# THE LYMPHOID ORGAN IN PENAEIDS AND ITS INTERACTION WITH MOULTING

Thesis submitted by

**R U S A I N I**

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**School of Veterinary and Biomedical Sciences**
**James Cook University**
**Townsville, northern Queensland**
**Australia**

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# DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outline in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A956).

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# DEDICATION

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

*Glory to Allah, who created in pairs all things that the earth produces, as well as their own (human) kind and (other) things of which they have no knowledge.*
*And a sign for them is the night: We withdraw there from the day, and behold they are plunged in darkness.*
*And the sun runs its course for a period determined, for it that is the decree of (Him), the exalted in might, the all knowing.*
*And the moon,, We have measured for it, mansions (to traverse) till it returns like the old (and withered) lower part of a date stalk.*
*It is not permitted to the sun to catch up the moon, nor can the night outstrip the day, each (just) swims along in (its own) orbit (according to law). (The Holy Qur'an, 36: 36 – 40).*

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*If there is*
*any goodness in this work,*
*I would dedicate it to*
*my beloved Mum and Dad,*
*and my lovely brother and sisters.*

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# ABSTRACT

Prawn immunity is still a mysterious puzzle in immunology. However, this knowledge is important in culture management in order to avoid the devastating impact of infectious pathogens and economic losses. Furthermore, since the effectiveness of vaccination and immunostimulants is unclear, the enhancement of immune capability of prawns might provide a bright light to this industry. Therefore, the objective of this thesis was to develop a simple modified method of quantifying the histopathological changes of a component of the lymphoid organ (LO), the spheroid cells, and apply this technique in a study of the influence of moult cycle, lunar rhythm, and viral infections on spheroid cells quantification.

Moult cycles of *P. monodon* were studied by using setal development (setogenesis) and retraction of epidermis from the setal bases (apolysis) in the inner uropod adjacent to the telson tip. Five stages and four substages of the moult i.e. postmoult (stage A and B), intermoult (stage C), and premoult (stage D0, D1, D2 and D3/D4) and ecdysis (stage E) could be determined by applying these two criteria. However, unsynchronised development of these two criteria in abnormal prawns led to the difficulties in differentiating between stage B, C and D.

A modified transect technique seemed to offer a simple, rapid, and accurate method in analysing the abundance of spheroid cells in the lymphoid organ. Furthermore, one half longitudinal section of the cephalothorax represented the abundance of the spheroid cells in the lymphoid organ of penaeid prawns. Based on this technique, the fluctuation of the lymphoid organ spheroid (LOS) cells during the life of *P.monodon* was investigated. It was found that animal size (weight and total length) had no significant effect ($P > 0.05$; ANOVA) on the spheroid to total tissue (STT) ratio, the prevalence of vacuolated spheroids and the number of vacuoles in the spheroid cells.

Unfortunately, the cyclic phenomena of the prawn's, moult cycle also showed no significant effect on any measure of spheroid cells ($P > 0.05$) rejecting the original hypothesis of this work. The effect of the prawn's sex was variably related to the spheroid cells during the experiments. In the first two trials (Chapter 5 and 6), evidently sex had no significant effect on any measure of spheroid cells ($P > 0.05$).

However, in the last experiment (Chapter 7) female bias on the ratio of STT was obvious ($P < 0.05$).

Lunar related patterns on the spheroid to total tissue ratio were evident during the experiments. It was found that the STT ratio was significantly lower at new moon than first quarter and full moons ($P < 0.05$). This indicated that increased activity of the prawns during the dark moon enhanced immunocompetence of the prawns to eliminate viral diseases. Moreover, apparently, the STT ratio of GAV-injected prawns was significantly higher than control prawns ($P < 0.05$). Together with this, the presence of distinct bacterial granulomas in the lymphoid organ implied that the formation of the spheroid cells in the haemal sinuses of the lymphoid organ was only associated with viral diseases not with bacterial infections.

