	1	4	6	9	5	9	8	10
% Total	65	53	65	47	6	24	35	53	29	53	47	59

In general, the water quality parameters for the 17 ponds examined showed different degrees of importance in relation to the amount of feed (Table 6.7.). The likely important parameters (based on the proportion of the ponds) were the temperature (53 – 65%), salinity (59%), morning or daily pH (53%), and morning DO and transparency (47%). The other parameters correlated significantly to the amount of feed only in 6 – 35% of the total ponds.

Of the 17 ponds included in the analyses, the amount of feed correlated significantly to the morning, or daily, water temperature in 11 ponds (65%), and the mean afternoon temperature in 9 ponds (53%) (Table 6.7.). In 7 ponds, the amount of feed correlated significantly to the morning, afternoon and mean daily temperature (Table 6.7.). A positive correlation between the amount of feed and the morning temperature occurred in 6 out of 11 ponds (55%), the afternoon temperature in 7 out of 9 ponds (78%), and the mean daily temperature in 6 out of 11 ponds (55%) (Table 6.8.). Thus, in the majority of the ponds (63% in a total mean), the feed amount correlated positively with the temperature during the high feeding phase.

Only 1 out of 17 ponds (6%) showed a significant correlation between the food amount and the dissolved oxygen (DO) in the afternoon (Table 6.7.). Thus, it was excluded from further analyses. However, the food amount correlated significantly to the morning DO in 8 ponds (47%), night DO in 4 ponds (24%) and daily mean DO in 6 ponds (35%) (Table 6.7.). Of these ponds, a negative correlation between the food amount and morning DO occurred in 7 out of 8 ponds (87%), night DO in all 5 ponds (100%), and daily mean DO in all 6 ponds (100%) (Table 6.8.). Thus, in the majority of the ponds (96% in the total mean), the feed amount related negatively with DO levels during the high feeding phase.

The feed amount correlated significantly with the morning pH in 9 out of 17 ponds (53%), the afternoon pH in 5 ponds (29%) and the mean daily pH in 9 ponds (53%) (Table 6.7.). Seventy-eight percent of the correlations with the morning pH, 60% with the afternoon pH, and 78% with the mean daily pH were negative (Table 6.8.). On

average, the majority of the ponds (72% of the total) had a negative correlation between the feed amount and pH (Table 6.8.).

Table 6.8. The modes of correlation between food amount and water quality parameters for all ponds based on Pearson's correlation analysis. *Only one datum available, thus excluded from the calculation of the pooled mean for DO.

Parameters	Groups	Mode	Count	% Total	Pooled Mean
Temperature	Morning	Positive (+)	6	55	63% (+)
		Negative (-)	5	45	
	Afternoon	Positive (+)	7	78	37% (-)
		Negative (-)	2	22	
	Daily mean	Positive (+)	6	55	
		Negative (-)	5	45	
Dissolved Oxygen	Morning	Positive (+)	1	13	4% (+)
		Negative (-)	7	87	
	Afternoon*	Positive (+)	0	0	96% (-)
		Negative (-)	1	100	
	Night	Positive (+)	0	0	
		Negative (-)	4	100	
	Daily mean	Positive (+)	0	0	
		Negative (-)	6	100	
pH	Morning	Positive (+)	2	22	28% (+)
		Negative (-)	7	78	
	Afternoon	Positive (+)	2	40	72% (-)
		Negative (-)	3	60	
	Daily mean	Positive (+)	2	22	
		Negative (-)	7	78	
Transparency		Positive (+)	3	38	38% (+)
		Negative (-)	5	62	62% (-)
Salinity		Positive (+)	4	40	40% (+)
		Negative (-)	6	60	60% (-)

Eight out of 17 ponds (47%) had a significant correlation between the feed amount and the transparency (Table 6.7.). Of these 8 ponds, 62% (5 ponds) showed negative correlation between the two variables (Table 6.8.).

The salinity and the amount of feed had a significant correlation in 10 out of 17 ponds (59%) (Table 6.7.). Among these 10 ponds, 60% (6 ponds) showed a negative correlation between both variables (Table 6.8.).

Overall, the daily temperature, salinity and pH were the major water quality parameters in the majority of the ponds relative to the amount of feed. Hence, in the majority of the ponds, the amount of feed correlated negatively with all water quality parameters, except for temperature, during the high feeding phase.

6.4.11. Content analysis of the biological and management data

The second source of written data from the farm contained records of biological and management parameters from different culture periods or crops (Table 6.9. and Appendix 6.6.). These data were sampled in the 20th of November 1991 and the 29th of April 1992 (Appendix 6.6.). The farm stocked the ponds with a total of 29 crops of *Penaeus monodon* from the 10th of January 1991 to the 18th of April 1992. Records of biological data exhibit the prawn age, individual growth rate, pond-weight gain, mean body weight and survival rate. The management factors cover the dates of stocking, total stocks, initial culture density, and feeding management (body weight-based feeding rate, feed amount in the last week before sampling, total running feed and food conversion ratio). Two other parameters, i.e., standing crop and biomass, were assigned as the outcomes of the interactions between other factors. Since no disease records were included in the written data, the surrogate measure of prawn health used to analyze these farm data was the biomass of the prawns. Biomass was assumed to reflect the productivity based on the prawn weight, which is readily altered by diseases.

Of those 29 data sets (Appendix 6.6.), only 69% (20/29) sets were considered valid and reliable (Table 6.10.). The 9 other data sets were excluded from analyses because:
1. The analyses only covered the increasing feeding phase (section 6.4.9.), i.e., older than 32 days in the pond (the 95% confidence upper bound for the mean initial feeding phase). Three 1991 data and three 1992 data failed to meet this criterion. The pond age of the

prawns in these data at the times of sampling ranged from 4 to 32 days whilst biomass ranged from 1 to 28 kg/ha (Appendix 6.6.).

2. The data set of the 8th of June 1991 had an estimated survival rate (SR) of 138%. Here, the SR reflects either an addition of stock during the culture period, or an incorrect initial estimate of the stocking density. Therefore these data were not used.

3. Data sets of the 10th and the 26th of January 1991 had records of the total running feed only, i.e., 12,711 kg and 28,239 kg, respectively (Appendix 6.6.). Thus, these two data sets were insufficient for further analysis.

Table 6.9. Statistical summary of the management and biological parameters.

Parameters	N	Min	Max	Mean	SE
Farming management					
(1) Stocking Date	29	None	None	None	None
(2) Number of Stock	26	250,000	730,000	506,235	21,619
(3) Density (No. of prawns/m^2)	26	8	25	17.62	0.86
(4) % Body Weight Fed	23	0.60	8.90	2.87	0.43
(5) Fed Amount in the last week (kg)	23	5	785	378	53
(6) Total Running Feed (kg)	25	8	28,239	5180	1252
(7) Estimated Running F. C. R.	23	0.46	2.49	1.44	0.10
Prawn biology					
(1) Age (days)	26	4	167	90.65	11.06
(2) Estimated Survival Rate (%)	26	20	100	63.81	3.47
(3) Pond Weight Gained (kg)	21	11	933	398	62
(4) Average Body Weight (g)	26	0.02	23.87	7.32	1.26
(5) Individual Growth Rate (g/week)	23	0.03	3.64	1.48	0.23
Outcomes					
(1) Standing Crop (kg)	26	4	6260	2288	392
(2) Biomass (kg/ha)	25	1	2159	843	141

The 20 valid and reliable data sets contained approximately 77% to 95% (mean±SE, 82 ± 2%) of the total data points from 13 parameters (Table 6.10.). The means of useful data points for each parameter group were 83 ± 2% (farming management), 83 ± 4% (prawn biology) and 79 ± 2% (outcomes). They did not significantly differ from each other ($F = 0.35; df = 2, 12; p >> 0.05$), although within group variability was high.

6.4.12. Correlation of the biomass to other management and biological parameters

The biomass of the harvested prawns was spread normally from 118 to 2159 kg/ha (Fig. 6.12.), with a mean ± SE of 1051 ± 141 kg/ha (N = 20) (Table 6.10.). The 95% confidence interval for the mean value of the biomass is from 1028 to 1074 kg/ha. Ten crops yielded a biomass below 1028 kg/ha (the lower bound of the 95% confidence interval).

Table 6.10. Statistical summary of 20 valid data sets from farm records. Useful %N = the proportion of data points within each parameter in the 20 valid data sets.

Parameters Farming management	Useful %N	Min	Max	Mean	SE	SD
(1) Number of stock	77%	350,000	730,000	516,680	22,771	101,837
(2) Stocking density (prawns/m^2)	77%	12	25	18.35	0.80	3.59
(3) % Body weight fed	87%	0.60	8.90	2.62	0.44	1.98
(4) Fed amount in the last week (kg)	87%	37	785	432	51	226
(5) Total running feed (kg)	80%	108	13142	4423	828	3702
(6) Estimated running F. C. R.	87%	0.46	2.49	1.51	0.10	0.46
Prawn biology						
(1) Age (days)	77%	39	167	113	10	42
(2) Estimated survival rate (%)	77%	20	100	64.55	4.30	19.21
(3) Average body weight (g)	77%	0.78	23.87	9.48	1.28	5.74
(4) Individual growth rate (g/week)	87%	0.41	3.64	1.68	0.23	1.01
(5) Pond weight gained (kg)	95%	137	1245	527	72.68	325
Outcomes						
(1) Standing crop (kg)	77%	342	6260	2967	398	1779
(2) Biomass (kg/ha)	80%	118	2159	1051	141	632

Of the 12 parameters tested with the Pearson's correlation analysis, 67% (8/12) correlated very significantly (6/12) and significantly (2/12) to the prawn biomass (Table 6.11.). The management factors that very significantly correlated to the biomass were total running feed, feed amount in the week before sampling and weight-based feeding rate. The age and mean body weight of prawns were two biological parameters that corresponded very significantly to the biomass. Other biological parameters, i.e., pond weight gain and survival rate, correlated significantly to the biomass. The standing crop and biomass of the prawns had a perfect linear correlation (Pearson's correlation coefficient = 1.00), which is not surprising as they are measures of the same thing.

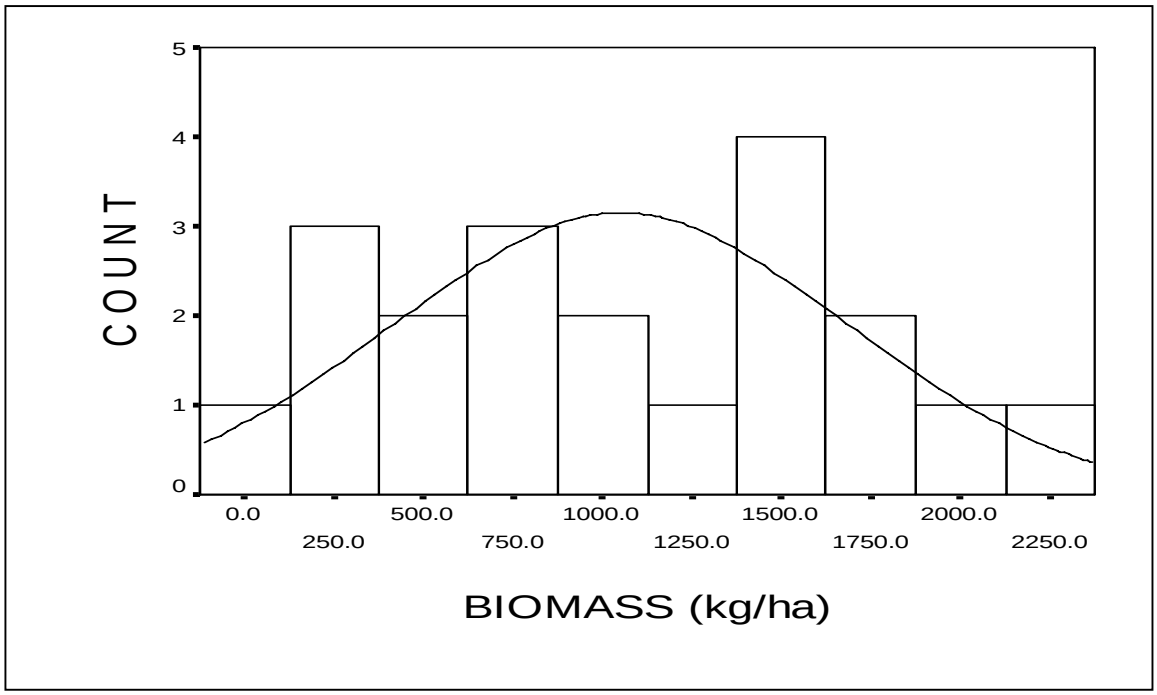


Figure 6.12. Frequency distribution of the biomass of the prawn crops. The line is the normal distribution curve.

Table 6.11. Correlation of the biomass to the other parameters based on Pearson's correlation analysis. P.c.c. = Pearson's correlation coefficient; P* = significant level at $p < 0.05$ (2-tailed).

No	Parameters	P.c.c.	P*	Correlations
1	Standing crop (kg)	1.00	<<0.05	*Very significant*
2	Prawn age (day)	0.89	<< 0.05	*Very significant*
3	Total running feed (kg)	0.82	<< 0.05	*Very significant*
4	Average body weight (g)	0.78	<< 0.05	*Very significant*
5	Feed amount in the last week (kg)	0.64	<< 0.05	*Very significant*
6	Weight-based feeding rate (%)	- 0.48	<< 0.05	*Very significant*
7	Pond weight gain (kg)	0.57	< 0.05	*Significant*
8	Survival rate (%)	0.56	< 0.05	*Significant*
9	Individual growth rate (%)	0.35	>> 0.05	Not significant
10	Food conversion ratio (FCR)	0.19	>> 0.05	Not significant
11	Stocking density (prawn/m^2)	- 0.20	>> 0.05	Not significant
12	Stocking number (prawn)	- 0.22	>> 0.05	Not significant

Based on the Pearson's correlation analyses, a diagrammatic relationship among the parameters was constructed (Fig. 6.13.). For convenience, the biomass represents the outcomes because the biomass and the standing crop had a perfect linear correlation and

correlated to the same factors. Likewise, the stocking numbers and stocking densities were perfectly correlated to one another (Pearson's correlation coefficient = 0.98 at $p << 0.05$). They also related to similar factors, except no correlation between the stock and total running feed (Pearson's correlation coefficient = - 0.39 at $p > 0.05$). Thus the stocking density appeared in the diagram for simplification only (Fig. 6.13.).

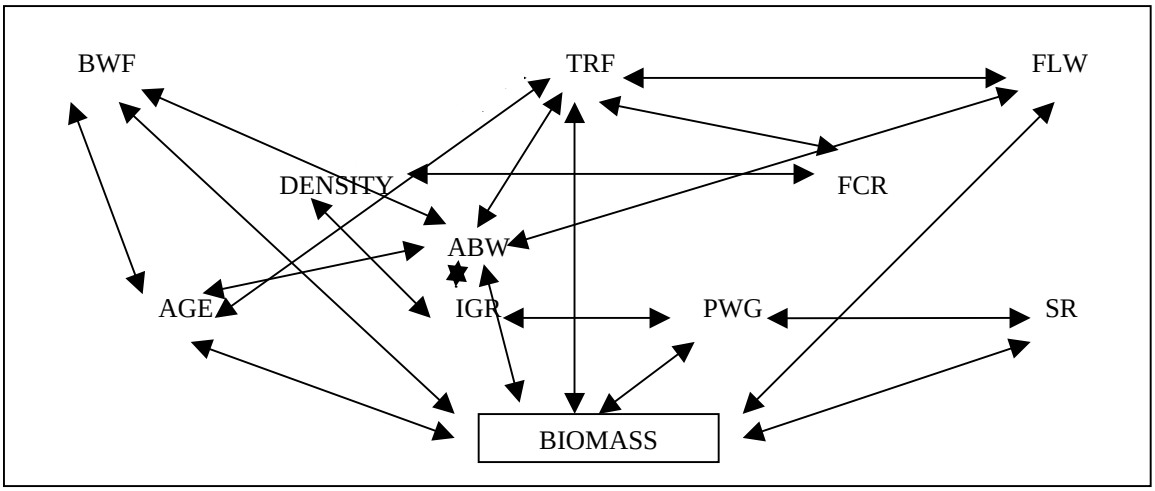


Figure 6.13. Diagrammatic correlations between management factors and biological factors in the production (biomass) of prawns in the farm based on Pearson's correlation analysis. BWF = body weight fed (%). TRF = total running feed (kg). FLW = the amount of feed in the last week before sampling (kg). FCR = estimated running food conversion ratio. DENSITY = number of prawns/m^2. ABW = average body weight. AGE = age of the prawns (day). IGR = individual growth rate (g/week). PWG = pond weight gain (kg). SR = estimated survival rate (%). SC = standing crop (kg). BIOMASS = biomass of prawns (kg/ha). Double-head arrows show significant correlations.

However, subsequent stepwise regression analyses showed that total running feed (TRF) and pond-weight gain (PWG) were the determinant factors of the outcomes (standing crop and biomass) (Table 6.12.). The models for standing crop (SC) and biomass (BIOMASS) both had similar multiple R and R^2, i.e., 0.99 (Table 6.12.). Thus, further analyses of explanatory variables were done on the BIOMASS model only.

Among the explanatory parameters, interestingly, TRF was determined by the AGE parameter alone ($R = 0.86$ and $R^2 = 0.74$) (Table 6.12). Apart from TRF, the age also positively associated with other management factor, i.e., the amount of feed given one week before the sampling (FLW). In addition, the AGE model alone can directly predict

the standing crop. Thus the age deserved further analyses in order to elaborate its roles in determining the biomass.

Table 6.12. Models of parameter correlations based on the stepwise regression analysis. For key to the abbreviations see the legend in Figure 6.13.

Parameters	Models	R	R^2
Farming management factors			
STOCK	4970.497 + 27886.076*DENSITY	0.98	0.97
DENSITY	0.468 + 0.0000346*STOCK	0.98	0.97
BWF	2.998 – 0.0029*BIOMASS + 0.006175*FLW	0.72	0.52
FLW	-248.156 + 4.747*AGE + 54.238*BWF	0.82	0.68
TRF	- 4115.265 + 75.293*AGE	0.86	0.74
FCR	2.267 – 0.0608*DENSITY	0.47	0.22
Prawn biological factors			
AGE	50.029 + 0.02136*SC	0.90	0.80
SR	93.142 + 0.0154*SC – 4.028*ABW - 1.968*DENSITY	0.95	0.91
ABW	20.582 - 0.212*SR + 0.00362*SC - 0.444*DENSITY	0.97	0.95
IGR	4.54 + 0.00288*PWG - 0.0308*SR - 0.121*DENSITY - 0.067*BWF	0.99	0.97
PWG	-1266.503+307.025*IGR+9.894*SR+34.84*DENSITY	0.98	0.96
Outcome factors			
SC	3.527 + 2.502*BIOMASS + 0.05036*TRF + 0.209PWG	0.99	0.99
BIOMASS	-0.079 + 0.396*SC – 0.019*TRF – 0.0774*PWG	0.99	0.99

In the PWG model, individual growth rate (IGR), survival rate (SR) and DENSITY parameters contribute positively to the outputs (Table 6.12.). Since IGR parameter did not affect other parameters, except PWG, it was not analyzed further. The DENSITY parameter affected 33% (2/6) of other management factors (STOCK and FCR) and 80% (4/5) of the biological factors (SR, ABW, IGR and PWG). Likewise, SR affected 60% (3/5) of biological factors (ABW, IGR and PWG). Therefore, subsequent analyses were carried out on the AGE, DENSITY and SR models to elaborate any possible links and interaction among these parameters with the biomass as the surrogate measure of the prawn health and disease status.

6.4.13. Contribution of the prawn age to the biomass outcomes

The pond-age of the prawn crops distributed from 39 – 167 days with a mean ± SE of 113 ± 10 days (N = 20) (Table 6.10.). The 95% confidence interval for the mean pond-age

was from 107 - 119 days. Ten crops had a mean pond-age higher than 119 days (the upper bound of the 95% confidence interval) (Fig. 6.14.).

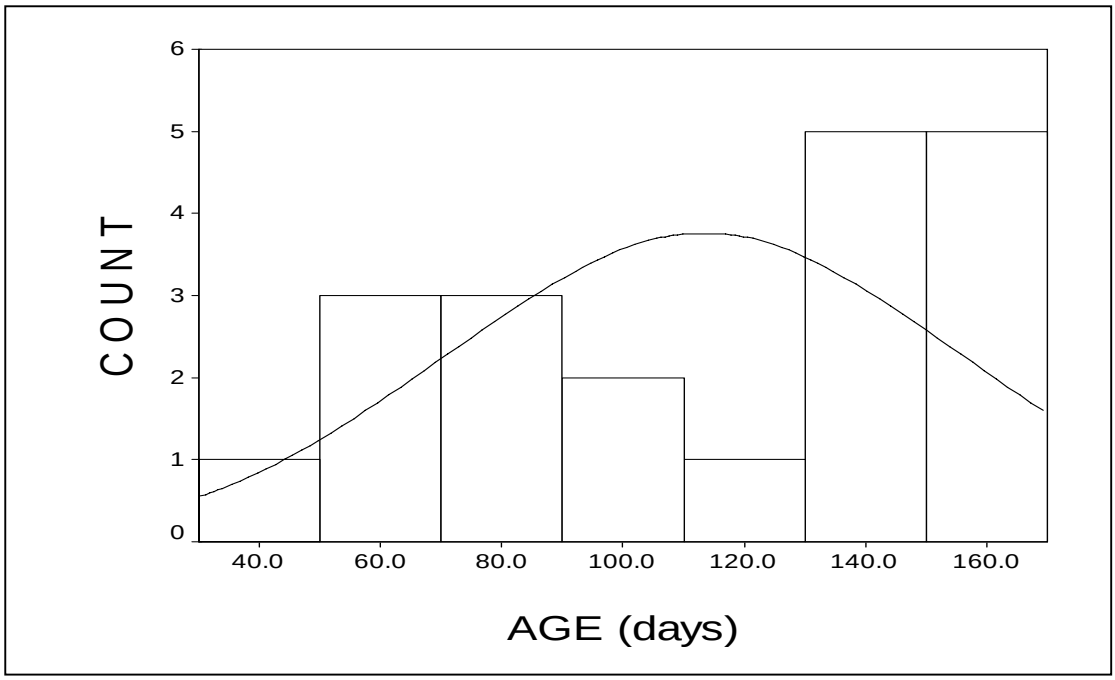


Figure 6.14. Frequency distribution of the pond-age of prawn crops. The line is the normal distribution curve.

Pearson's correlation analysis showed that the pond-age correlated very significantly ($p << 0.05$) to PWG, SC, TRF, FLW and BIOMASS parameters (Table 6.13.). However, the stepwise regression analysis showed that only the SC parameter determines the outputs of the AGE model (Table 6.12.). Obviously, describing age as dependent on SC model is illogical. Nevertheless, the SC model can be rewritten with age as the independent variable based on the AGE model (Table 6.12.), i.e., SC = - 2342.18 + 46.817*AGE. Consequently, in the BIOMASS model, two variables can be substituted with the AGE variable, i.e. TRF (total running food) and SC (standing crop), in which: TRF = - 4115.265 + 75.293*AGE (Table 6.12.) and SC = - 2342.18 + 46.817*AGE. Subsequently, the BIOMASS model can be rewritten based on the AGE variable, i.e.,

BIOMASS = - 849.392 + 17.11*AGE – 0.0774*PWG.

Table 6.13. Parameters with a significant correlation to the pond-age.

	PWG (kg)	SC (kg)	FLW (kg)	TRF (kg)	BIOMASS (kg/ha)
Pearson's correlation coefficient	0.80	0.90	0.70	0.86	0.89
Probability (2-tailed)	<< 0.05	<< 0.05	<< 0.05	<< 0.05	<< 0.05
N	20	20	20	20	20

The age-based BIOMASS model yielded a predicted biomass of 1043 kg/ha when the AGE and PWG variables were substituted with their respective mean values (Table 6.10.). The predicted value was 0.8% lower than the estimated mean of the prawn biomass (1051 kg/ha) (Table 6.10.) and within the 95% confidence interval for the biomass mean (section 6.4.13.). Thus, during the increasing feeding phase (section 6.4.9.), the age of the prawns corresponded very strongly to the prawn biomass as indicated by Pearson's correlation and stepwise regression analyses.

Both analytical approaches also confirmed that the age of the prawns did not correlate directly to the other two major explanatory parameters, i.e. DENSITY (as logic should dictate) and SR. However, prawn age has an indirect correlation to SR through the SC variable in the SR model (Table 6.12.). Here, the SC variable can be substituted with the AGE variable, i.e. SC = - 2342.18 + 46.817*AGE. Thus the AGE parameter can correlate positively and directly to SR, which in turn, directly and positively influences the PWG variable in the BIOMASS model. Therefore, the AGE parameter can affect the BIOMASS model indirectly by altering the PWG variable through the SR parameter. Nevertheless, the age contributes positively to the outputs of the BIOMASS model, i.e., the prawn biomass and age increase in parallel.

6.4.14. Contribution of the stocking density to the biomass outcomes

The stocking density of the cultured prawns ranged from 12 to 25 prawns/m^2, with a mean ± SE of 18 ± 0.8 prawns/m^2 (N = 20) (Table 6.10.). The 95% confidence interval for the mean density was from 17 - 20 prawns/m^2. Six crops had a stocking density higher than 20 prawns/m^2 (the upper bound of the confidence interval) (Fig. 6.15.).

Pearson's correlation analysis showed a significant correlation between the stocking density (DENSITY) with the initial stocking number (STOCK), individual growth rate (IGR), or food conversion rate (FCR) (Fig. 6.13. and Table 6.14.). However, the stepwise regression analysis showed that STOCK was the only parameter that determines the output of the DENSITY model with R = 0.98 and R^2 = 0.97 (Table 6.12.).

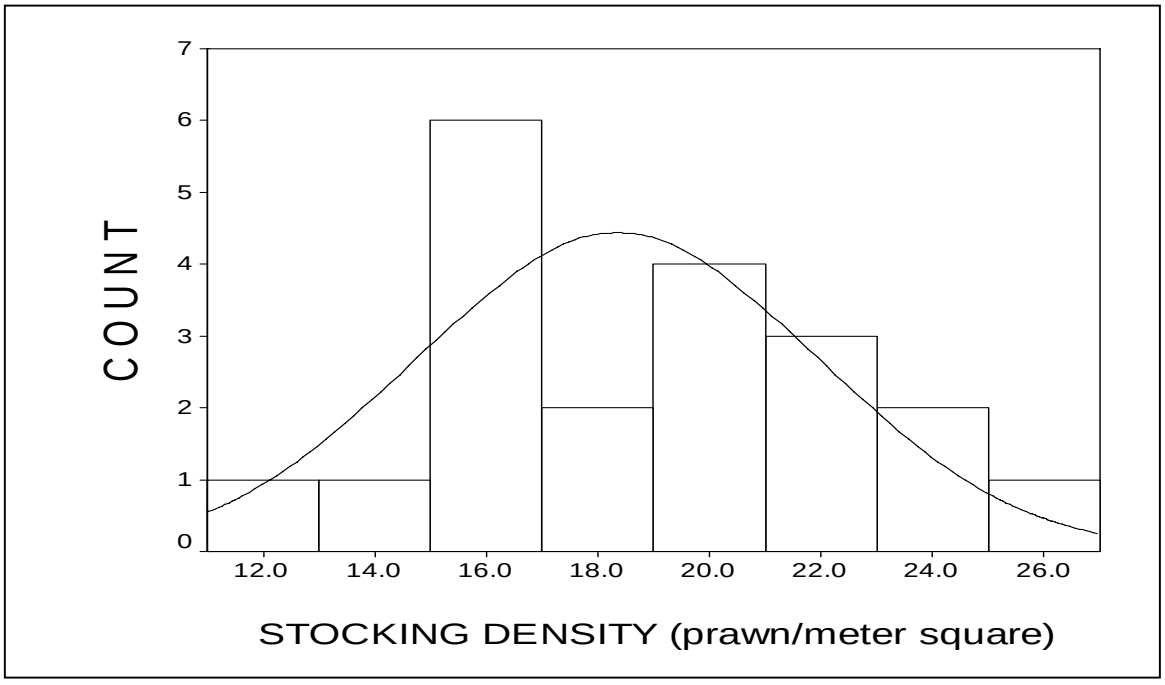


Figure 6.15. Frequency distribution of the stocking density of prawn crops. The line is the normal distribution curve.

Table 6.14. Parameters with a significant correlation to the stocking density.

	STOCK	IGR (g/week)	FCR
Pearson's correlation coefficient	0.98	-0.46	-0.47
Probability (2-tailed)	<< 0.05	< 0.05	< 0.05
N	20.00	20.00	20.00

With the BIOMASS model, the density correlates indirectly through its positive effects on the pond-weight gain (PWG) model with R = 0.98 and R^2 = 0.96 (Table 6.12.). Thus, if all variables in PWG and BIOMASS models are kept constant, an increase in the prawn density will increase the PWG, that in turn, increases the biomass via the SC model.

Except in the PWG model, prawn density has negative effects on the outputs of other biological models, i.e., SR, average body weight (ABW) and IGR models (Table 6.12.).

6.4.15. Contribution of the survival rate to the biomass outcomes

The estimated SR of the cultured prawns ranged from 20 to 100 %, with a mean ± SE of 64.6 ± 4.3 % (N = 20) (Table 6.10.). The 95% confidence interval for the mean SR was from 60.5 to 68.6 %. Eleven crops had SR values under the lower bound of the confidence interval (Fig. 6.16.), in which 5 crops had SR values ≤ 50%.

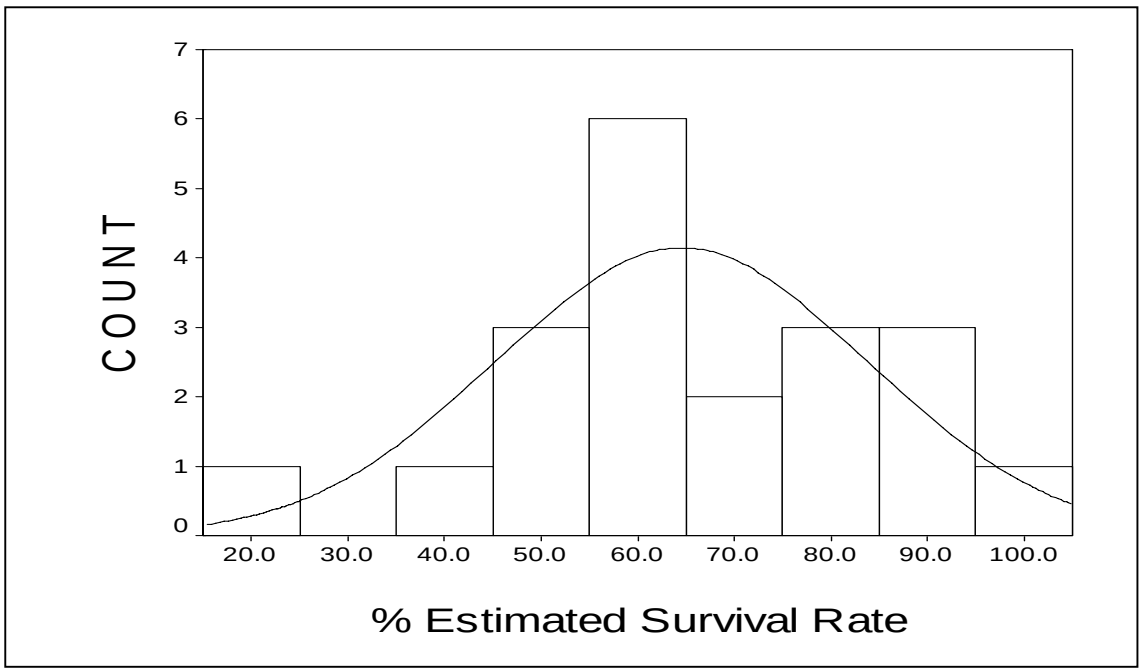


Figure 6.16. Frequency distribution of the survival rates of the cultured prawns. The line is the normal distribution curve.

Of the 12 parameters tested, only 3 (three) parameters related significantly to the SR, namely: pond-weight gain (PWG), standing crop (SC) and biomass (Table 6.15.). However, the stepwise regression analysis revealed that SR was determined by SC, ABW (average body weight) and DENSITY variables with R = 0.95 and R^2 = 0.91 (Table 6.12.). Hence, the stocking density can produce negative outputs for the SR model given that other variables are kept constant. However, the SC and ABW variables in the SR model can be substituted with the AGE variable, that is:

$$SR = 57.1348 + 0.281*AGE – 1.232*DENSITY$$

The age-based SR model produced a mean SR value of 66.3 % when the AGE and DENSITY variables were substituted with their mean values, i.e., 113 days and 18.35 prawns/m^2, respectively (Table 6.10.). The predicted SR value was within the 95 % confidence interval for the SR mean. Thus, it is very likely that the AGE and DENSITY parameters that predominantly determine the outputs of the SR model. The age-based SR model shows that an increasing age corresponds positively to an increasing survival rate via the SC variable, whereas an increased stocking density can reduce the survival rate.

Table 6.15. Parameters with a significant correlation to the survival rate.

	PWG (kg)	SC (kg)	BIOMASS (kg/ha)
Pearson's correlation coefficient	0.49	0.57	0.56
Probability (2-tailed)	< 0.05	< 0.05	< 0.05
N = crops	20	20	20

In relation to the biomass, SR parameter can have direct effect by substituting the PWG variable in the BIOMASS model (Table 6.12.). Here, SR correlates positively to the PWG outputs, which in turn, can increase the prawn biomass via the SC variable. Since in PWG model the coefficients for DENSITY variable (34.84) and SR variable (9.89) were positive (Table 6.12.), an increased survival rate and stocking density can synergically increase the PWG outputs. In turn, a higher PWG output can increase the prawn biomass during the increasing feeding phase, via the SC variable in the BIOMASS model (Table 6.12.).

6.4.16. The availability of anatomical organs or tissues

The organs or tissues of the animals examined histologically included the lymphoid organ, heart, anterior mid-gut, hepatopancreas, gut, gills, and muscle. However, not all slides contained the entire selected organs.

On the average, 13 out of 35 slides examined (around 38% of the samples) did not contain one, or more than one, of the organs (Table 6.16.). Organs not available on most

of the slides were gut (in 26 slides), gills (in 25 slides), lymphoid organ (in 17 slides), heart (in 12 slides) and anterior mid gut (in 12 slides). On the contrary, muscle tissue was seen in all slides, while the hepatopancreas was seen in 32 slides (91% of the samples). Thus, they were used to assess the association of diseases and abnormalities in section 6.4.20.

For convenience, the term "normal" condition in Table 6.16. was loosely defined as "no observable abnormal anatomy" (e.g., inflammation, oedema, encapsulation, and degradation), and "no pathogen" (e.g., virus, protozoa, or helminthes) seen under the light microscope. Therefore, any condition that varies from the defined "normal" condition is categorized as "abnormal". Any organ or tissue with only small portion of it seen was categorized as "uncertain" condition since so little evidence was available to properly suggest the condition of the respective organ or tissue. Thus these categories did not reflect the severity of the abnormality.

Table 6.16. The frequency of availability and observable anatomy in 35 slides.

Organs or Tissues	**Observed Condition (count and %)**			**Not available (count and %)**
	"Normal"	**"Abnormal"**	**Uncertain**	
Lymphoid organ	13 (37%)	5 (14%)	0 (0%)	17 (49%)
Heart	10 (29%)	9 (26%)	4 (11%)	12 (34%)
Anterior mid-gut	10 (29%)	8 (23%)	5 (14%)	12 (34%)
Hepatopancreas	18 (51%)	14 (40%)	0 (0%)	3 (6%)
Gut	3 (9%)	6 (17%)	0 (0%)	26 (74%)
Gills	10 (29%)	0 (0%)	0 (0%)	25 (71%)
Muscle	3 (9%)	32 (91%)	0 (0%)	0 (0%)
Mean	**10**	**11**	**1**	**13**
Mean percentage	**27%**	**31%**	**4%**	**38%**

On the average, only 4% of the samples were grouped under "uncertain" condition (Table 6.16.). Of the 22 slides that contained one, or more than one, organs or tissues, 46% contained "normal" and 50% "abnormal" anatomy. These numbers represent 27% and 31% of the samples, respectively (Table 6.16.).

The organ with the highest count for the “normal” condition was the hepatopancreas, i.e., 51% of the pooled samples (Table 6.16.). The lowest counts for the “normal” category were shown by muscle tissue (9%), and the midgut (9%). However, 91% of the samples contained “abnormal” muscle tissue, while 74% of them did not contain the midgut.

6.4.17. Anatomical abnormalities

As per the definition of “abnormal” in Table 6.16., the anatomical abnormalities in the samples were varied as manifested by various cellular or cytological changes (Table 6.16.). Haemocytic infiltration, encapsulation, inflammation or degradation was common manifestation in various observed organs and muscle tissue (Table 6.17.).

Table 6.17. Types of anatomical abnormalities in selected organs and tissue.

Organs or Tissue	Cellular and Cytological Abnormalities
Lymphoid organs	Many have spheroids with inclusion bodies, karyolytic cells, pyknotic cells, chromatin-marginated cells, encapsulation.
Anterior mid-gut	Localized haemocytic encapsulation, or focal damaged
Midgut	Haemocytic enteritis, epithelium detached
Hepatopancreas	Necrosis, oedema, injury, or septicaemia
Heart	Inflamed pericardium, localized haemocytic encapsulation, dirt inside the heart muscle, oedema, or injury.
Muscle tissue	Degradation, haemocytic infiltration, injury, or oedema

The vast majority of the samples sustained muscular abnormality, such as the breaking of muscle fibers (Fig. 6.17.a.) and haemocytic infiltration (Table 6.17.). In one sample, spheroids were found in the connective tissue of antennal gland (Fig. 6.17.b.). Some samples had oedemous hepatopancreas with haemocytic encapsulation of necrotic tubules. Granulized hepatopancreatocytes also occurred with intact myoepithelial layers of the tubules (Fig. 6.17.c.). The lymphoid organs of some samples contained numerous spheroids (Fig. 6.17.d.). Few samples contained anterior midgut with haemocytic enteritis and detachment of the epithelium from the basal membrane.