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| | |
|---|---|
| AMP | Anti-microbial peptide |
| BGBP | Beta-glucan binding protein |
| CL | Carapace length |
| CP | Clotting protein |
| CPUE | Catch per unit of effort |
| DHC | Differentiated haemocyte count |
| DNA | Deoxyribonucleic acid |
| dpi | Day post-injection |
| EST | Expressed sequence tag |
| GAV | Gill associated virus |
| HDL | High density lipoprotein |
| HH | Hyaline haemocyte |
| HLF | Haemocyte lysate fraction |
| HLS | Haemocyte lysate supernatant |
| hpi | Hour post-injection |
| HPLC | High performance liquid chromatography |
| HPT | Haematopoietic tissue |
| HST | Head soft tissue |
| ICC | Immunocytochemistry |
| ISH | *In situ* hybridization |
| KBr | Potassium bromide |
| kDa | kiloDalton |
| LGBP | Lipopolysaccharide and β-1,3-glucan-binding protein |
| LGH | Large granular haemocyte |
| LM | Light microscope |
| LO | Lymphoid organ |
| LOS | Lymphoid organ spheroid |
| LOV | Lymphoid organ virus |
| LOVV | Lymphoid organ vacuolization virus |
| LPS | Lipopolysaccharides |
| LPV | Lymphoidal parvovirus |

| | |
|---|---|
| LSD | Least significant difference |
| LSNV | Laem-Singh virus |
| MCMS | Midcrop mortality syndrome |
| ME | Mercaptoethanol |
| MSGS | Monodon slow growth syndrome |
| PG | Peptidoglycan |
| pH | Puissance d'hydrogene |
| pI | Isoelectric point |
| PNR | Peripheral neuropathy and retinopathy |
| PO | Phenoloxidase |
| ppA | Prophenoloxidase activating enzyme |
| proPO | Prophenoloxidase |
| proppA | Pro-form of prophenoloxidase activating enzyme |
| RBC | Red blood cell |
| rER | Rough endoplasmic reticulum |
| RNA | Ribonucleic acid |
| ROI | Reactive oxygen intermediate |
| RV-PJ | Rod-shaped nuclear virus of *Penaeus japonicus* |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SGH | Small granular haemocyte |
| SPF | Specific pathogen free |
| $TCID_{50}$ | 50% tissue culture infective dose |
| TEM | Transmission electron microscopy |
| TGase | Transglutaminase |
| THC | Total haemocyte count |
| TMMS | Three main moult stages |
| TSV | Taura syndrome virus |
| TUNEL | Terminal deoxynucleotidyl transferase (TdT) – mediated dUTP Nick-End Labelling |
| VHDL | Very high density lipoprotein |
| VTG | Vitellologenin |
| WSBV | White spot associated baculovirus |
| WSSV | White spot syndrome virus |
| YHV | Yellow head virus |

# CHAPTER 1
# GENERAL INTRODUCTION

## 1.1. Overview

The devastating impact of viral and bacterial diseases in prawn culture has led to many investigations over many years into the control and elimination of diseases. In early work, the application of therapeutic agents including antibiotics resulted in high cost of production, contamination of final products and harmful impacts on the environment. Antibiotics also caused mortality and abnormalities in prawns. In addition, antibiotic resistant pathogens resulted from long periods of application. Finally, this practice was banned in many countries (Smith *et al.*, 2003). Improvements in the immunocompetence in the stock became an important step in the prawn industry to help reduce the use of therapeutic agents. However, production of genetically modified animals still has a long way go to overcome the effects of harmful pathogens (Bachere, 2003; Smith *et al.*, 2003). Recently, vaccination or application of immunostimulants has become an alternative method to sustain prawn production. Some studies revealed the success of these methods in enhancing the crustacean immunity against pathogens, but the lack of statistical analysis and the temporary nature of the stimulation did not support the claims (Smith *et al.*, 2003).