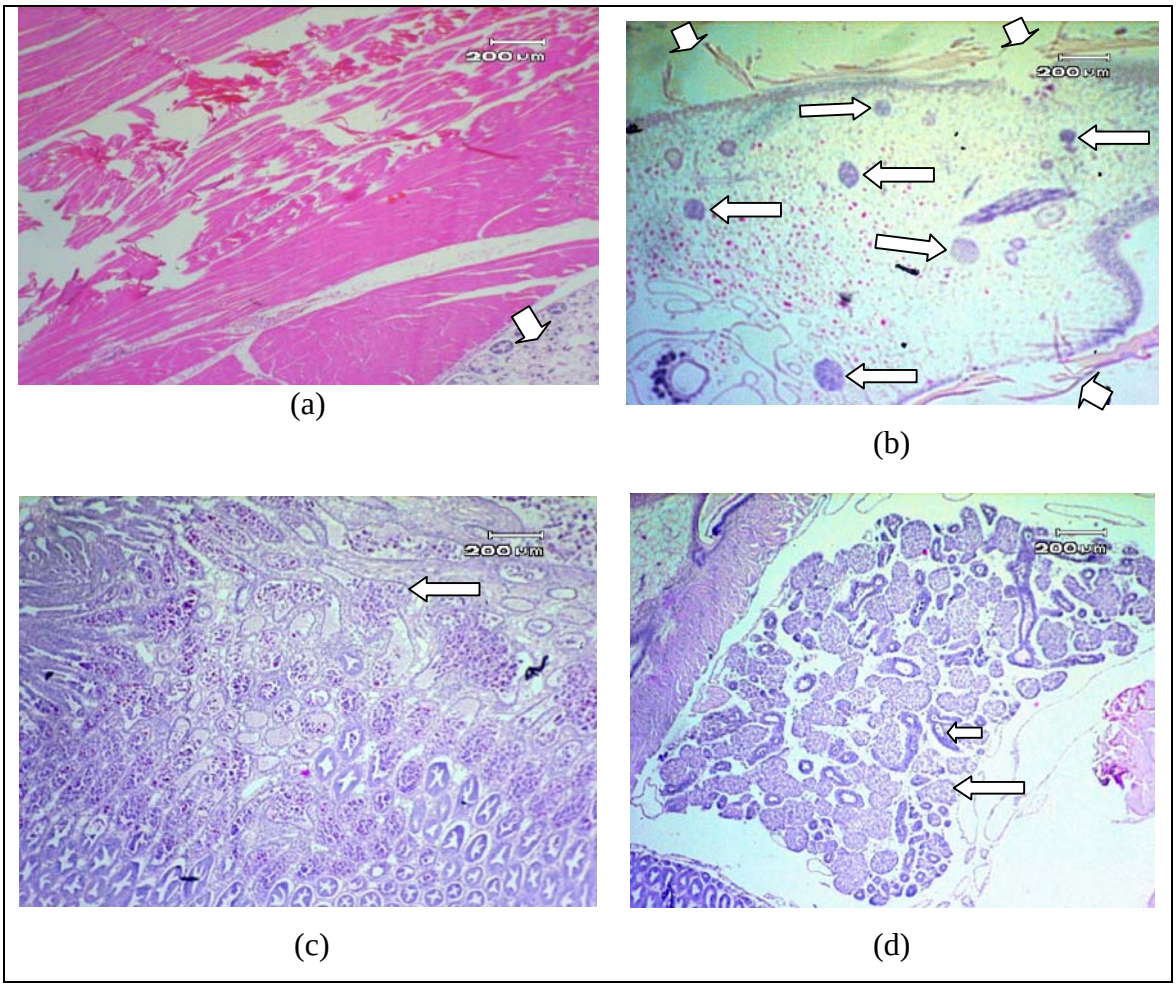


Figure 6.17. Anatomical abnormalities. (a) Typical muscle necrosis. Small arrow is hepatopancreas. (b) Spheroids (long arrows) in spongy connective tissue of antennal gland with broken columnar epithelium (short arrows). (c) Granulation of hepatopancreatic tubules with intact myoepithelial layers (arrow). (d) Lymphoid organ dominated by spheroids (long arrow) among normal tubules (short arrow) suffering a grade 4 severity of infection. H&E stained.

6.4.18. Types of diseases

Several viruses, including monodon baculovirus (MBV) (Fig. 6.18.a and c.), hepatopancreatic bacilliform virus (Fig. 6.18.b.) and an unknown pathogen (Fig. 6.18.d.) infected the hepatopancreas. Suspected hepatopancreatic parvo-like virus (HPV) was seen in the anterior midgut ceca (Table 6.18. and Appendix 6.7.). Based on histological profiles, suspected viruses that may be found in the lymphoid organ include gill-associated virus (GAV), lymphoidal parvo-like virus (LPV) and lymphoid organ

vacuolization virus (LOVV). A suspected bacterial infection in the organs was indicated mostly by massive septicaemia, oedema and haemocytic encapsulation (Table 6.18.).

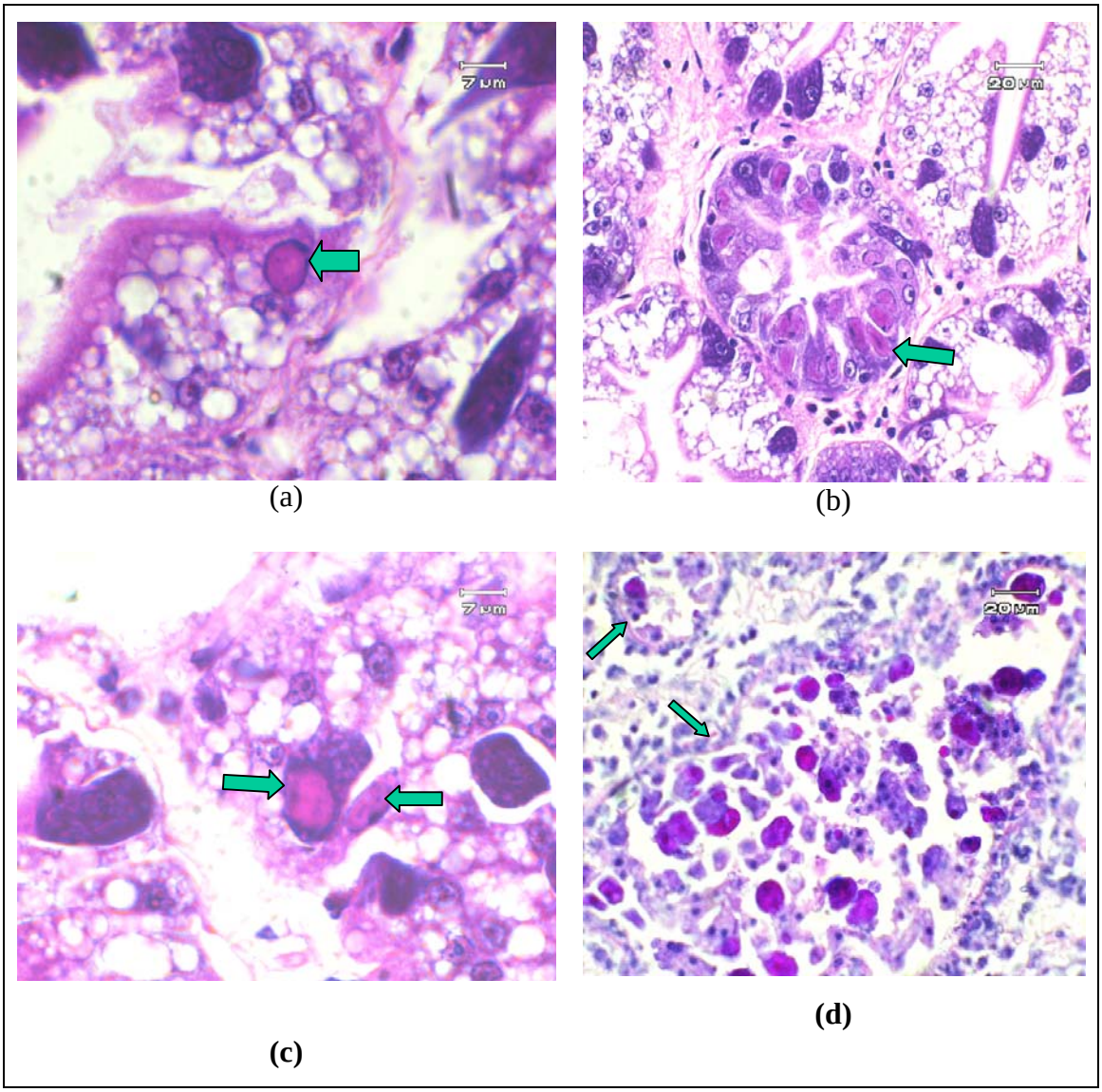


Figure 6.18. Pathogens in the hepatopancreas. (a, b and c) Stage 1 infection of MBV-like virus (arrows). (d) Granulation of hepatopancreas of unknown etiology with intact myoepithelial layers (thin arrows). H&E stained.

Evidence of virus was not seen in the heart and muscle, whereas bacteria infected all organs and the muscle, and both pathogens could at the same time infect lymphoid organ, anterior midgut, and hepatopancreas (Table 6.18. and 6.19.).

Non-infectious changes, probably due to processing of the samples, included an algal remnant in the heart, a mass of muscle embedded inside the hepatopancreas, and a tubule of hepatopancreas inside the muscle (Table 6.18.).

Table 6.18. Diseases found in the prawns.

Pathogen	Organ or tissue	Assessment
Bacilliform virus	Hepatopancreas	3 samples with grade 1 severity and very localized.
GAV-like	Lymphoid organ	4 samples have massive eosinophilic spheroids.
HPV	Anterior midgut caeca	Grade 1 infection in the basal cells.
LOVV-like	Lymphoid organ	5 samples have vacuolated spheroids.
MBV	Hepatopancreas	1 sample with a stage 1 occlusion body in an F-cell.
Unknown viruses	Lymphoid organ	Many inclusion bodies seen in the majority of the lymphoid samples.
Suspected bacterial infection	Hepatopancreas, heart, anterior midgut, muscle	15 samples show massive oedema, haemocytic encapsulation and septicaemia.
Non-infectious agent	Heart, hepatopancreas, muscle	1 sample contains algae encapsulated by haemocytes in the myocardial cells, 1 sample with a chunk of muscle inside the hepatopancreas, and 1 sample with a hepatopancreatic tubule inside the muscle.

6.4.19. Prevalence of infection

Of the 35 samples, 10 were infected by viruses only, 14 by bacteria only, and 2 by both pathogens. Accordingly, the prevalence for the infection by viruses only was 28.57%, by bacteria only was 40%, and by both types of pathogen in the same animals was 5.71%. In total, the prevalence of infection, regardless of the pathogens, was 74.29%.

Since not all samples contained all organs, the prevalence of the infection was also categorized according to the infected organs, and the occurrence of the pathogens. The lymphoid organ (LO) had the highest prevalence of infection by viruses only (33.33%), and by bacteria and viruses (5.56%) (Table 6.19.). The hepatopancreas (HP) had the highest prevalence of infection by bacteria only (25%) (Table 6.19.). In total, organs and tissues with the highest prevalence to the lowest prevalence were the lymphoid organ

(LO) (50%), hepatopancreas (HP) (37.51%), anterior midgut caeca (AMC) (25%), muscle (22.86%), heart and anterior midgut (AMG) (21.74% each) (Table 6.19.).

Table 6.19. Prevalence of disease agents in each organ and for the total sample. The denominator is the number of slides containing the respected organ (see Table 6.16). * Two samples contain both bacteria and viruses. See the texts for the key to abbreviations.

Pathogen	LO	Heart	AMC	AMG	HP	Muscle
Virus only	33.33%	0	8.33%	8.70%	9.38%	0
Bacteria only	11.11%	21.74%	16.67%	8.70%	25.00%	20.00%
Both*	5.56%	0	0	4.34%	3.13%	2.86%
Total	50.00%	21.74%	25.00%	21.74%	37.51%	22.86%

6.4.20. Association between abnormalities and diseases

The attributable risk (AR) of the prawns getting oedema in the hepatopancreas due to viral infection of any kind, in any organ of their bodies, was 13.64%, and the odds ratio (OR) was 1.44 (Table 6.20.).

Table 6.20. Attributable Risk (AR) and Odds Ratio (OR) of oedema in the hepatopancreas due to viral infection in any part of the body of the prawns. **AR3** = [(7/32) – 3/16]/(7/32) = 13.64%; **OR** = (4 x 13)/(3 x 12) = 1.44.

	Oedema	No oedema	TOTAL
Infected by any virus	4	12	16
Not infected by any virus	3	13	16
TOTAL	7	25	32

However, the AR of the prawns getting any abnormality in their hepatopancreas increased to 39% with the combination of viruses and bacteria in their bodies, and with an OR of 2.54 (Table 6.21.).

Table 6.21. Attributable Risk (AR) and Odds Ratio (OR) of abnormality in the hepatopancreas by the combination of viruses and bacteria in the prawn body. **AR3** = [(13/32) – (2/8)]/(13/32) = 39%; **OR** = (11 x 6)/(2 x 13) = 2.54

	Abnormal	Normal	TOTAL
Viruses and bacteria present	11	13	24
No infection	2	6	8
TOTAL	13	19	32

Of the total 32 hepatopancreata, 8 with suspected bacterial infection also showed massive oedema. The other 24 samples without bacterial infection did not show oedema in that organ, even though in some samples their hepatopancreas were injured by muscle infiltration or other causes.

The risk of muscular degradation was not attributable to bacterial infection (AR3 = 0% and odds ratio = 1) (Table 6.22.).

Table 6.22. Attributable Risk (AR) and Odds Ratio (OR) of muscular damage by bacteria. **AR3** = [(30/35) – (24/28)]/(30/35) = 0%; **OR** = (6x4)/(24x1) = 1

	Degradation	Normal	TOTAL
Bacterial infection occurs	6	1	7
No bacterial infection	24	4	28
TOTAL	30	5	35

6.5. Discussion

Quantitative approaches are not only useful for diagnostic purposes, but also for far-reaching purposes, such as health management of animals in a farm. This is because statistical epidemiology can identify risk factors of diseases in animal population within natural environment (Thrusfield, 1986; Thompson *et al*., 1997; Lotz *et al.*, 2001). However, the types of collected data are crucial for making inferences about the risk factors of diseases in population (Thrusfield, 1986; Dawson-Saunders and Trapp, 1994).

The present study used data originally collected for daily management of water quality related to food budgets, and tracking down prawn production in the grow-out ponds. Therefore, this study suffered from the lack of records of prawn diseases in the farm. Although the written data were independent from each other, they can be related by the dates of sampling and stocking. The data of environment and feeding budget were taken from the 30th of August 1991 to the 30th of May 1992 (section 6.4.1.). The data of farming management and prawn biology were sampled in the 20th of November 1991 and the 29th of April 1992 (section 6.4.11.). The latter covered a stocking period from the 10th of January 1991 to the 18th of April 1992. However, the 20 crops analyzed in this

study were stocked between the 6th of June 1991 to the 21st of March 1992. Therefore, it is most likely that both written records correlate to the same prawns.

Unfortunately, the prawn samples provided were only of May 1992 without any label related to the pond of origin or the stocking date. Thus it is impossible to relate the histopathological data with the water quality and biological data from the ponds. Nevertheless, the prawn samples can be used, though very cautiously, to identify possible pathogenic risk factors for the farmed prawns. The prawn samples (juveniles and sub-adults) taken in May 1992 might represent the health conditions of prawns stocked since January 1992. Since the farm was bankrupt when the data were analyzed, it was impossible to validate the data.

The data also lacked pointers as to which pond(s) a particular management, biological and biomass data belonged to. Likewise, the presence of some unusual data of water quality across the parameters (Table 6.3.), such as DO levels higher than the saturation, suggest a technical or human error. However, corrections can be made using the table of DO levels relative to temperature and salinity in fish ponds according to American Public Health Association (APHA) (Delincé, 1992). Eventually, the majority of the water quality data (93%) were useable (section 6.4.2.). Likewise, 82% of the management and biological data appeared valid and reliable (section 6.4.11.). Thus, overall the data were good as inputs for statistical epidemiology, although they lacked the study designs for epidemiology.

Despite those limitations, the records of water quality parameters and feed amount were remarkably complete because they were taken regularly twice or more each day. Therefore, with limited data, the present study has demonstrated that the amount of feed can serve as a good proxy (indirect) measure of the prawn health status. Hence, the amount of feed correlated strongly with the temperature, pH and salinity of the pond water, but not with DO levels and transparency. The temperature also significantly correlated to pH (Fig. 6.2.) and salinity (Fig. 6.4.). Therefore, the water temperature seems to be a significant descriptive and possible risk factor for prawn diseases in the

farm. Indeed, water temperature affects the metabolism, food consumption and conversion, molting, growth, maturity and survival of crustaceans (Maguire and Allan, 1992; Hoang *et al*., 2002 a, b).

The pond water in this study had a range of means for temperature from 24.5 to 28.2 ^{o}C (Appendix 6.3.) with an average of 26 ^{o}C. Except in December 1991 and January 1992, the mean values of monthly water temperature were below 28 ^{o}C (Fig. 6.1.). Under laboratory experimentation, the optimal growth temperature for *P. monodon* juveniles from China (Chen, 1985) or Australia (Maguire and Allan, 1992) ranges from 28 ^{o}C to 33 ^{o}C. Thus, the mean water temperature of 26 ^{o}C in the present study might imply a problem of suboptimal growth of *P. monodon* in the farm. Indeed, Maguire and Allan (1992) and Hoang *et al.* (2002 a) have pinpointed low water temperature as a major problem for penaeid farming in Australia. Therefore, more research has been directed to find penaeid species suitable for culture during winter in Australia. To substitute *P. monodon* during winter months in Australia, Hoang *et al*. (2002 b) proposed *P. merguiensis* as the candidate based on its ability to grow, mature and spawn in outdoor covered ponds at temperature between 19-23 ^{o}C with salinities of 30 - 35 ppt. This species can even be acclimated to as low as 4.7 ^{o}C at a cooling rate of 3 ^{o}C h^{-1} (Hoang *et al*., 2002 a).

However, temperature is not the only factor for prawn growth. Maguire and Allan (1992) reported poor food conversion rates or FCRs (2.3 to 2.6) for Australian *P. monodon* juveniles in laboratory experiments at a temperature range of 24 ^{o}C - 30 ^{o}C. The poor FCRs in that study might correlate to high food consumption rates and high salinity (34 – 36 ppt). However, in the present study, the FCR ranges from 0.46 – 2.49 with a mean of 1.51 (Table 6.9.). Thus, an increase of feed with an increasing water temperature in the farm did not necessarily correspond to an increase in the health condition of the prawns. It is most likely that beside temperature, other water quality factors, such as salinity, also contributed combined risks for prawn diseases in the present study.

On a monthly basis, the range of water salinity in the farm was from 24 – 37 ppt, in which 6 out of the 9 month's data showed a salinity range between 30 – 37 ppt (Table 6.4.). In ponds, the salinity ranged from 24 – 37 ppt (Appendix 6.3.), in which 67% (12/18) of the ponds had water salinity above 31 ppt. In Thailand, a successful production of *P. monodon* in grow-out ponds requires a salinity range of 10 – 30 ppt (Chanratchakool *et al*., 1998). Moreover, Shariff *et al*. (2001) from Malaysia found that *P. monodon* did not grow well in grow out ponds (0.6 ha each) with a salinity of 35 ppt, although bioremediation products were used to improve quality of the pond water and pond bottom. In that study, reduced appetite along with black-gill syndrome, gill clogging and poor growth forced a premature harvest of the prawns at day 72 (instead of 120 days as planned). Thus, the significant correlation between salinity and feed amount in the present study might indicate high water salinity, like low water temperature, was a risk factor for prawn health in the farm.

Indeed, several experimental studies with other penaeids confirmed that temperature and salinity are the most important abiotic factors in penaeid aquaculture. Kumlu *et al*. (2000) found that *P. semisulcatus* larvae, well-adapted to the cool waters of the subtropical Mediterranean Sea, grew best in waters with a combination of 30 ppt salinity and 30 ^{o}C temperature. Juveniles and subadult *P. stylirostris* from Tahiti suffered from sudden changes in water temperature at the beginning and end of cold season (Lemaire *et al*., 2002). These prawns lost their ability to maintain osmotic homeostasis at all levels of salinity, especially at low (10 ppt) and at high (43 ppt) salinity levels. Cold-adapted *P. merguiensis* from Australia gained weight much more rapidly at 24 ^{o}C (86.7%) than at lower temperatures of 15 – 21 ^{o}C (5.1 – 23.3%) (Hoang *et al*., 2002 a).

In the present study, since the salinity increases 0.6 ppt with a 1 ^{o}C increment of water temperature in the ponds (Fig. 6.4.), the salinity might pose a greater risk to the prawn health in December to January. These were months with higher water temperature. On the contrary, during lower-temperature months, the salinity might be favorable for the prawns. In reality, the salinity in the pond water remained high (31.5 to 37.2 ppt) during lower-temperature months from September to November 1992 (Table 6.4.). This

indicates that low temperature and high salinity might prevail during those months. In Australia, Keys (2003) promoted *P. esculentus*, instead of *P. monodon*, aquaculture in ponds with salinities between 30 – 40 ppt and temperatures between 20 – 28 ^{o}C.

Although the water pH corresponds significantly with the feed amount, the range of mean pH values in the ponds (7.4 – 8.5; Appendix 6.3.) and at monthly basis (8.3 – 8.7; Fig. 6.1) was within the optimal range for *P. monodon* farming (7.5 – 8.3; Chanratchakool *et al*., 1998). Therefore, the water pH might not be a risk factor in the present study.

Likewise, DO levels and water transparency might not be risk factors in the present study. The range of the mean values of DO levels in the ponds (6.6 – 10.8 ppm; Appendix 6.3.) and at a monthly basis (5.7 – 7.1 ppm; Fig. 6.5.) was favorable for farmed *P. monodon* (5 – 6 ppm; Chanratchakool *et al*., 1998). Those values are much higher than the critical oxygen pressure for *P. monodon*, i.e., 3.7 ppm (Chen, 1985) probably because the farm employed 2-4 paddlewheel aerators in each pond. The range of mean transparency values in the present study was 38 – 66 cm for the pond basis (Appendix 6.3.) and 34 – 71 cm for the monthly basis (Table 6.4.). In Thailand, the recommended water transparency for *P. monodon* ponds is 30 – 50 cm (Chanratchakool *et al*., 1998). Around 61% (11/18) of ponds in the present study had a mean of transparency within the recommended values for Thailand. Again, this extrapolative comparison is conditional.

Moreover, low temperature and high salinities might induce reduced appetite, slow metabolism and osmoregulatory stress as well as increased susceptibility to diseases (Moullac and Haffner, 2000; Hoang *et al*., 2002 a). An experiment by Villarreal *et al*. (2003) on penaeid *Farfantepenaeus californiensis* showed that at high salinities (above 24 ppt), the prawns diverted its energy more for metabolic function than for tissue growth. In addition, the prawns became lethargic and lost their appetite. Therefore, the presence of a mixture of viruses and septicemic necrosis of the prawns in the present study is not unique. Infection of individual prawns by a cocktail of viruses, epicomensals and/or bacteria seems common in grow-out ponds. In Malacca Straits coast (Indonesia), Turnbull *et al*. (1994) reported that of 30 prawn farms surveyed, 10% contained *P.*

monodon with dual infections by MBV and HPV and 20% by MBV and lymphoid organ pathology (LOP). The pathology of LOP seems similar to that of LPV (Owens *et al*., 1991) or LOV (Spann *et al*., 1995) from *P. monodon* in Australia. Turnbull *et al*. (1994) also found that most of the prawns infected by those viruses were also infected by bacteria and epicomensal fouling organisms. Flegel *et al*. (1999) reported dual infection of pond reared *P. monodon* in Songkhla Province (southern Thailand) by MBV and HPV causing stunted growth. Farmed *P. monodon* and *P. indicus* from south-east coast of India also carried a combination of MBV and protozoan *Zoothamnium* sp., although the protozoans did not cause internal damage to the host surface or gills (Vijayan et al., 1995). In addition, few prawns also had white spots on the shells. Vijayan *et al*. (1995) did not elaborate the cause of the spots, but Karunasagar *et al*. (1998) reported severe mortalities of both prawn species in that area between 1994-1995 due to WSSV. This suggests a dual infection of MBV and WSSV was present. Vijayan *et al*. (1995) also observed massive necrosis of the lymphoid organ of MBV-infected prawns. The lymphoidal histopathology seems to resemble LPV or LOV pathology in *P. monodon* from Australia suggesting an increasing risk due to a mixture of pathogenic viruses occurred in *P. monodon* and *P. indicus* from east coast of India. Likewise, in India, YHV was found *in P. monodon* samples containing MBV, IHHNV (infectious hypodermal and haematopoietic necrosis virus), vibriosis and protozoan *Zoothamnium* (Panchayuthapani, 1997). Combined infectious diseases were also prevalent in cultured *P. monodon* in Taiwan where MBV or WSSV together with *Vibrio* sp. caused most of the mortalities (Chen, 1995). Therefore, simultaneous infection by different viruses and bacteria increases the risk of diseases for cultured prawns.

In the present study, however, the direct correlation between these pathogens with the feed amount or production (biomass) was impossible to establish due to the limited farm records. The only way to correlate those factors is through a deductive reasoning based on coherence, analogy and strength of association regarding diseases and internal pathology. Fortunately, 91% of the samples contained hepatopancreas and muscles (Table 6.16.). Therefore, both tissues can be used to assess the correlation between their abnormalities with biological factors and water quality.

The hepatopancreas is the major digestive gland of penaeids (Vogt *et al*., 1985; Dall, 1992; Vogt, 1992). In the hepatopancreas, F (fibrillar) cells secrete digestive enzymes, B (blazenzellen or vacuolar) cells perform pinocytosis of nutrients, and R (restzellen) cells absorb lipid, glycogen, metals, and other molecules (Ceccaldi, 1990; Dall, 1992). Therefore, the hepatopancreas is a very important organ in relation to feed consumption and the subsequent production of prawns in the farm. Vogt (1992) suggests that during MBV infection, the F-cells may stop enzyme production, while the virus may use up the lipid content of the R-cells as energy for its replication. Therefore, a combining infection by viruses will most likely result in serious reduction of energy for immune system and growth, and subsequently drop the farm production.

Despite the number of prawns examined being small, some conclusions are possible. Bacterial diseases might be an important risk to the farm since bacterial prevalence in the time of sampling (May 1992) was quite high. The bacterial infection alone had a 40% prevalence as compared to a 29% prevalence of viral infection alone, on top of 5.7% prevalence in dual infection by both pathogens. In fact, the presence of necrosis, oedema, injury or septicemia (Table 6.17.) in the hepatopancreas was mostly independent of viral presence. In addition, all the viruses found in the hepatopancreas were in the early stage (stage 1) of infection (Table 6.18. and Fig. 6.18 a, b and c). The severity of viral infection in hepatopancreas was negligible judged from the small number of focalized infected cells (Table 6.18.). Besides, an odds ratio of 1.4 for oedema in hepatopancreas associated with viral infection alone (Table 6.20.) was much smaller than that of 2.5 for dual infection by viruses and bacteria (Table 6.21.). Likewise, the incidence rate of oedema might only drop by 14% with the elimination of viral disease alone as compared to 39% reduction by the removal of both viral and bacterial diseases. While in the lymphoid organ the prevalence of viral infection (33%) was three times higher than that of bacterial infection (11%) (Table 6.19.), this organ is not involved in food digestion and nutrient absorption. Moreover, Anggraeni and Owens (2000) argue that lymphoid organ is a major immunological organ for viral replication or eviction from the prawn body. Moreover, bacterial infections as shown by typical haemolytic aggregations and

melanized lesions can occur in several tissues such as the subcuticular connective tissue, lymphoid organ, and other tissues (Jiravanichpaisal *et al*., 1995).

The high prevalence of bacterial infection alone in the anterior mid-caeca (17%) and hepatopancreas (25%) (Table 6.19.) indicates that bacterial infection might seriously reduce the capacity of these organs to digest nutrients. Indeed, atrophy of muscle (Fig. 6.17.a.) and hepatopancreas (Fig. 6.17.c.) in prawns suggest starvation due to pathogenic bacterial infections (Owens and Hall-Mendelin, 1990). Chronic vibriosis or septic hepatopancreatic necrosis also yields a small and discoloured hepatopancreas (Chanratchakool *et al*., 1998). Jiravanichpaisal *et al*. (1995) found that in the east and central coasts of Thailand (between July 1989 to November 1992) around 59% of juvenile and adult *P. monodon* had bacterial infections in their hepatopancreas. Thus, it is most likely that bacterial infection alone was a potential pathogenic risk factor for prawn health in the farm.

However, caution should be taken because the relationship between bacterial infection with the growth and survival rate of the prawns were not recorded. In addition, many bacteria commonly live in marine environment, thus regarded as opportunistic pathogens in prawns (Baticados, 1988; Lightner *et al*., 1992; Lavilla-Pitogo, 1995). They only cause severe effects following adverse environmental stresses that lower the resistance of the cultured prawns against diseases (Baticados, 1988). Lemaire *et al*. (2002) reveal that mass mortalities of cultured *P. stylirostris* at the beginning and end of cold seasons in New Caledonia (“syndrome 93”) arise from sudden temperature changes that cause osmotic stress combined with a high prevalence of vibriosis. In Taiwan, *Vibrio* sp. is the most deadly secondary infection for *P. monodon* (Chen, 1995). However, under stressful conditions, vibriosis may become the primary and deadly infection (Lightner *et al*., 1992). Thus, suppose the prawns in this present study were stressed by low temperature and high salinities, combined with low level viral infection and reduced feed consumption, the risk of bacterial infection in prawns might be greatly exacerbated.

Beside hepatopancreatic abnormalities, the prawns in the farm also suffered from muscular atrophy with 91% prevalence (Table 6.16.) as indicated by muscular

degradation (Fig. 6.17.), injury, haemocytic infiltration or oedema (Table 6.17.). Although the muscle had a 20% prevalence of bacterial infection (Table 6.19.), the muscular damage did not correspond to the bacterial presence (odds ratio = 1 and attributable risk = 0%; Table 6.22.). Some of the muscle damage my have been artifact due to processing. However, not all of the muscle changes were. The presence of suspected viral infection (3% prevalence) lacked back-up by confirmative diagnosis. Thus, the prevalence of muscle atrophy without bacterial infection could reach 80% (28/35). Lightner (1985) considers spontaneous muscle necrosis, especially in the distal portion of the abdomen, as a common chronic condition in all penaeids affected by environmental shocks. Muscle necrosis of cultured penaeids is reportedly prevalent in Indonesia, Malaysia, Taiwan, Thailand and the Philippines under poor environmental conditions and very high stocking densities (Baticados, 1988). The same problem has also been found in other cultured and wild-caught crustaceans from freshwater and marine water, including the Norway lobsters *Nephrops norvegicus* (Stentiford and Neil, 2000). Chronic starvation due to diseases or nutritional problems or handling stress can also cause muscle degeneration and associated signs of poor health in the cultured prawns (Chanratchakool *et al*., 1998). Chen (1992) reports that muscle necrosis occurs in *P. monodon* in grow-out ponds during summer season due to overcrowding, low oxygen, sudden changes in temperature or salinity and other unstable conditions. Chen (1992) suggests that high water quality in the ponds and moderate stocking densities may prevent muscle necrosis and secondary bacterial and fungal infections. In the present study, the underlying mechanisms of muscle atrophy were unknown. At this point, it is only hypothesized that the prevailing low water temperature and high salinity might increase the risks of muscle atrophy, especially when the prawns were starving due to diseases in their hepatopancreas, lethargy and loss of appetite. In turn, a prevailing muscle necrosis, like bacterial infection, seems to be a potential risk factor in the decline of productivity in the farm.

Nevertheless, the biological factors of the prawns need to be considered. In this study, the age of the prawns seems to govern the variability in the productivity and survival rate as measured by the BIOMASS model (section 6.4.13.) and SR model (section 6.4.15.).

Here, the productivity and survival rates increased in parallel with the age. It seems that the impacts of environmental stresses and diseases in the prawns in younger life stages were greater than in older stages. Jiravanichpaisal *et al*. (1995) reported that the proportion of hepatopancreas invaded by vibriosis in *P. monodon* juveniles (72%) was much bigger than that of adult prawns (23%). Lightner (1983) reported that MBV prevalence and the severity and mortality due to MBV infection decrease with an increasing age. Thus, Flegel and Pasharawipas (1998) coined the term "viral accommodation".

On the other hand, the stocking density was the likely risk factor of the farming management and had negative effects on productivity (section 6.4.14.) and survival rates (section 6.4.15.). However, the effects of host density on the dynamics of diseases in this study might not be linear. Here, the interactions between survival rate (which determines the numbers of the hosts susceptible for infection) and the pathogen infectivity and genetic variability of the hosts could alter the effects of host density on the BIOMASS model. Lafferty and Holt (2003) theorized that stress can reduce the host density and abundance of the pathogens, thus reducing the impacts of diseases.

Overall, the present study has proved that quantitative approaches, such as statistical epidemiology, can point out the potential risk factors and possible coherent correlations among them. Even with less than perfect data such as in the present case, the analytical power of quantitative analyses made it possible to produce some interesting findings. Here, the potential risk factors have been identified, i.e., low temperature and high salinity (environmental risk factors), prawn age (biological risk factor), stocking density (farming management risk factor), and bacterial diseases and muscle atrophy (disease risk factors). Therefore, it is most likely that viral diseases observed in histopathology did not correlate to the declining productivity in the farm. It is also well established that these risk factors might have dynamic interactions with one another to determine the productivity in the prawn farm. However, to improve the power and accuracy of this kind of quantitative analyses, the study designs and data collection should be corrected accordingly.

CHAPTER 7

COMPARISON OF DIAGNOSTIC TOOLS FOR MONODON BACULOVIRUS (MBV) FROM AUSTRALIAN PENAEIDS

7.1. Introduction

Diagnosis of the advanced stages of MBV infection in penaeids traditionally relies on histology (Lightner, 1996). However, since 1980s, molecular diagnosis in prawn virology has developed rapidly (Vaughan, 1996; Benzie, 1998; Cunningham, 2002). Nowdays, screening for various important viruses, including MBV, in prawn larvae depends on molecular genetic techniques, especially *in situ* hybridization (ISH) and polymerase chain reaction (PCR) assays. These diagnostic techniques provide sensitive and specific results, although technical difficulties and inter-laboratory inconsistencies occur (Cunningham, 2002). Unfortunately, third world countries, like Indonesia, lack facilities, funding and expertise to apply these techniques. However, there is no reason to avoid these technologies. In fact, commercial probes and PCR systems are available worldwide. Thus in this study, diagnosis of advanced MBV infection was carried out using different methods, i.e. routine histology, commercial ISH assay and reverse-transcriptase PCR (RT-PCR) assay using published primers.

7.2. Objectives of the Study

Since advanced stages of MBV infection are easily recognized using histology and light microscopy, the present study focuses primarily at:

1. Assessing the sensitivity and specificity of a commercial ISH probe and published PCR primers for detecting MBV in Australian *P. monodon* and *P. merguiensis*, and
2. Evaluating the procedures of molecular diagnosis of MBV.

7.3. Materials and Methods

MBV-infected postlarvae screened with histology using light microscopy in Chapter 4 were subjected to genetic diagnoses for MBV using non-isotopic *in situ* hybridization (NISH) and polymerase chain reaction (PCR). No fresh clinical samples of MBV-infected prawns were obtained for this study. The samples for PCR were fixed in 70% alcohol or methanol.

7.3.1. Non-radioactive *in situ* hybridization (NISH) assay

This assay utilized MBV-InSitu Shrimp Probe® Detection Test Kit version 07/00 (DiagXotics, Inc., USA). The probe contained dixogenin-11-dUTP (DIG)-labeled DNA of MBV.

Several initial attempts of ISH failed to produce meaningful results. It might be because the probe was developed for juvenile prawns, but was used with small postlarvae in this study. Therefore, some changes were made to the manufacturer's protocol based on the advice of Dr. Catriona McElnea (Microbiology and Immunology JCU, *pers. comm.*). Here, the paraffin-embedded tissues in the slides were heated in an oven at 60 ^{o}C for 1 hour (not at 65 ^{o}C for 30 minutes). The paraffin was removed by two soaks in xylene of 2 minutes each (not three soaks of 5 minutes each). Then, the xylene was removed by one 2 minute soak followed by two 1 minute soaks in 100% ethanol (not two soaks of 1 minute each). After that, the tissues were directly rehydrated in tap water for 1 minute (bypassing the rehydrating procedure using 95%, 80% and 50% ethanol respectively as stated in the manufacturer's protocol). The incubation time for proteinase K in this study was varied between 15 minutes (for postlarvae of *P. merguiensis*) to 20 minutes (for juveniles) to stop the tissues falling off during this enzymatic treatment. Instead of boiling the hybridization solution separately as outlined in the manufacturer's protocol, the probe was boiled together with the tissue on the slide. Moreover, the incubation time for colour development in the hybridized tissue was increased to 4 hours (two hours longer than the manufacturer's protocol).

To enhance the attachment of the tissues to a glass slide, the glass slides were silanised before being used. Prior to the silanization procedure, the slides were washed in 2N HCl for 5 minutes followed by rinsing in distilled water for 1 minute and then in high-grade acetone for 1 minute before air-dried. After drying, the slides were washed in a 2% solution of organosilane in high-grade acetone for 1 minute with gentle agitation, then rinsed with high-grade acetone for 1 minute, air-dried and stored at room temperature in a box (Dr. Catriona McElnea, Microbiology and Immunoloy JCU, *pers. comm.*).

Based on routine histological assessment, slides for the negative controls were prepared from tissues of juveniles of *P. monodon* (archive number 00-342B) and *P. merguiensis* (archive number 00-342A). These prawns were caught in Kupang Bay, west Timor, Indonesia, in May 2000, and had no MBV infection seen with light microscopy. Likewise, a subadult *P. monodon* (archive number 91-342-6) collected from a prawn grow-out facility in central Java, Indonesia, in 1991 served as the positive control (Fig. 7.1.). This prawn suffered grade 4 severity of infection with 6 - 12 prominent occlusion bodies per nucleus.

7.3.2. Viral DNA extraction for PCR assay

The postlarvae used in the PCR assay originated from a hatchery in northern Queensland and were fixed in 70% alcohol. The hot phenol method (Belcher and Young, 1998) was employed to extract the viral DNA from a whole animal.

Briefly, prawn tissues were grounded in pre-heated digestion buffer, then incubated at 65 ^{o}C for 5-10 min. The resulting suspension was homogenised, buffered phenol added and incubated for 2 h at 65 ^{o}C. Following extraction with phenol:chloroform (a ratio of 1:1), the viral DNA was precipitated at –20 ^{o}C overnight. The DNA pellet obtained was washed with ethanol, resuspended in a dilution of buffer at 37 ^{o}C for 2 h, and finally diluted to a working concentration after spectrophotometric analysis.