There are two types of immunity to deal with foreign materials, innate (natural immunity) and adaptive (acquired) immunity (Lee & Soderhall, 2002). Like other invertebrates, crustaceans do not have antigen-specific lymphocyte and do not produce immunoglobulin (acquired immunity) (Roch, 1999). Their small body size does not allow them to have the large number of cells required for the operation of the anticipatory immune system (Klein, 1997). Their defence mechanisms depends on the innate immunity consisting of cellular and humoral components that can recognise and eliminate foreign particles (Bachere, 2003). Thus, the probability of vaccination has been debated by those who proposed the absence of adaptive immunity in these animals (Arala-Chaves & Sequeira, 2000).

In early work, many aspects of crustacean immunity were studied in crayfish, *Pacifastacus leniusculus*, some marine decapods such as lobster (*Homarus vulgaris*) and crab (*Carcinus maenas*). Recently, some information has emerged on the penaeid species including the ridgeback prawn (*Sicyonia ingentis*), the kuruma prawn (*Penaeus japonicus*), the brown prawn (*P. californiensis*) and sergestid prawns (Bachere *et al.*, 1995; Smith *et al.*, 2003). Interestingly, it seemed that each species had different cell types for immune reactivity (Smith *et al.*, 2003). Without doubt, haemocytes were the primary cells involved in the defence mechanisms of prawns (Soderhall & Cerenius, 1992; Rodriguez & Le Moullac, 2000).

In penaeid prawns, haemocytes were involved in the clotting system (Hose *et al.*, 1990; Yeh *et al.*, 1999), phagocytosis (Lee *et al.*, 2001), and took an important part in the production of microbicidal proteins such as prophenoloxidase (Sung *et al.*, 1998), penaeidins (Destoumieux *et al.*, 1997), and lectins/agglutinins (Fragkiadakis & Stratakis, 1995). Most of these immunological reactive factors were stored in an inactive form and became active when stimulated by the presence of foreign material (Smith *et al.*, 2003). Whether haemocytes originate from one or several tissues, is still open to question. Most researchers believe that circulating haemocytes of crustaceans do not divide, therefore old cells should be replaced by new cells released into the haemolymph (Soderhall & Cerenius, 1992). Some researchers claimed that haemocytes originated from haematopoietic tissue. This tissue is composed of densely packed lobules located anterior to the stomach and posterior to the brain, surrounding the lateral arterial vessels and the proximal maxillipedes near the epigastric region (Bell & Lightner, 1988; Van de Braak *et al.*, 2002b; Bachere *et al.*, 2004).

Another organ that it is believed to have an important role in immunodefence against foreign material in penaeid prawns is the lymphoid organ (LO). This organ is located between the hepatopancreas and stomach (Oka, 1969; Bell & Lightner, 1988). The lymphoid organ has roles in trapping foreign substances, elimination or degradation of pathogens and phagocytosis of invaders (Kondo *et al.*, 1994; Martin *et al.*, 1996; Anggraeni & Owens, 2000; Van de Braak *et al.*, 2002c). It was proposed that this organ might be the major defence mechanism against viral diseases (Anggraeni & Owens, 2000).

There are some viral diseases of penaeids that cause changes in the lymphoid organ. These include lymphoidal parvovirus (LPV) in *P. monodon, P. merguiensis,* and *P. esculentus* (Owens *et al.*, 1991), lymphoid organ vacuolization virus (LOVV) in cultured *P. vannamei* (Bonami *et al.*, 1992), lymphoid organ virus (LOV) later called gill associated virus (GAV) in broodstock and cultured adult *P. monodon* from four farms in Queensland, Australia (Spann *et al.*, 1995; Spann *et al.*, 1997), Taura syndrome virus (TSV) in *P. vannamei* (Hasson *et al.*, 1995), bacilliform virus infection in cultured Chinese prawn, *P. orientalis* in China (Guoxing *et al.*, 1997), an infectious viral diseases in *P. orientalis* in Korea (Park *et al.*, 1998). The lymphoidal changes were also found when yellow head virus (YHV) was injected into *P. vannamei* in Hawaii (Lu *et al.*, 1995) and white spot syndrome associated bacilliform virus (WSBV) was injected into *P. monodon* (Chang *et al.*, 1996).