7.3.3. Primers and reaction conditions for PCR assay

The primers for PCR assay were constructed by Sigma® Genosys based on primers designed by Belcher and Young (1998) (primary amplicon: 533 bp; nested amplicon: 361 bp) and Hsu *et al.* (2000) (511 bp amplicon). All PCR were carried out in 0.5-ml tubes in a PTC-100™ programmable thermal controller (MJ Research, Inc.). The PCR conditions were described in the De*f*initive TAQPAQ PCR Kit (Fisher Biotech®). Briefly, the PCR reaction contained 5 µl of polymerisation buffer, 2 µl of 25 mM $MgCl_2$, up to 1 µM of primers, up to 100 ng of template DNA, 0.5 – 1 U of *TaqPaq* DNA polymerase and added with PCR grade water to a final volume of 25 µl before overlaying with 30 µl of paraffin oil. The cycling conditions consisted of one primary denaturation cycle (5 min in 94 ^{o}C, 1 min in 55 ^{o}C, 2 min in 72 ^{o}C); 38 repetitive cycles (30 sec in 94 ^{o}C, 1 min in 55 ^{o}C, 2 min in 72 ^{o}C), one chase cycle (30 sec in 94 ^{o}C, 1 min in 55 ^{o}C, 10 min in 72 ^{o}C, 1 min in 25 ^{o}C) and holding temperature at 4 ^{o}C.

Under these conditions, JumpStart™ Taq DNA Polymerase (Sigma®) for a hot start PCR assay was compared to the DNA polymerase from the De*f*initive TAQPAQ PCR Kit (Fisher Biotech®). The hot start assay prevents *Taq* polymerase from reacting with the reaction mix prior to the first high temperature step in the PCR cycle.

All PCR reagents were handled in a laminar flow cabinet using aerosol-resistant tips to avoid contamination. The reliability of the PCR conditions and the quality of the DNA extraction were tested using the primers for 18s RNA of decapods (Lo *et al*., 1996 b), which produced an 848 bp amplicon.

7.3.4. Electrophoresis

Any DNA sample with a concentration of >50 ng/µl was subjected to electrophoresis to check the integrity of the DNA. Likewise, PCR products were visualised using electrophoresis procedure (Trudel and Payment, 1993; Belcher and Young, 1998), as follows:

1. A 1.5% w/v agarose gel was prepared by boiling a mixture of 0.9 g agarose in 60 ml TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing 0.5 g/l ethidium bromide.
2. The melted agarose solution was cooled to about 50 ^{o}C and then poured carefully to avoid bubbles into a 10 x 7 cm glass plate. The glass plate was fitted with a well-forming comb placed parallel to the top of the glass plate. A minigel of 0.6 cm thick was formed in the glass plate.
3. After the gel had solidified, the comb was removed and the glass plate was mounted into a Plexiglass platform (30 x 7 x 4.5 cm) connected by electrodes to a two outlet power supply. TAE buffer was poured into the platform until flooding the entire gel.
4. For analysis, 1 µl of gel-loading buffer (0.25% w/v bromophenol blue, 30% glycerol, 10 mM EDTA pH 8.0) was added to 10 µl of each reaction mixture before injected the mixture into a well in the gel. Four µl of a molecular weight standard (GIBCO BRL 1 kb DNA Ladder; Life Technologies) was included in each gel.
5. The minigel was electrophoresed at 80 V (0.05 A) for 2 hours.

The result of the electrophoresis was photographed using GeneSnap and analyzed using GeneTool for Windows 95 (Synoptics® Limited 1998). The output was then edited using Corel Photo Paint for Windows 95.

7.3.5. DNA sequencing analysis

DNA sequencing process was carried out using a sequencing system of Beckman Coulter Inc. (USA) in the Department of Biochemistry of JCU. The result was analyzed using a sequencer program (SEQUENCER^{TM} 4.0 for Windows 1999, Gene Codes Corp., USA).

7.4. Results

7.4.1. ISH assay

Initially, the ISH assay did not produce repeatable results due to very low sensitivity. The problems either arose from the loss of tissues during the processing or, very weak

reaction to the probe. Tissues of postlarvae of *P. merguiensis* frequently eroded during the ISH processing. However, after optimization of the ISH protocol, such as by varying the incubation period of proteinase-K, the integrity of the tissues was stabilized. Also, the sensitivity and specificity of the probe increased greatly with the boiling the tissue and probe at the same time on the slide and two-fold increase of incubation time for color development.

The positive controls provided with the ISH kit as well those prepared from an archived paraffin-embedded prawn samples showed a strong positive reaction to the probe (Table 7.1. & Fig. 7.1.). On the contrary, the negative controls indicated no cross-reaction of the probe to other tissues.

Table 7.1. Results of ISH. (a) Positive controls: cultured prawns of central Java, Indonesia and ISH kit. (b) Negative controls: wild prawns of Kupang Bay, west Timor, Indonesia, and ISH kit. + + + : very severe MBV infection, or react very strongly to the probe. + + : severe MBV infection, or react strongly to the probe. + : weak reaction to the probe. - : no MBV found, or no reaction to the probe.

Prawn species	**Archive number**	**Life stage**	**Origin**	**Histology**	**ISH assay**
P. monodon	89-226-3	Postlarvae	Australia	+ + +	-
	90-126-55	Postlarvae	Australia	+ + +	-
	90-216-56	Postlarvae	Australia	+ + +	-
	91-342-6[a]	Juvenile	Indonesia	+ + +	+ +
	99-125 C	Postlarvae	Australia	++	-
	00-28-K	Postlarvae	Australia	++	-
	00-134-B2[b]	Juvenile	Indonesia	-	-
	(+) control[a]	Adult	ISH kit	+ + +	+ + +
	(-) control[b]	Adult	ISH kit	-	-
P. merguiensis	88-618-C	Postlarvae	Australia	++	-
	99-125 B	Postlarvae	Australia	+ + +	-
	99-125 I	Postlarvae	Australia	+ + +	-
	99-125 O	Postlarvae	Australia	-	-
	00-28-B	Postlarvae	Australia	+++	-
	00-134-A2[b]	Juvenile	Indonesia	-	-

The ISH indicated that every MBV-infected postlarvae of *P. monodon* from Australia tested in this study reacted negatively to the probe. Likewise, all Australian MBV-infected samples of *P. merguiensis* reacted non-specifically or negatively to the probe.

Nevertheless, the results of the ISH for all samples of *P. monodon* and *P. merguiensis* from Indonesia agreed with the histological assessment (Table 7.1.).

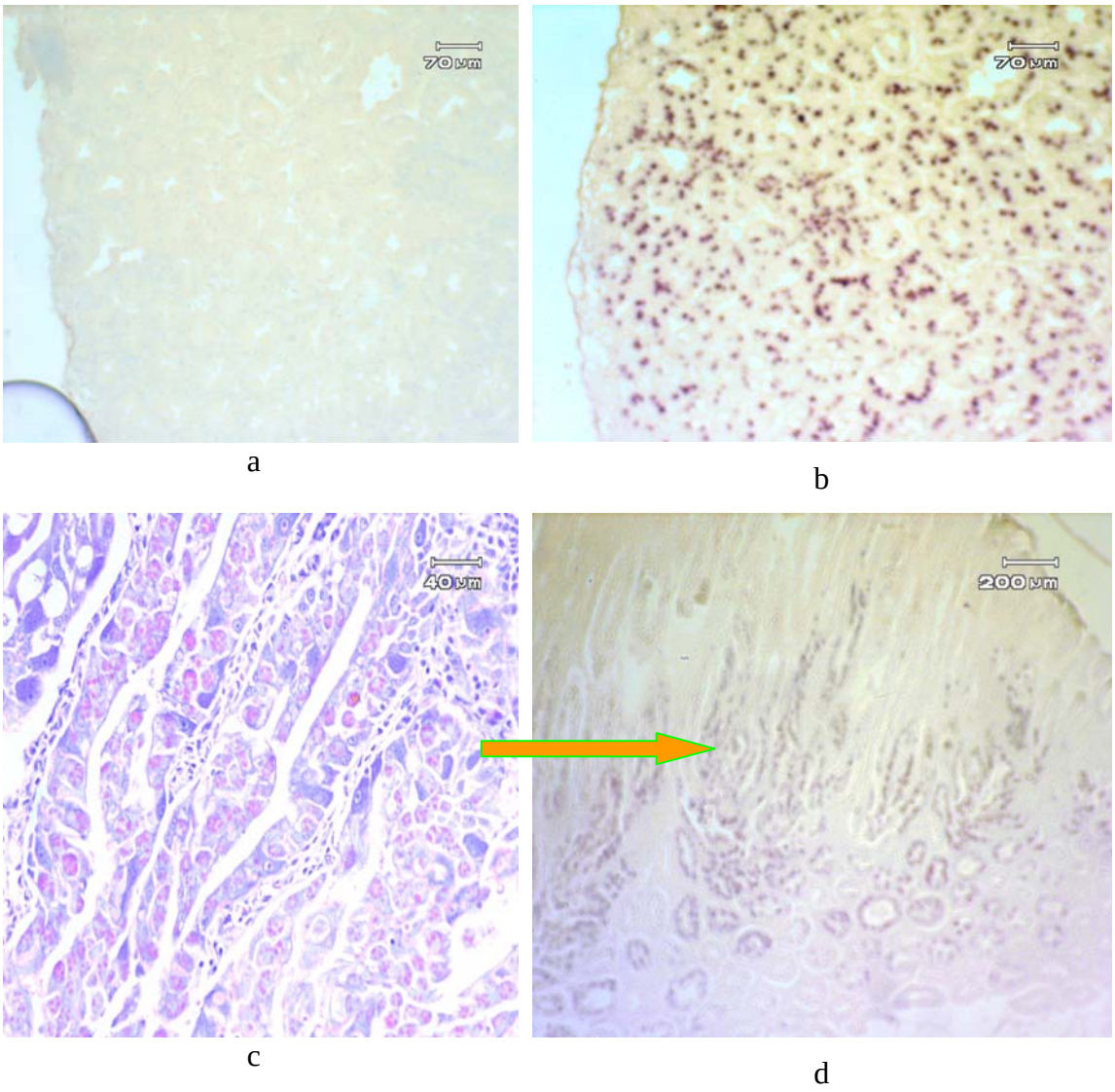


Figure 7.1. Results of ISH. (a) negative control from the kit, (b) positive control from the kit, (c) the hepatopancreas of sub-adult *P. monodon* from central Java (Indonesia) heavily infected by MBV as a positive control (H&E stained), and (d) the same slide in (c) subjected to ISH assay.

7.4.2. DNA extractions using hot phenol method

The prawns used in the DNA extraction were from 1998 to 2000. They were submitted to Microbiology and Immunology, JCU in 1999 and 2000 (Table 7.2.). Only 2 out of 18

batches of samples were fixed in alcohol. The concentrations of DNA obtained varied from 0 to 1248 ng/µl (Table 7.2.). However, some DNA samples were sheared, thus unsuitable for PCR assay.

Table 7.2. DNA concentrations based on 260 nm wavelength.

Date of Assay	Prawn Species	Sample Code	Necropsy Number	Fixative	MBV Status	[DNA] in ng/µl
22/06/00	*P. monodon*	None	99-125J	Methanol	Heavy	1146
	P. monodon	None	99-125P	Methanol	Heavy	1248
12/12/00	*P. merguiensis*	A3A5	00-28D	Alcohol	None	245
	P. merguiensis	C30/10/6/99	00-28J	Alcohol	None	164
14/12/00	*P. monodon*	A5/99-154	00-28K	Methanol	Heavy	214
	P. monodon	A10-116/98	None	Methanol	None	204
02/02/01	*P. monodon*	A5/99-154	00-28K	Methanol	Heavy	0
	P. merguiensis	C10/00-020	None	Methanol	None	0
07/02/01	*P. monodon*	A5/99-154	00-28K	Methanol	Heavy	56
	P. merguiensis	C10/00-020	None	Methanol	None	119
	P. merguiensis	C11/00-021	None	Methanol	None	62
14/02/01	*P. monodon*	A5/99-154	00-28K	Methanol	Heavy	57
27/02/01	*P. merguiensis*	A1/00-042	None	Methanol	None	85
27/02/01	*P. monodon*	A11/00-218	None	Methanol	None	74
06/03/01	*P. monodon*	00-27/6/00	None	Methanol	None	12
	P. monodon	C11/00-217	None	Methanol	None	575

7.4.3. PCR assays

Any DNA template showing DNA integrity after the gel electrophoresis was used for PCR assay. Nearly all PCR conditions produced 848 bp amplicons for 18s rRNA of decapods using primers of Lo *et al*. (1996 b) (Fig. 7.2.). No primary or nested amplicons of MBV PCR were detected in *P. monodon* and *P. merguiensis* using the primers of Young & Belcher (1998) (Fig. 7.3. and Appendix 7.1.). Two assays using the Taq polymerase in the De*f*initive TAQPAQ PCR kit and primers of Hsu *et al*. (2000) on *P. monodon* samples produced 5 bands of amplicons between around 150 to 450 bp (Fig. 7.3. and Appendix 7.1.). The hot start PCR assay on *P. monodon* and *P. merguiensis* using MBV primers of Hsu *et al*. (2000), JumpStart Taq polymerase and the De*f*initive TAQPAQ PCR kit produced an amplicon at around 400 bp (Fig. 7.4.). Thus, the expected amplicon of 511 bp was not seen.

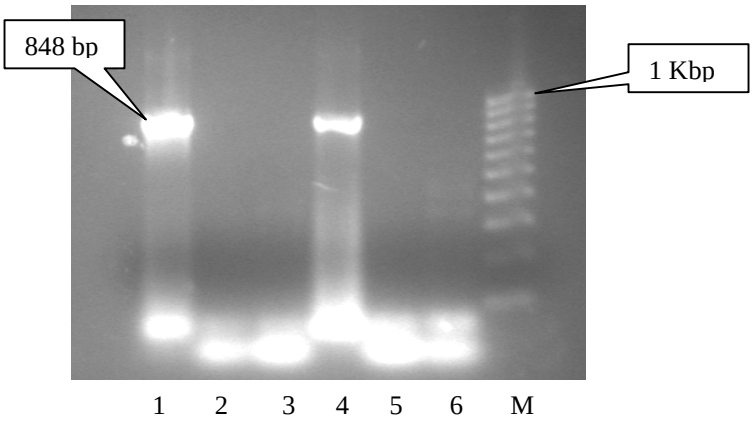


Figure 7.2. PCR results using *P. merguiensis* samples from northern Queensland as the templates, 18s RNA primers of Lo *et al*. (1996 b) and Taiwan-based MBV primers of Hsu *et al*. (2000). A13/01-050 batch: 18s RNA (lane 1), template without MBV primer (lane 2), and template with MBV primer (lane 3). A14/00-051 batch: 18s RNA (lane 4), template without MBV primer (lane 5), and template with MBV primer (lane 6). M = 1 kbp DNA ladder.

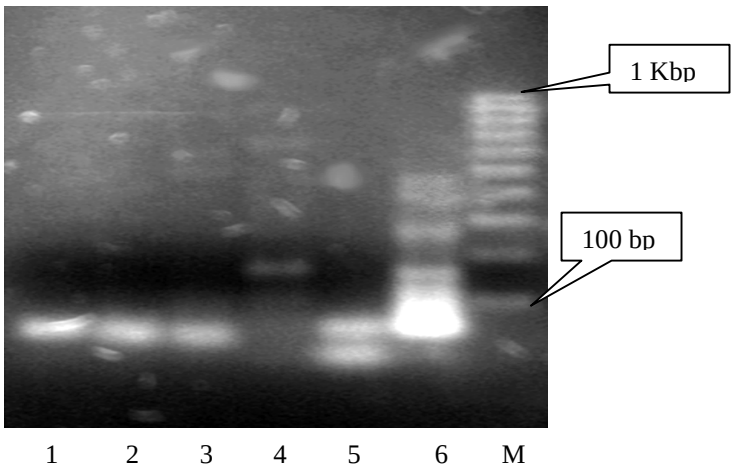


Figure 7.3. Non-specific products of PCR assay for MBV in *P. monodon* using two different primers. Belcher and Young (1998) primers: nested without template (lane 1), nested with template (lane 2), primary without template (lane 3), primary with template (lane 4). Hsu *et al*. (2000) primers: without template (lane 5), with template (lane 6). M = 1 kbp DNA ladder.

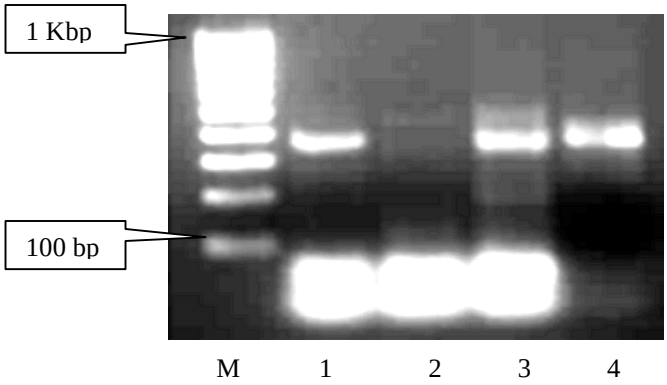


Figure 7.4. Amplicons of MBV primers (Hsu *et al*., 2000) from 'hot start' PCR assay. M = 1 kbp DNA ladder. Lane 1: 300 ng *P. monodon* DNA template. Lane 2: no template. Lane 3: 300 ng *P. merguiensis* DNA template. Lane 4: purified gel agarose of products in lane 3.

7.5. Discussion

MBV strains from Australian *P. monodon* and *P. merguiensis* not only differed from each other in histology as revealed in Chapter 4, but they might also vary from MBV strains from Indonesia based on ISH (Table 7.1.). Spann and Lester (1996) have reported that baculovirus found in wild *Metapenaeus bennettae* in Australia also did not react with a DNA probe for MBV. Moreover, the DNA probes used in these studies might be produced from clones of MBV DNA from a Taiwanese isolate or Hawaii isolate. J. Cowley (CSIRO Australia, *pers. comm*.) also could not generate DNA amplicons of Australian MBV with real time PCR using published primers of non-Australian MBV strain(s). Thus, whilst preliminary, the findings of the present study seem to support the existence of a variation of MBV strains across hosts and geographical range as suggested by Lightner (1996). At this stage, it is hypothesized that MBV strain from Australia is distinct from those from Asia (Indonesia and Taiwan).

Although PCR assays in this study were done around 1 – 2 years after the sample acquisition, the prawn DNA was extractable as shown by the amplification of 18s RNA of the prawns (Table 7.2. and Fig. 7.2.). However, DNA of MBV was not detectable using the published primers. Although the primers of Hsu *et al*. (2000) gave positive amplifications of DNA, the products were non-specific as the expected amplicon of 511

bp did not develop (Figs. 7.2, 7.3. and 7.4.). Nevertheless, the results show that the condition of PCR assay was optimal to produce amplicons using the 'hot start' DNA polymerase and primers by Hsu *et al*. (2000). The lack of clinical samples prevented the tests upon the primers of Belcher and Young (1998) using the 'hot start' PCR assay. Therefore, the inability of PCR assay to detect MBV DNA in the present study might not be associated with the PCR condition.

Perhaps the amount of MBV DNA was insufficient for the PCR assay. Hsu *et al*. (2000) overcome this problem by isolating and purifying the OBs of MBV using centrifugation before attempting phenol/chloroform extraction of MBV DNA for PCR assay. Likewise, Lu *et al*. (1993) isolated and purified virions and OBs of MBV from 5000 fresh hepatopancreas of *P. monodon* PLs in their attempts to sequence MBV DNA. Again, due to the limitation of prawn samples, this procedure was not attempted in the present study. In addition, the prawns in this study were fixed in methanol or alcohol, while Vickers *et al*. (1992), Belcher and Young (1998) and Hsu *et al*. (2000) used snap frozen prawns in their study. Possibly, this difference also yielded variability in the outcomes of the extraction of MBV DNA and subsequent PCR.

Since the PCR using Taiwanese MBV-based primers from Hsu *et al*. (2000) produced non-specific amplicons, while the histology showed heavy MBV presence in some samples, it is likely that MBV in northern Queensland differed from that from Taiwan. Indeed, geographic differences among MBV strains occur (Lightner, 1996).

Nevertheless, this study has demonstrated that molecular diagnosis of prawn viruses was greatly simplified by the availability of commercial ISH probes, published PCR primers, commercial PCR reagents and hot start *Taq* polymerase. The extraction of viral DNA from the whole body of the prawn (Belcher and Young, 1998) also curtails the procedure. However, Cunningham (2002) argues that more research needs to be carried out to make PCR applicable and cost-effective for routine diagnosis at the farm level. In this regard, the present study shows that strain variation of prawn viruses also needs to be taken into consideration in the application of molecular diagnosis.

CHAPTER 8
GENERAL DISCUSSION

Quantitative evaluation has long been used to analyse the interactions between hosts, pathogens and the environment in the development of viral diseases of prawns. Most of the epidemiological, ultrastructural, immunological and genetic studies report the prevalence, physical dimensions, abundance and other quantitative features of the viruses. Usually, the histopathology associated with the viral infection is also described quantitatively. Well-defined environmental data generally accompany such studies. Obviously, quantitative analyses in this thesis are familiar to microbiologists and pathologists. However, this study exhibits a novel and genuine attempt to assess the multiple interactions using simple and cost-effective approaches within the limits of light microscopy and histology (section 1.1.). This approach may find its' significant relevance in countries and regions lacking advanced diagnostic facilities.

The primary limitations of this study revolve around sampling. Indeed, this study depended on samples from other sources (Chapters 4, 6 and 7), was limited in sample size (Chapters 4 -7) and lacked clinical samples (Chapter 7). However, throughout the study, statistics, coherence and analogy have improved the power and quality of the analyses, and reliability of the conclusions. Hence, the associations between statistics, simple methods and literature analogy have generated a matrix of simple and robust quantitative parameters for describing the intra- and interrelationship between hosts, viruses and the environment (Table 8.1.). The matrix shows that the only relationship not assessed in this study was between the pathogens and the environment. This is because the main focus of this study was the hosts, at individual and population levels (section 1.1.).

In general, from cellular to population levels, quantitative analyses in this study were sensitive enough to describe the viruses (e.g. MBV from two penaeid species), suitable to quantify the histopathological conditions of virus-infected organs (e.g. hepatopancreas

Table 8.1. A matrix of simple and robust parameters for assessing the relationship between prawns, pathogens and the environment.

FACTORS	**Prawns**	**Pathogen**	**Environment**	**ALL**
Prawns	DESCRIBE & COMPARE: Prevalence Ratio of infected nuclei Ratio of cytolytic cells Stages of infective nuclei Ratio of stages of infective nuclei Severity of infection indices Severity of infection grading Ratio of spheroid-total length (LO) Number of spheroids (LO) Morphotype score (LO)	CORRELATE: Size of occlusion bodies in cells Number of occlusion bodies Prevalence of abnormal tissues Prevalence of diseases in tissues Attributable risk (AR) Odds ratio (OR)	CORRELATE: Water quality parameters Biomass & farming management Biomass & biological parameters Food budget & water quality	
Pathogen	COMPARE: Size of occlusion bodies in cells Number of occlusion bodies Prevalence of abnormal tissues Prevalence of pathogens in tissues Grades of severity of infection Attributable risk (AR) Odds ratio (OR)	DESCRIBE & COMPARE: Size of occlusion bodies in cells Number of occlusion bodies in nuclei Attributable risk (AR) Odds ratio (OR) PCR & ISH for MBV		COHERENCE AND ANALOGY
Environ-ment	COMPARE: Water quality parameters of crops Farming management of crops Biological parameters of crops		DESCRIBE & COMPARE: Water quality parameters (daily and monthly for each crop)	
ALL	COHERENCE AND ANALOGY			CONCLUSIONS

and lymphoid organ) and robust enough to identify risk factors associated with farm production (Table 8.1.).

With regard to MBV, quantitative analyses based on histopathology clearly indicate that host-specific and tissue-specific MBV exist in Australia. Again, this finding has not been reported before. Perhaps the major findings from this study are the tissue tropism of MBV and host factors that control the development of MBV occlusion bodies (OBs). It implies that the diagnosis of MBV in *P. merguiensis* should focus on the anterior midgut or the entire digestive track. However, to avoid killing prawns, the examination of fecal strands using malachite green method to detect MBV OBs or PCR to detect non-Australian MBV DNA (Lightner, 1996), are suitable for MBV diagnosis.

While histopathology demonstrated differences among MBV from two Australian penaeid species, molecular methods (i.e., ISH and PCR) yielded inconclusive results. However, the molecular diagnosis of MBV supported the hypothesis of strain differences based on geography. In fact, gene probes (DiagXotics, Inc., USA) and primers (Hsu *et al*., 2000) used in the current study did not originate from Australian MBV strain(s). Unfortunately, this hypothesis was slightly confounded by the fact that Australian-based primers by Belcher and Young (1998) yielded no amplicon from the PCR. Since the reasons behind these phenomena are beyond the boundaries of the current study, it is appropriate to suggest further research should be directed towards the differences in the genetics and serology of MBV from various penaeids in the world as suggested by Doubrovsky *et al*. (1988), Natividad and Lightner (1992) and Lightner (1996). This approach needs a complementary and parallel step towards the study of biochemistry, anatomical evolution, immunological mechanism and genetics of the host's digestive system.

Nevertheless, despite of the sampling problems, the present study has highlighted the simplicity and power of quantitative histopathology. Here, only three tissues were probed as examples, i.e. hepatopancreas and anterior mid-gut (digestive tissues) and lymphoid organ (immunological tissue). Several common quantitative parameters used to

assess these tissues, e.g. disease prevalence, severity grades and ratio of infective nuclei, might be applicable for different tissues. In addition, tissue-specific parameters, such as the number and size of occlusion bodies, ratio of cytolytic cells, spheroid-total length ratio and morphotype scores, show that any tissue can be assessed quantitatively according to the healthy (normal) and disease (abnormal) features of that tissue.

These parameters will be meaningful for disease assessment and management when data of pathogen diagnosis and environmental factors are available. Hence, the sensitivity and reliability of the histopathological parameters can be assessed against the infection of a particular pathogen or cocktail of pathogens (e.g. proportional attributable risk and odds ratio analyses). Likewise, the same histopathological data can be assessed against environmental factors to find histological parameters most relevant and sensitive to environmental changes for that particular tissue infected by a particular pathogen or cocktail of pathogens. In either case, statistics provides the tool for analyses. For example, the retrospective longitudinal study in Chapter 4 based on archival samples from 1988 - 2002 (15 years span) was able to differentiate two host-specific MBV-type viruses and identify tissue tropism of MBV in Australian penaeids. The severity index, ratio of infected nuclei and ratio of stages of nuclear changes due to MBV infection clearly support the conclusion. This type of study is cost effective and applicable for places lacking molecular diagnostic facilities. The study also emphasizes the benefit of histological archives for longitudinal study since the archives demand low-cost maintenance and can be analysed at anytime.

The quantitative analyses of tissues in the current study also convey a general idea about biological responses of the hosts to pathogen infection and environmental stress. Hence, the degree of disruption or destruction of digestive tissues (e.g., hepatopancreas) and immunological tissues (e.g., lymphoid organ) in individual prawn or population can cast a light on the implications of the infections and environmental pressures on the health, survival and growth of the hosts. Such implications can be analysed using coherence and analogy in cases where there are no farm records of diseases as demonstrated in Chapter 6. Here, proxy measures of prawn health, such as biomass and food budget, provide a

limited means for analyses of the diseases. The variability of these proxy methods means strong correlations are not often found. However, the analytical approaches and models in this particular case will be widely applicable since most of prawn hatcheries and farms, especially small farmers in third world countries, like Indonesia, rarely record the disease in their facilities. However, they usually record other data, such as the food budget, growth, survival rate and biomass. Moreover, simple methods, such as the transect method for describing lymphoidal condition (Chapter 5), might improve the quality of the analysis when the sampling is valid and reliable.

Given the cost of molecular diagnosis (Cunningham, 2002), the majority of prawn farmers in the third world countries, like Indonesia, appear to opt for growing their prawns under disease conditions. Moreover, the presence of viral infection does not necessarily cause disease outbreaks as long as the farming practices sustain high quality of the environment (Horowitz and Horowitz, 2001). Even after an outbreak of viral diseases, "viral accommodation" by the prawns might occur. Therefore, it is realistic to expect that the majority of prawn farmers in third world countries, like Indonesia, will not rely on molecular diagnosis. They may even not attempt to detect prawn viruses in their hatcheries or ponds, and drain the infected prawns from their facilities to the local environment due to lack of awareness and control as suggested by Subashinge and Barg (1998) and Karunasagar *et al*. (1998).

Under such circumstances, a policy of quarantine and health certification for prawn hatcheries and farms may encourage the application of molecular diagnosis in third world countries down to the farm level. For many countries in southeast Asia, it implies that funding, training, expertise and facilities are needed for the diagnosis of aquatic pathogens including prawn viruses (Liao, 1989; Fulks and Main, 1992; Arthur, 1995). Again, commercial gene probes, published primers, commercial reagents, DNA purification system and simplified procedures combined with government initiatives, market competition and human resource development will overcome the challenges of cost benefit and technical problems associated with the application of molecular diagnosis.

Along that line of progress, conventional histology is and will be a compatible tool for molecular methods. Therefore, in places lacking molecular diagnostic capabilities, histology combined with quantitative logical reasoning appear to be a reasonable and preeminent option for diagnosis and epidemiological studies related to decision making process to control viral diseases in cultured or wild prawns. Hence, the present study on quantitative histopathology and statistical epidemiology has demonstrated the usefulness and power of quantitative approach in analyzing prawn viral diseases.

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APPENDICES

Appendix 3

3.1. Davidson's Fixative

Acetic Acid	110	mL
95% ethanol (EtOH)	330	mL
Tap water	335	mL
Formalin	220	mL

3.2. Mayer's Haematoxylin

Haematoxylin	2	g
Sodium Iodate	0.4	g
Aluminium Ammonium Sulphate ($Al\ NH_4(SO_4)_2.12H_2O$) (Ammonium Alum)	100	g
Citric Acid	2	g
Chloral Hydrate	100	g
Water	2	L

3.3. Scott's tap water

Distilled water	2.5	L
$NaHCO_3$	8.75	g
$MgSO_4.7H_2O$	50	g

3.4. Young's Eosin

Eosin	15	g
Erythrosin	5	g
Calcium Chloride	5	g
Water	2	L

Appendix 4. Data of prawn samples in the archive of Discipline of Microbiology and Immunology, James Cook University, Australia.

Year	Case No.	Origin	No. of Slides	Prawn Species and Diagnosis
1988	88/517	Mackay Prawn Farm (PF)	2	*P. monodon* juveniles; heavy MBV in hepatopancreas [HP]; bacilliform virus present
1988	88/618	Seafarms PF	3	*P. monodon* juveniles; 2 slides with no MBV present, 1 slide with possible MBV present.
1988	88/835	Jkelenec	15	Larvae of unknown prawn species; Possible MBV or bacilliform virus.
1989	89/226	Seafarms PF	12	*P. monodon* larvae; 20 larvae seen; Possible to very low MBV present in HP.
1989	89/445	Sunsun PF	15	Juveniles of unknown prawn species; No MBV present, but very low to very heavy bacilliform virus present in HP.
1990	90/143	Seafarms PF	5	Larvae of unknown prawn species. Very heavy bacilliform virus in HP & low in the midgut
1990	90/146	Seafarms PF	26	P. monodon larvae; all, except 1, slides have no MBV present; very low to heavy load of bacilliform virus in HP; the gut is clean.
1990	90/154	Seafarms PF	7	*P. monodon* larvae; Possible BMN-like present in 1 slide only. 3 slides with heavy S1 MBV in HP, 2 with no MBV and 1 with light MBV; the gut is clean.
1990	90/216	Seafarms PF	14	*P. monodon* larvae; all slides, except 1, contain larvae with low to heavy MBV infection;
1990	90/219	Seafarms PF	15	*P. monodon* larvae; moderate to heavy MBV in HP
1990	90/23	Sunsun PF	8	Juveniles of unknown species; no MBV
1990	90/223	Seafarms PF	17	Spawners of *P. monodon*; no MBV
1990	90/235	Seafarms PF	3	*P. monodon* larvae; No MBV, bacteriacemia
1991	91/341	Seafarms PF	18	*P. monodon* postlarvae (PLs); No MBV
1991	91/324	Ika Muda PF Indonesia	24	*P. monodon* adult; None to heavy MBV.
1992	92/143	Cooktown PF	32	*P. monodon* juveniles; MBV, HPV, etc.
1999	99/125	Seafarms PF	6	*P. monodon* PLs; none to moderate MBV; used for PCR assay
			13	*P. merguiensis* PLs; None to heavy MBV; used for PCR assay

Appendix 4. Continued.

Year	Case No.	Origin	No. of Slides	Prawn Species and Diagnosis
2000	00/28	Seafarms PF	10	*P. merguiensis* larvae; no to heavy MBV; used for PCR assay
			1	*P. monodon* larvae; heavy MBV in HP; used for PCR assay
2000	00/134	Kupang Bay, Indonesia	12	Wild *P. merguiensis* juveniles; no MBV; in situ hybridisation assay
			12	Wild *P. monodon* juveniles; no MBV; in situ hybridisation assay
2001	01/321	Ayr PF	20	*P. monodon* juveniles; no MBV
2001	01/325	Ayr PF	34	*P. monodon* juveniles; no MBV; bioassay
2002	02/128	Ayr PF	15	*P. monodon* juveniles; no MBV; bioassay
2002	02/370	Bali PF	15	*P. vannamei* juveniles; no MBV; survey in Bali
2002	02/371	Bali PF	21	Mixture of *P. vannamei* and *P. monodon* juveniles; no MBV; survey in Bali

Appendix 5. Histology for slide No.: 02-128 from the 16-h bioassay of AcNPV induced-infection on *P. monodon* sub-adults (reared in laboratory from 12 December 2001 to 16 April 2002).

Time	Slides	Sites	Observations
0 h	A1	HP	No viral infection; massive, spreading bacterial septicemia
		Heart	Normal pericardium and myocardium
		LO	Empty lumens: + (very few); with hematocytes (+++); SMC +++; spheroids + (but huge); hemorrhage.
	A2	None!	Damaged slide
	A3	Heart	2 sites with focal haemocytic accumulation in myocardium; one OB? (round eosinophilic body) in one of the sites.
		LO	Empty lumens ++ (50%); lumens with haemocytes:++; no hemorrhage; seems normal overall.
	A4	Heart	One focal haemocytic infiltration + encapsulation
		LO	No hemorrhage; empty lumens +; filled lumens +++
2 h	B1	HP	Normal
		Heart	Heavy hemorrhage; massive blood infiltration, nodules
		LO	Broken spheroids; scattered haemocytes; heavy hemorrhage; few vacuolated spheroids
	B2	Heart	Massive hemorrhage + haemocytic encapsulation/infiltration
		LO	Heavy hemorrhage; some vacuolated spheroids; broken spheroids.
		HP	Normal
	B3	Heart	Not seen
		LO	Open lumens +++ closed +; focal disarray of cells
	B4	Heart	Focal haemocytic encapsulation in the myomers
		HP	Focal septicemia
		LO	Open lumen + (few)
4 h	C1	Heart	One big wound in myocardium; massive disruption and haemocytic encapsulation (**size?**) in the myomers.
		LO	Broken tubules (no walls); haemocyte-filled lumens ++++; hemorrhage & eosinophilic materials filled the hemal sinuses
		HP	Normal
	C2	Heart	Spreading haemocytic encapsulation in the myomers (probably the nerve ganglion in the heart).
		LO	Same as C1
		HP	Normal
6 h	D1	HP	Focalized septicemia
		LO	Hemorrhage, tubular lumens filled with haemocytes +++; no spheroids
		Heart	Normal (seen valves and subgastric artery leading to LO)
	D2	All	Same as D1
	D3	Heart	Only a remnant
		LO	Same as D1 & D2
		HP	Dissolved (post-mortem fixation)
	D4	Heart	Normal
		LO	Broken tubules; hemorrhage; lumens filled with haemocytes +++
		HP	Normal.

Appendix 5. Continued

Time	Slides	Sites	Observations
8 h	E1	Heart	Normal.
		LO	Hemorrhage; broken tubules; strongly eosinophilic materials in the hemal sinuses of empty intertubular spaces
		HP	Normal.
	E2	Heart	Focalized haemocytic encapsulation of myomers.
		LO	Heavily disrupted [loss defined tubules]. Hemorrhage; few spheroids
		HP	Normal.
	E3	Heart	Normal.
		HP	Normal.
		LO	Not seen.
	E4	Heart	Normal.
		HP	Normal.
		LO	Less hemorrhage; more tubules with empty lumens; few spheroids; one small hemal sinus with eosinophilic substance inside
		Heart	Normal.
10 h	F1	LO	Two small vacuolated spheroids; most of the lumens filled with cells; hemorrhage; eosinophilic materials in the hemal sinuses, loss of defined tubules
		Heart	Massive haemocytic accumulation in one place.
		HP	Normal.
	F2	Heart	A massive heart wound in the middle with haemocytic encapsulation (probably from the injection)
		LO	Not clear
		HP	Normal.
	F3	Heart	Normal.
		LO	Large vacuolated spheroids, hemorrhage; most of lumens filled with cells.
		HP	A small septicemic wound with melanization/pigmentation
	F4		All organs same as F3
12 h	G1	LO	Abundant hemal sinuses with eosinophilic substances inside. Some tubules are broken. More tubules with open lumens
		Heart	Normal.
		HP	Normal.
	G2	Heart	Normal.
		HP	Normal.
		LO	Not seen.
	G3	Heart	Not seen.
		LO	More opened lumen, few closed ones.
	G4	Heart	Accumulation of haemocytes in one place
		LO	Scattered.
14 h	H1	Heart	Haemocytic accumulation in one place [mostly elongated haemocytes]. Damage in the anterior of the heart.
		LO	Only one small spheroid seen. Most tubules intact, some ruptured, hemorrhage; mot lumens filled with cells.
		HP	Not seen.
	H2		Damaged slide!
	H3	LO	Some spheroids; open lumens higher than closed ones
		HP	Normal.
		Heart	Not seen.
	H4		Damaged slide!