Generally, changes associated with these viruses include spheroid cell production as a result of LO changes at the cellular level. The spheroids displayed hypertrophy of nuclei, margination of chromatin and more basophilic cytoplasm (Owens *et al.*, 1991; Bonami *et al.*, 1992; Hasson *et al.*, 1999b; Van de Braak *et al.*, 2002c). Both in the spheroids (Owens *et al.*, 1991; Van de Braak *et al.*, 2002c) and the matrix (Anggraeni & Owens, 2000) mitotic activity was never observed. This confirmed that the lymphoid organ is not a haematopoietic tissue; contrary to the descriptions by Martin *et al.* (1987) and Hose *et al*. (1992).

Most studies of the spheroid cells were carried out by using routine histological, cytological and histochemical techniques (Owens *et al.*, 1991; Hasson *et al.*, 1999b; Anggraeni & Owens, 2000; Van de Braak *et al.*, 2002c; Shao *et al.*, 2004). However, quantitative techniques have also been introduced to analysed the spheroid area (Anggraeni and Owens, 2000), the abundance and the size of the spheroid cells (Littik, 2003) in penaeid prawn. Unfortunately, the methods that they used were skilled labour, expensive, time consuming (Anggraeni and Owens, 2000) and resulted in bias (Littik, 2003). Therefore, the need for the development of a simple, rapid and accurate method in assessing this immune reactivity is important in early detection of pathogen infection.

The physiological functions of prawn are affected by moulting and it may also affect the immunological state of the prawns. Some studies were carried out to investigate the association between defence mechanisms and moulting stages in prawns. It seems that haematological variation in penaeid prawns appear during the moult cycle. During the moulting stages, the total haemocyte count (THC) and differentiated haemocyte count (DHC) differed in individuals and between species (Hose *et al.*, 1992; Sequeira *et al.*, 1995; Le Moullac *et al.*, 1997; Cheng & Chen, 2001; Liu *et al.*, 2003). Prophenoloxidase (proPO) (Hose *et al.*, 1987), phenoloxidase (Le Moullac *et al.*, 1997; Liu *et al.*, 2003) and phagocytic activity were affected by this physiological activity. The clearance efficiency of prawns was also influenced by the moult cycle (Liu *et al.*, 2003). Furthermore, the sensitivity of prawns to pathogens was supposed to be related to the stage of the moult (Le Moullac *et al.*, 1997; Liu *et al.*, 2003).

However, there is no study of the association between the lymphoid organ spheroids (LOS) and moulting stages. Anggraeni & Owens (2000) argued that the area of spheroid cells might increase with increasing the animal size, if the spheroid cells were concentrated in the haemal sinus of the LO during the life of a prawn. However, there is no correlation between the area of spheroid cell and the prawn size. Therefore, there must be a mechanism for removing or breaking down the spheroids and possibly moulting (ecdysis). All above, the current investigation is significant to pursue.

### 1.2. Hypothesis

The working hypothesis for this research is that the lymphoid organ spheroid cells are disposed of during ecdysis (Figure 1.1). It is also hypothesised that these special cells are only formed as a result of viral infection.