Appendix 5. Continued

Time	Slides	Sites	Observations
16 h	I1	Heart	Normal.
		LO	No spheroids, but loss defined tubules (cells everywhere)
	I2	Heart	Normal.
		LO	Not seen.
		HP	Normal.
	I3	Heart	Normal.
		LO	Some vacuolated spheroids with OBs. Most lumens closed. One large wound. Some OBs. Hemal sinuses apparent.
		HP	Mostly normal. A small septicemic wound.
	I4	Heart	Normal.
		LO	50:50 filled and empty lumens. OBs in spheroids. No hemorrhage.
		HP	Three small focalized septicemic wounds. Mostly healthy tubules.

Appendix 6.1. Descriptive statistics of weather condition in the farm location from September 1991 to May 1992.

Parameters	N	Min.	Max.	Mean	SE	SD	Variance	Skewness	Kurtosis
Morning rainfall (mm)	570	.00	108.00	7.00	.72	17.20	295.66	4.11	19.16
Afternoon rainfall (mm)	611	.00	150.00	6.45	.73	18.11	327.89	6.29	43.35
Morning temperature (^{o}C)	1086	16.80	33.00	23.74	6.79 E-02	2.24	5.02	.82	2.91
Afternoon temperature (^{o}C)	875	21.40	35.00	26.92	9.43 E-02	2.79	7.78	.48	.19
Morning cloud cover (%)	1056	.00	100.00	51.83	1.03	33.37	1113.25	.04	-1.35
Afternoon cloud cover (%)	994	.00	100.00	57.37	1.09	34.28	1175.40	-.19	-1.41
Morning wind speed (knot)	1145	.00	30.00	9.09	.22	7.29	53.09	.86	.04
Afternoon wind speed (knot)	1015	.00	30.00	8.31	.20	6.32	39.97	1.41	2.22

Appendix 6.2. Summary of monthly weather condition (September 1991 to May 1992).
*) 1 = morning; **) 2 = afternoon.

Month	Parameter	N	Min.	Max.	Mean	SE	SD	Skewness	Kurtosis
Sept	Rain 1*	22	.00	5.00	.2273	.2273	1.0660	4.690	22.000
91	Rain 2**	20	.00	.00	.0000	.0000	.0000	.	.
	Temp 1	2	22.30	23.60	22.9500	.6500	.9192	.	.
	Temp 2	0							
	Cloud 1	22	.00	70.00	22.8864	4.9935	23.4216	.876	-.485
	Cloud 2	22	.00	60.00	23.6591	4.0094	18.8056	.200	-1.136
	Wind 1	23	.00	20.00	3.8696	1.1075	5.3113	1.812	3.159
	Wind 2	23	.00	20.00	5.7826	1.0907	5.2306	1.379	1.296
Oct 91	Rain 1	46	.00	.00	.0000	.0000	.0000	.	.
	Rain 2	26	.00	.00	.0000	.0000	.0000	.	.
	Temp 1	50	16.80	24.80	22.6360	.2191	1.5492	-2.298	6.939
	Temp 2	30	22.90	30.60	27.2933	.4453	2.4392	-.474	-.792
	Cloud 1	56	.00	80.00	25.0714	3.1933	23.8966	1.227	.233
	Cloud 2	52	.00	80.00	31.6923	3.5215	25.3938	.654	-.792
	Wind 1	60	.00	20.00	6.2667	.7083	5.4861	1.022	-.194
	Wind 2	56	.00	20.00	7.5714	.7345	5.4966	.971	-.168
Nov	Rain 1	78	.00	59.50	2.3077	1.3036	11.5129	4.894	22.531
91	Rain 2	81	.00	10.00	.7407	.2928	2.6352	3.314	9.212
	Temp 1	81	19.60	26.62	24.8304	.1507	1.3562	-2.328	6.558
	Temp 2	87	25.00	33.00	28.3897	.2464	2.2980	.237	-1.126
	Cloud 1	81	.00	95.00	45.0000	3.7485	33.7361	.060	-1.510
	Cloud 2	81	1.00	90.00	40.8889	3.2252	29.0271	.247	-1.344
	Wind 1	84	.00	10.00	4.1607	.2768	2.5367	.706	.244
	Wind 2	81	1.00	17.50	5.5926	.4498	4.0482	1.707	3.277
Dec 91	Rain 1	76	.00	25.00	4.3684	.8618	7.5133	1.633	1.507
	Rain 2	60	.00	16.00	5.4000	.7362	5.7022	.486	-1.138
	Temp 1	100	21.20	28.00	25.2000	.1539	1.5387	-.631	.408
	Temp 2	64	24.80	32.90	29.1438	.2560	2.0477	-.389	-.040
	Cloud 1	92	.00	100.00	56.0435	3.2795	31.4562	-.380	-1.093
	Cloud 2	68	5.00	90.00	58.5294	3.5170	29.0015	-.809	-.759
	Wind 1	92	.00	15.00	4.6087	.3757	3.6039	1.953	3.617
	Wind 2	68	1.50	15.00	6.1176	.5805	4.7866	.727	-1.031
Jan 92	Rain 1	65	.00	108.00	12.0000	3.5878	28.9261	2.903	7.290
	Rain 2	55	.00	17.50	5.0000	.8582	6.3647	.873	-.726
	Temp 1	105	23.40	33.00	27.4286	.2695	2.7617	.719	-.539
	Temp 2	115	25.00	35.00	29.6174	.2353	2.5232	.464	-.371
	Cloud 1	105	.00	100.00	46.6667	3.4647	35.5023	-.006	-1.486
	Cloud 2	105	5.00	100.00	52.6190	3.5697	36.5790	-.066	-1.658
	Wind 1	110	.00	15.00	5.8182	.3892	4.0821	.892	.055
	Wind 2	105	2.00	15.00	6.2857	.3769	3.8623	1.090	.181

Appendix 6.2. Continued.

Month	Parameter	N	Min.	Max.	Mean	Std. Error	Std. Dev.	Skewness	Kurtosis
Feb 92	Rain 1	138	.00	103.00	13.9348	2.0313	23.8624	2.472	6.237
	Rain 2	150	.00	150.00	15.2800	2.7885	34.1526	3.081	8.701
	Temp 1	150	20.00	27.00	23.6080	.1382	1.6928	-.389	-.257
	Temp 2	132	21.60	33.00	27.6045	.1936	2.2248	-.232	1.677
	Cloud 1	150	.00	100.00	54.5000	3.3408	40.9165	-.001	-1.762
	Cloud 2	150	.00	100.00	64.0000	2.9606	36.2597	-.531	-1.202
	Wind 1	168	.00	20.00	3.7500	.3433	4.4503	1.839	4.209
	Wind2	150	.00	15.00	3.6800	.2992	3.6643	1.325	1.809
March 92	Rain 1	70	.00	31.00	5.0000	1.0848	9.0762	2.364	4.370
	Rain 2	84	.00	10.00	2.5000	.3338	3.0596	1.140	.570
	Temp 1	168	22.50	26.00	24.0375	7.385E-02	.9571	.625	-.560
	Temp 2	147	23.20	30.30	26.3619	.1343	1.6287	.319	.087
	Cloud 1	168	.00	100.00	52.9167	2.5517	33.0732	.211	-1.275
	Cloud 2	154	5.00	100.00	47.9545	2.7216	33.7746	.457	-1.195
	Wind 1	175	.00	15.00	6.6800	.2890	3.8238	1.017	.348
	Wind 2	161	1.00	20.00	6.5652	.3287	4.1710	1.722	2.921
April 92	Rain 1	48	1.00	18.00	6.6667	.9280	6.4291	.841	-.976
	Rain 2	72	.00	15.00	7.5556	.6080	5.1591	.091	-1.157
	Temp 1	232	20.30	24.90	22.7552	8.242E-02	1.2555	.091	-.910
	Temp 2	192	22.10	34.60	25.1417	.1729	2.3963	2.388	7.847
	Cloud 1	184	10.00	100.00	62.1739	2.1129	28.6614	-.221	-1.155
	Cloud 2	200	10.00	100.00	67.2000	2.4435	34.5562	-.552	-1.278
	Wind 1	208	5.00	30.00	14.6154	.5093	7.3456	.651	-.086
	Wind 2	200	5.00	30.00	12.0000	.5112	7.2292	1.370	.920
May 92	Rain 1	27	.00	7.50	3.8333	.6009	3.1225	-.086	-1.560
	Rain 2	63	.00	10.00	3.7143	.3883	3.0818	.954	.010
	Temp 1	198	18.10	24.40	21.8773	.1109	1.5607	-.839	.497
	Temp 2	108	21.40	27.00	24.5417	.1817	1.8882	-.321	-1.311
	Cloud 1	198	10.00	100.00	53.6364	1.9517	27.4631	.176	-1.322
	Cloud 2	162	20.00	100.00	71.6667	1.9527	24.8536	-.702	-.296
	Wind 1	225	5.00	30.00	16.4000	.3841	5.7609	-.406	.342
	Wind 2	171	10.00	30.00	13.6842	.4459	5.8304	1.541	1.344

Appendix 6.3. Summary of daily records of weather and water quality parameters, and total feed amount given in each pond within the relevant culture period. Rainfall (mm), temperature (^{o}C), cloud cover (%), wind speed (knot), dissolved oxygen (ppm), turbidity (cm), salinity (ppt).

Pond No.	Culture period	Weather quality (mean±SD)	Water quality (mean±SD)	Total running feed (mean±SD)
1	30/8/91-2/11/91 (62 days)	Rainfall: 0.1±0.7 Temp.: 24.4±4.8 Cloud cover: 26.0±20.4 Wind speed: 5.7±5.0	Temp.: 25.2±1.5 DO: 6.9±1.1 pH: 8.4±0.2 Turbidity:39.6±24.0 Salinity: 31.3±3.0	5320 kg (99.2±26.6 kg/day)
3	30/8/91-11/11/91 (68 days)	Rainfall: 0.1±0.7 Temp.: 25.2±4.3 Cloud cover: 27.1±21.1 Wind speed: 5.5±4.8	Temp.: 25.3±1.6 DO: 6.9±0.9 pH: 8.4±0.2 Turbidity: 46.5±21.2 Salinity: 33.8±3.3	6761 kg (95.2±47.6 kg/day)
4	16/9/91-3/4/92 (194 days)	Rainfall:5.6±16.9 Temp.:26.2±3.0 Cloud cover: 46.4±29.8 Wind speed: 5.5±3.9	Temp.: 27.7±2.1 DO: 7.7±3.3 pH: 7.9±0.4 Turbidity: 57.8±26.2 Salinity: 34.2±4.2	9449 kg (48.7±38.8 kg/day)
5	30/8/91-16/12/91 (105 days)	Rainfall: 1.4±4.7 Temp.: 26.0±3.4 Cloud cover: 46.4±29.8 Wind speed: 5.5±3.9	Temp.: 26.4±2.2 DO: 6.7±1.1 pH: 8.5±0.2 Turbidity: 52.4±26.3 Salinity: 34.6±3.3	8068 kg (76.8±25.3 kg/day)
6	30/8/91-16/12/91 (104 days)	Rainfall:1.4±5.9 Temp.: 26.4±4.9 Cloud cover: 33.8±28.3 Wind speed: 5.5±4.8	Temp.: 26.5±2.5 DO: 6.7±1.0 pH: 8.5±0.2 Turbidity: 49.2±23.4 Salinity: 34.9±3.8	7972 kg (76±29.2 kg/day)
7	30/8/91-7/12/91 (96 days)	Rainfall:1.4±6.1 Temp.: 26.3±5.2 Cloud cover: 32.7±27.8 Wind speed: 5.4±4.8	Temp.: 26.0±2.4 DO: 7.1±1.7 pH: 8.2±0.2 Turbidity: 40.5±20.1 Salinity: 34.8±3.0	7067 kg (73.6±24.8 kg/day)
8	01/12/91-16/5/92 (160 days)	Rainfall: 8.7±20.0 Temp.:25.7±3.0 Cloud cover: 56.2±34.4 Wind speed: 7.8±6.5	Temp.: 28.2±3.0 DO:8.5±3.8 pH: 8.2±0.2 Turbidity: 62.3±24.6 Salinity: 28.5±7.8	12,863.5 kg (80.4±59.5 kg/day)

Appendix 6.3. Continued

Pond No.	Culture period	Weather quality (mean±SD)	Water quality (mean±SD)	Total running feed (mean±SD)
10A	03/10/91-27/12/91 (85 days)	Rainfall: 2.0±6.8 Temp.: 26.4±4.8 Cloud cover: 41.5±31.2 Wind speed: 5.8±4.6	Temp.: 27.5±2.5 DO: 7.0±1.9 pH: 8.5±0.2 Turbidity: 64.3±33.7 Salinity: 36.9±3.0	736.8 kg (8.7±8.5 kg/day)
10B	07/3/92-30/5/92 (82 days)	Rainfall: 5.6±5.2 Temp.: 23.9±2.3 Cloud cover: 59.8±31.4 Wind speed: 12.2±6.9	Temp.: 25.6±2.0 DO: 10.8±2.6 pH: 8.4±0.2 Turbidity: 37.8±23.7 Salinity: 25.4±3.3	2300 kg (28.0±22.6 kg/day)
11A	30/8/91-28/11/91 (87 days)	Rainfall: 0.6±5.3 Temp.: 26.1±5.5 Cloud cover: 31.1±27.4 Wind speed: 5.4±4.9	Temp.: 25.7±2.3 DO: 6.9±1.2 pH: 8.4±0.2 Turbidity: 66.3±34.4 Salinity: 35.8±3.0	1530 kg (17.6±13.9 kg/day)
11B	11/4/92-30/592 (48 days)	Rainfall: 4.4±4.2 Temp.:23.3±2.4 Cloud cover: 62.4±31.8 Wind speed: 14.7±7.0	Temp.: 24.5±1.4 DO: 10.0±2.3 pH: 8.2±0.4 Turbidity: 45.3±21.3 Salinity: 25.5±2.6	393 kg (8.2±7.7 kg/day)
12A	30/8/91-18/12/91 (103 days)	Rainfall: 1.5±5.9 Temp.: 26.3±4.8 Cloud cover:35.0±29.3 Wind speed:9.5±7.3	Temp.: 26.3±2.6 DO: 6.8±1.2 pH: 8.4±0.2 Turbidity: 49.2±14.7 Salinity: 35.1±3.1	7679 kg (74.628.3 kg/day)
12B	01/2/92-30/5/92 (117 days)	Rainfall: 9.8±22.1 Temp.: 24.4±2.5 Cloud cover: 59.1±34.0 Wind speed: 9.5±7.3	Temp.: 26.5±2.2 DO: 8.6±3.3 pH: 8.3±0.3 Turbidity: 39.9±18.4 Salinity: 24.4±3.0	7552 kg (64.550.3 kg/day)
13A	30/8/91-20/12/91 (109 days)	Rainfall: 1.5±5.9 Temp.:26.3±4.8 Cloud cover:35.7±29.6 Wind speed:5.4±4.7	Temp.: 26.4±2.5 DO:6.6±1.2 pH: 8.2±0.3 Turbidity: 62.8±22.6 Salinity: 35.2±3.0	4381 kg (40.226.0 kg/day)
13B	30/1/92-30/5/92 (117 days)	Rainfall: 9.7±21.9 Temp.:24.4±2.5 Cloud cover: 59.3±34.0 Wind speed: 9.5±7.2	Temp.: 26.3±2.2 DO: 8.7±3.2 pH: 8.3±0.3 Turbidity: 41.7±22.0 Salinity: 24.4±2.9	4579.5 kg (39.135.1 kg/day)

Appendix 6.3. Continued

Pond No.	Culture period	Weather quality (mean±SD)	Water quality (mean±SD)	Total running feed (mean±SD)
14	13/11/91-12/5/92 (176 days)	Rainfall: 7.7±19.0 Temp.:25.9±2.9 Cloud cover:55.5±34.3 Wind speed: 7.4±6.3	Temp.: 28.2±2.4 DO: 8.1±3.6 pH: 7.8±0.5 Turbidity: 58.9±25.3 Salinity: 32.8±6.8	12,690 kg (72.160.5 kg/day)
15A	30/8/91-10/10/91 (38 days)	Rainfall: 0.1±0.7 Temp.:22.4±2.0 Cloud cover: 20.4±2.0 Wind speed: 20.4±18.5	Temp.: 4.7±4.9 DO:6.8±1.3 pH:8.3±0.2 Turbidity:41.1±13.0 Salinity: 32.4±5.3	1315 kg (34.620.3 kg/day)
15B	15/12/91-30/5/92 (162 days)	Rainfall: 9.2±21.4 Temp.:25.3±3.0 Cloud cover: 58.4±34.1 Wind speed: 8.6±6.9	Temp.: 27.9±2.5 DO: 9.6±3.7 pH: 7.4±0.4 Turbidity: 46.6±24.4 Salinity: 26.8±4.9	6689 kg (41.8±27.9 kg/day)

Appendix 6.4. Pearson's correlation between water quality parameters. P.c.c. = Pearson's correlation coefficient.

Parameters		**PMTEMP**	**DAILY-TEMP**	**AMDO**	**PMDO**	**NIGHTDO**	**Daily DO**
AMTEMP (Morning temperature)	P.c.c.	0.89	0.96	-0.24	-0.18	-0.40	-0.36
	Probability	1.43E-22	6.82E-23	2.23E-15	2.8E-07	5.8E-22	1.34E-22
	N	1068	1635	1051	824	616	1104
PMTEMP (Afeternoon temperature)	P.c.c.		0.97	-0.32	-0.13	-0.32	-0.31
	Probability		1.28E-22	1.18E-21	1.43E-04	1.27E-16	3.5E-22
	N		1127	859	844	625	917
DAILY-TEMP (Daily temperature)	P.c.c.			-0.28	-0.16	-0.35	-0.32
	Probability			3.83E-20	3.42E-06	1.04E-19	1.25E-22
	N			1061	857	650	1141
AMDO (Morning DO)	P.c.c.				0.24	0.25	0.66
	Probability				1.18E-11	5.45E-10	1.41E-22
	N				766	591	1076
PMDO (Afternoon DO)	P.c.c.					0.36	0.77
	Probability					1.09E-19	2.42E-22
	N					590	847
NIGHTDO (Midnight DO)	P.c.c.						0.71
	Probability						4.75E-22
	N						659

Parameters		**AMPH (Morning pH)**	**PMPH (Noon pH)**	**Daily pH**	**Turbidity**	**Salinity**
AM-TEMP	P.c.c.	0.13	0.28	0.17	0.35	0.19
	Probability	2.57E-07	2.68E-20	1.75E-10	2.53E-22	3.85E-08
	N	1530	1025	1445	832	859
PM-TEMP	P.c.c.	0.21	0.29	0.25	0.34	0.22
	Probability	7.2E-12	1.31E-21	4.31E-17	1E-20	1.36E-09
	N	1030	1030	1087	701	732
DAILY-TEMP	P.c.c.	0.14	0.30	0.19	0.41	0.28
	Probability	3.49E-08	1.5E-22	1.13E-13	2.46E-22	1.15E-17
	N	1541	1045	1484	841	876
AMDO	P.c.c.	-0.06	-0.12	-0.08	-0.05	-0.14
	Probability	5.34E-02	3.90E-04	9.02E-03	0.21	1.37E-04
	N	1030	823	1045	625	699
PMDO	P.c.c.	0.17	0.26	0.20	-0.21	0.03
	Probability	1.33E-06	9.79E-14	1.46E-08	3.89E-07	0.48
	N	780	795	829	560	630
NIGHT DO	P.c.c.	0.03	0.10	0.05	-0.21	-0.07
	Probability	0.46	1.38E-02	0.18	1.04E-05	0.13
	N	585	587	630	423	489
Daily DO	P.c.c.	0.04	0.08	0.07	-0.18	-0.10
	Probability	1.70E-01	1.27E-02	2.74E-02	3.29E-06	6.31E-03
	N	1078	863	1114	648	719

Appendix 6.4. Continued.

Parameters		PMPH	Daily pH	Turbidity	Salinity
AMPH (Morning pH)	P.c.c.	0.60	0.93	0.01	-0.02
	Probability	1.7E-22	8.06E-23	0.74	0.57
	N	985	1463	789	820
PMPH (Afternoon pH)	P.c.c.		0.83	-0.11	0.07
	Probability		1.49E-22	3.84E-03	0.07
	N		1046	652	676
Daily pH	P.c.c.			-0.02	-0.03
	Probability			0.53	0.46
	N			780	815
Turbidity	P.c.c.				0.35
	Probability				5.82E-22
	N				728

Appendix 6. 5. The significant Pearson's correlation between the food amount given and water quality parameters in each pond during the high feeding phase.

Pond #	Parameter	Group	Coefficient	p	N
1	DO	Daily mean	- 0.24	0.04	53
	Salinity	N/a	- 0.33	0.02	40
3	Temperature	Morning	- 0.24	0.03	63
		Daily mean	- 0.30	8.5E-03	65
	DO	Morning	0.22	0.04	62
	pH	Morning	- 0.40	4.0E-04	66
		Mean daily	- 0.23	2.9E-03	66
	Salinity	N/a	- 0.48	2.4E-04	50
4	Temperature	Morning	0.29	1.8E-04	144
		Afternoon	0.35	2.5E-04	93
		Mean daily	0.14	0.04	93
	DO	Afternoon	0.54	2.0E-06	64
		Midnight	0.55	2.2E-04	37
	Salinity	N/a	- 0.75	3.2E-11	51
5	Temperature	Morning	0.43	4.6E-06	101
		Afternoon	0.51	4.4E-08	99
		Mean daily	0.46	4.0E-07	104
	DO	Morning	- 0.43	3.2E-06	101
		Midnight	- 0.20	0.04	80
		Mean daily	- 0.27	2.3E-03	105
	Turbidity	N/a	0.27	9.0E-03	74
	Salinity	N/a	0.56	4.0E-08	80
6	Temperature	Afternoon	0.14	0.04	33
	DO	Morning	- 0.30	0.04	34
	pH	Morning	0.34	0.03	34

Appendix 6.5. Continued.

Pond #	Parameter	Group	Coefficient	p	N
7	Temperature	Morning	0.46	3.3E-06	90
		Afternoon	0.45	4.2E-06	91
		Mean daily	0.44	4.5E-06	95
	DO	Morning	- 0.44	6.9E-06	90
		Mean daily	- 0.24	8.8E-03	96
	Salinity	N/a	0.56	3.0E-07	70
8	Temperature	Afternoon	- 0.28	0.04	39
	DO	Mean daily	- 0.18	0.03	99
	pH	Afternoon	- 0.46	2.8E-03	35
	Turbidity	N/a	- 0.37	0.02	33
10A	Temperature	Morning	- 0.33	0.03	36
	DO	Morning	- 0.75	2.2E-05	23
		Mean daily	- 0.70	1.1E-04	23
	pH	Morning	0.30	0.04	36
10B	DO	Midnight	- 0.69	3.0E-03	14
	pH	Morning	0.27	0.05	40
11A	Temperature	Morning	0.60	1.3E-06	53
		Afternoon	0.52	2.5E-05	55
		Mean daily	0.56	2.9E-06	57
	DO	Morning	- 0.49	1.3E-04	52
		Midnight	- 0.28	0.03	44
		Mean daily	- 0.34	4.1E-03	58
	pH	Morning	- 0.42	8.1E-04	53
		Afternoon	- 0.54	1.6E-05	52
		Mean daily	- 0.54	7.3E-06	56

Appendix 6.5. Continued.

Pond #	Parameter	Group	Coefficient	p	N
12A	Temperature	Morning	0.50	9.2E-08	97
		Afternoon	0.62	4.2E-12	96
		Mean daily	0.56	5.1E-10	102
	DO	Morning	- 0.47	8.3E-07	96
		Afternoon	- 0.21	0.02	89
		Mean daily	- 0.36	1.2E-04	102
	pH	Morning	0.22	0.02	93
		Afternoon	0.19	0.03	94
		Mean daily	0.25	5.4E-03	101
	Turbidity	N/a	0.36	9.4E-04	72
	Salinity	N/a	0.54	2.6E-07	76
12B	Temperature	Morning	- 0.66	4.9E-10	67
		Afternoon	- 0.61	2.9E-02	60
		Mean daily	- 0.56	2.4E-07	69
	DO	Mean daily	0.26	2.8E-02	54
	pH	Morning	- 0.42	2.5E-04	65
		Mean daily	- 0.48	4.2E-04	66
	Turbidity	N/a	- 0.39	1.2E-02	33
13A	Temperature	Morning	0.66	4.3E-12	82
		Afternoon	0.58	1.3E-08	78
		Mean daily	0.65	6.0E-12	85
	DO	Morning	- 0.37	3.3E-04	81
		Afternoon	- 0.28	1.8E-02	56
		Mean daily	- 0.34	7.6E-04	84
	pH	Afternoon	- 0.31	3.7E-03	74
	Salinity	N/a	0.21	5.6E-03	61

Appendix 6.5. Continued.

Pond #	Parameter	Group	Coefficient	p	N
13B	Temperature	Morning	- 0.66	6.4E-09	58
		Afternoon	- 0.53	5.7E-02	10
		Mean daily	- 0.50	1.7E-05	61
	pH	Morning	0.31	8.2E-03	58
		Afternoon	0.78	1.2E-02	8
		Mean daily	0.48	5.5E-05	59
	Turbidity	N/a	- 0.54	2.6E-03	25
14	Temperature	Morning	- 0.28	2.1E-03	102
		Afternoon	- 0.45	1.7E-03	41
		Mean daily	- 0.39	2.1E-05	105
	DO	Morning	- 0.22	1.9E-02	93
		Mean daily	- 0.28	2.5E-03	98
	pH	Morning	- 0.19	2.7E-02	109
		Afternoon	0.51	6.0E-04	38
		Mean daily	- 0.18	3.0E-02	110
	Turbidity	N/a	- 0.57	7.4E-05	39
	Salinity	N/a	- 0.60	2.0E-02	12
15A	Temperature	Morning	0.26	8.2E-02	31
		Afternoon	0.30	6.0E-02	29
		Mean daily	0.36	2.4E-02	31
	DO	Night	- 0.40	1.5E-02	30
	Salinity	N/a	- 0.45	1.5E-02	23
15B	Temperature	Morning	- 0.58	3.5E-12	115
		Afternoon	- 0.52	2.9E-04	41
		Mean daily	- 0.62	3.0E-14	117
	DO	Morning	0.13	9.7E-02	97
		Mean daily	0.14	8.8E-02	100
	pH	Morning	- 0.15	4.8E-02	119
		Mean daily	- 0.15	4.7E-02	119
	Turbidity	N/a	- 0.58	3.6E-05	41
	Salinity	N/a	- 0.49	2.9E-03	30

APPENDIX 6.6. Biological parameters from 29 crops. **Stock'g date** = stocking date; **Sampl'g date** = sampling date; **STOCK** = total number of animals; **DEN**= stocking density (number of prawns/m2); **AGE** = age of the prawns (days); **SR** = estimated survival rate (%); **ABW** = average body weight (g); **IGR** = individual growth rate (g/week); **PWG** = pond weight gain (kg); **SC** = standing crop (kg); **FLW** = amount of feed in the last week (kg); **BWF** = body weight fed (%); **TRF** = total running feed (kg); **FCR** = estimated running food conversion ratio; **BIO** = biomass (kg/ha).

No.	Stock'g Date	Sampl'g Date	STOCK	DEN	AGE	SR	ABW	IGR	PWG	SC	FLW	BWF	TRF	FCR	BIO
1	6/6/91	20/11/91	430000	16	167	85	13.17	1.72	629	4800	643	1.90	6,268	1.31	1778
2	8/6/91	20/11/91	440000	16	165	138	9.50	0.58	354	5800	678	1.70	6,354	1.10	2148
3	15/6/91	20/11/91	660000	24	158	79	10.12	1.79	933	5300	597	1.60	6,201	1.17	1963
4	25/6/91	20/11/91	560000	21	148	49	14.21	0.50	137	3900	577	2.14	5,374	1.38	1444
5	9/8/91	20/11/91	650000	24	103	20	14.97	1.26	164	1900	216	1.60	1,255	1.66	704
6	22/8/91	20/11/91	580000	21	90	75	9.23	1.70	740	4000	395	1.40	2,236	1.56	1481
7	11/8/91	20/11/91	366500	14	70	70	4.48	2.05	526	1150	122	1.50	445	1.39	426
8	25/9/91	20/11/91	479000	18	56	50	3.91	2.29	548	940	37	0.60	108	1.11	348
9	1/11/91	20/11/91	500000	19	19	70	0.07	0.03	11	20	5	3.40	8	1.40	7
10	15/11/91	20/11/91	250000	9	5	75	0.02			4					
11	15/11/91	29/4/92	438000	15	166	50	23.87	3.57	782	5228	760	2.10	12496	1.72	1803
12	16/1191	20/11/91	600000	22	4	73	0.02			10					4
13	16/11/91	29/4/92	350000	12	165	100	17.89	1.77	620	6260	355	0.80	13142	2.10	2159
14	26/11/91	29/4/92	495000	17	155	70	8.82	0.83	288	3056	570	2.70	6524	2.13	1054
15	11/12/91	29/4/92	540000	19	140	80	8.59	0.51	220	3711	705	2.70	6630	1.79	1280
16	14/12/91	29/4/92	421000	15	137	85	12.36	3.48	1,245	4423	550	1.80	4964	1.12	1525
17	21/12/91	29/4/92	460000	16	130	60	8.76	1.72	475	2418	315	1.90	6011	2.49	834
18	21/12/91	29/4/92	424000	15	130	55	12.73	3.64	849	2969	380	1.80	4299	1.45	1024
19	28/12/91	29/4/92	608100	21	123	90	7.42	1.90	1,040	4061	585	2.10	4949	1.22	1400
20	10/1/92	20/11/91											12711		
21	26/1/92	20/11/91											28239		
22	31/1/92	29/4/92	580000	20	89	37	8.03	2.17	466	1723	433	3.60	1658	0.96	594
23	1/2/92	29/4/92	555000	19	88	56	6.23	0.97	301	1936	785	5.80	3790	1.96	668
24	28/2/92	29/4/92	557000	19	61	60	2.32	0.70	234	775	297	5.50	1201	1.55	267
25	7/3/92	29/4/92	450000	16	53	60	1.63	0.61	165	440	274	8.90	753	1.71	152
26	21/3/92	29/4/92	730000	25	39	60	0.78	0.41	180	342	45	1.90	156	0.46	118
27	28/3/92	29/4/92	360000	12	32	50	0.46	0.28	50	80	20	3.60	38	0.48	28
28	11/4/92	29/4/92	598500	21	18	50	0.15	0.10	30	45	21	6.70	46	1.02	16
29	18/4/92	29/4/92	520000	8	11	50	0.02			4					1

Appendix 6.7. Diseases found in the prawn samples from the archival slides from the histological batch encoded TVS 92-143 (Microbiology and Immunology, JCU, Australia)

Pathogen*	Slide #	Histological Diagnosis
Hepatopan-creatic bacilliform virus	7A	Hepatopancreas (HP): grade 1 severity, only 1 tubule contains cells with all stages of the infection.
	9B	HP: grade 1 severity, 2 cells in 1 tubule with fused viruses
	9D	HP: grade 1 severity, 4 tubules in dorso-posterior region containing bacilliform virus-infected cells.
Possibly Gill-associated Virus (GAV)	5D	Lymphoid organ (LO): extensive eosinophilic spheroids.
	19A	LO: extensive eosinophilic spheroids.
Possibly Hepatopan-creatic Parvo-like Virus (HPV)	9C	Anterior midgut (AMG): some epithelial cells contain prominent single spherical eosinophilic occlusion bodies in the hypertrophied nuclei with a ring of marginated chromatin around the periphery.
	9D	Anterior midgut ceca (AMC): 6 occlusion bodies in the epithelium.
	19A	AMG: possibly HPV in the epithelium.
Possibly Lymhpoidal Parvo-like Virus (LPV) and Lymphoid Organ Vacuoliza-tion Virus (LOVV)	7B	LO: massive prominent vacuolated spheroids
	7E	LO: massive prominent vacuolated spheroids
	12A	LO: massive prominent vacuolated spheroids
	19F	LO: massive prominent vacuolated spheroids
Monodon Baculovirus (MBV)	9B	HP: stage 1 MBV infection indicated by a newly developing spherical eosinophilic occlusion body in the hypertrophied nucleus of an F-cell.
Unidentified viruses	7B	LO: some non-occluded viruses in the stromal matrix of cells and spheroids.
	15C	LO: occlusion bodies inside the spheroids.
	19B	LO: few occlusion bodies inside the spheroids.
	19D	LO: occlusion bodies inside the spheroids.

Appendix 6.7. Continued.

Pathogen*	Slide #	Histological Diagnosis
Suspected bacterial infection	3B	Heart: vocalized hemocytic encapsulation in the heart. HP: massive oedema and heavy necrosis of HP tubules. AMG: suspected bacterial infection in the epithelium. Muscle: few vocalized hemocytic encapsulation.
	3C	Heart: small vocalized hemocytic encapsulation. HP: massive oedema.
	3D	HP: massive injury conataining granulized necrotic tubules.
	5A	Muscle: few localized hemocytic encapsulation.
	7B	Muscle: few lesions in the dorsal region of the body.
	7C	Muscle: some lesions swarmed by hemocytes.
	9C	HP: massive oedema in area with degraded tubules.
	15C	LO: heavy necrosis. HP: hemocytic encapsulation of an extensive injury.
	19B	Heart: a vocalized hemocytic encapsulation.
	19C	Heart: massive hemocytic accumulation in the heart.
	19D	HP: massive oedema, necrotic tubules encapsulated by hemocytes, possible septicemia.
	19E	HP: massive oedema and necrotic tubules. Muscle: small wounds encapsulated by hemocytes.
	19G	LO: extensive hemocytic encapsulation of tubules. Heart: the ventro-posterior end slightly inflamed. HP: massive septicemia, oedema, and an extensive lesion encapsulated by hemocytes. AMG: heavy hemocytic swarming in the basal cells.
Non-infectious agents	3A	Heart: vocalized hemocytic encapsulation of algae and fine particles in the myocardial cells, and massive oedema of the epicardium.
	5B	Muscle: few HP tubules infiltrated the muscle.
	5D	Heart: fine particles encapsulated by hemocytes in the epicardium.
	9A	Heart: an algal remnant encapsulated by hemocytes.
	15C	HP: hemocytic encapsulation of a chunk of muscle inside the HP.

Appendix 7. Results from PCR assay for MBV on *P. monodon* and *P. merguiensis.* B&Y = Belcher and Young (1998). Hsu = Hsu *et al*. (2000). *P. mono* = *P. monodon*. *P. merg* = *P. merguiensis*. Neg.(-) = negative. Pos.(+) = positive.

Date of Assay	Prawn Species	MBV Status	Sample Code	Necropsy Number	Primer	PCR Result	Comments
23/06/00	*P. mono.*	Heavy	None	99-125J	B&Y	Neg.(-)	All dNTPs were separated from the polymerisa-tion buffer.
	P. mono.	Heavy	None	99-125P	B&Y	Neg.(-)	
29/06/00	*P. mono.*	Heavy	None	99-125J	B&Y	Neg.(-)	
	P. mono.	Heavy	None	99-125P	B&Y	Neg.(-)	
03/07/00	*P. merg.*	None	A4	None	B&Y	Neg.(-)	
	P. mono.	Heavy	A5/99-154	00-28K	B&Y	Neg.(-)	
23/01/01	*P. merg.*	Suspect	A3A5	00-28D	Hsu	Neg.(-)	Biotech™ Definitive TAQPAQ PCR kit
	P. merg.	Suspect	C30/10/6/99	00-28J	Hsu	Neg.(-)	
	P. mono.	Heavy	A5/ 99-54	00-28K	Hsu	Neg.(-)	
	P. mono.	None	A10-116/98	None	Hsu	Neg.(-)	
09/02/01	*P. mono.*	Heavy	A5/ 99-154	00-28K	B&Y	Neg.(-)	Eye stalks of the prawns were cut off.
	same	Same	Same	Same	Hsu	Neg.(-)	
	P. merg.	None	C10/00-020	None	B&Y	Neg.(-)	
	same	Same	Same	Same	Hsu	Neg.(-)	
	P. merg.	None	C11/00-021	None	B&Y	Neg.(-)	
	Same	Same	Same	Same	Hsu	Neg.(-)	
14/02/01	*P. mono.*	Heavy	A5/99-154	00-28K	B&Y	Neg.(-)	5 bands with Hsu primers
	Same	Same	Same	Same	Hsu	Pos.(+)	
19/02/01	*P. mono.*	Heavy	A5/99-154	00-28K	**B&Y**	Neg.(-)	Repeat above assay
	same	Same	Same	Same	Hsu	Pos.(+)	
02/03/01	*P. mono.*	Heavy	A5/99-154	00-28K	**Hsu**	Neg.(-)	Repeat above assay
	P. merg.	None	A1/00-042	None	Hsu	Neg.(-)	
05/03/01	*P. mono.*	Heavy	A5/99-154	00-28K	Hsu	Neg.(-)	Same templates with varied PCR condition
	P. merg.	None	A1/00-042	None	Hsu	Neg.(-)	
09/03/01	*P. mono.*	None	None	99-125J	Hsu	Neg.(-)	Same as above
	P. mono.	None	None	99-125P	Hsu	Neg.(-)	

Appendix 7. Continued.

Date of Assay	Prawn Species	MBV Status	Sample Code	Necropsy Number	Primer	PCR Result	Comments
29/03/01	*P. merg.*	None	A13/01-050	None	Hsu	Neg.(-)	Prawn DNA too high; not enough MBV DNA template
	P. merg.	None	A14/00-051	None	Hsu	Neg.(-)	
01/05/01	*P. merg.*	None	A13/01-050	None	Hsu	Neg.(-)	Use JumpStart Taq DNA polymerase; PCR cycle as Young & Belcher (1998)
	P. merg.	None	C12/01-145	None	Hsu	Neg.(-)	
	P. merg.	None	C21/01-053	None	Hsu	Neg.(-)	
04/05/01	*P. merg.*	None	A13/01-050	None	Hsu	Neg.(-)	C12 template was purified using spin column.
	P. merg.	None	C12/01-145	None	Hsu	Neg.(-)	
	P. merg.	None	Purified C12	None	Hsu	Neg.(-)	
09/05/01	*P. merg.*	None	C12/01-145	None	Hsu	Neg.(-)	Use ordinary Taq DNA polymerase
17/05/01	*P. merg.*	None	C12/01-145	None	Hsu	Pos.(+)	JumpStart Polymerase & Biotech™ Definitive TAQPAQ PCR kit
	P. merg.	None	Purified above product	None	Hsu	Pos.(+)	
	P. mono.	Heavy	None	99-125J	Hsu	Pos.(+)	