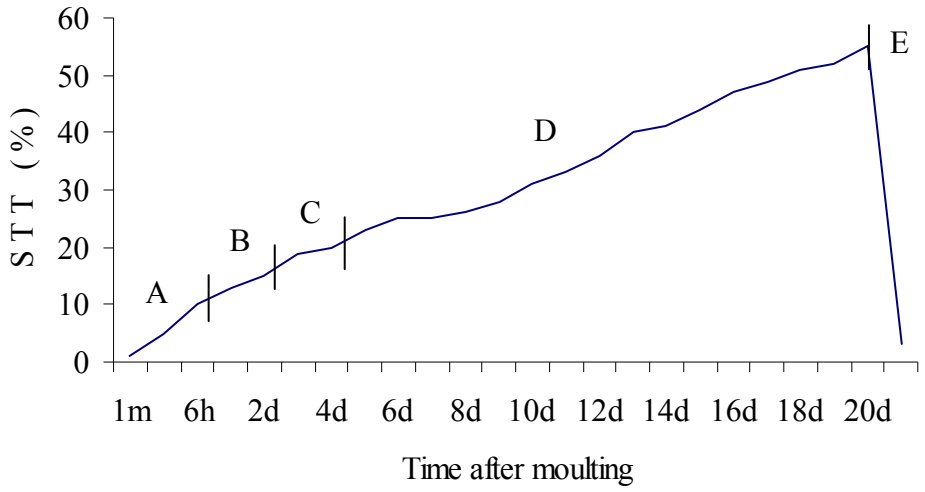



**Figure 1.1**. The hypothetical association between the lymphoid organ spheroid cells and moulting stages in penaeid prawns. The ratio of the spheroids to total tissue (STT) increases with progression through the moulting stages. During ecdysis, the lymphoid organ spheroid (LOS) cells are disposed of.

### 1.3. Research Aims

There are four main objectives in this study:

1. Modify a simple method of quantifying the histological changes in the lymphoid organ.
2. Investigate the relationship between the lymphoid organ spheroid cells and moulting stages in penaeid prawns in controlled aquaria.
3. Study the relationship between the LOS cells and moulting stages in penaeid prawns from commercial farms.
4. Determine the association between the LOS and moult cycle in virally challenged prawns.

This study will validate a simple, rapid, and accurate method of observing histopathological changes (spheroid cells) in the lymphoid organ of penaeids and use this method in investigating the effect of moult cycle, lunar rhythm, and viral infection on the spheroid cells quantification. It is hoped that this method can be used in early detection of pathogen infection, particularly viral infections in cultured penaeid prawns.

# CHAPTER 2
# REVIEW OF LITERATURE

## 2.1. Introduction

Crustaceans inhabit marine or freshwater environments that may also contain an abundance of parasites and pathogens. Therefore, it is crucial for crustaceans to develop an efficient biological self-defence to deal with foreign substances through their immune systems. Two types of immunity against foreign materials have evolved: innate immunity (natural immunity) and adaptive (acquired) immunity (Soderhall & Cerenius, 1992; Lee & Soderhall, 2002). Since crustaceans, like other invertebrates, do not possess adaptive immunity, their defence systems depend on innate immunity. This natural immunity consists of cellular and humoral components that are remarkably effective (Roch, 1999; Bachere, 2003; Loker *et al.*, 2004; Iwanaga & Lee, 2005).

In invertebrates, the major host defence systems consists of the following systems: coagulation; prophenoloxidase (proPO); agglutinin-lectin; antimicrobial peptides mediated by toll-like receptors and peptidoglycan binding protein (PGBP) and phagocytic. These cellular and humoral factors are mainly in haemolymph plasma and haemocytes (Iwanaga & Lee, 2005). In this review, general crustaceans' immunity will be discussed then the role of the lymphoid organ in immunity and finally, the interaction between moulting and immunity. Understanding crustacean immunity may lead to the development of immunointervention strategies to help sustain the aquaculture which is very vulnerable to infectious pathogens.

## 2.2. Immunity

### 2.2.1. Haemocytes

Crustacean defence mechanisms are generally based on activities of the blood cells, the haemocytes (Soderhall & Cerenius, 1992; Rodriguez & Le Moullac, 2000). Haemocytes display various physiological functions. Direct sequestration and killing of infectious agents, synthesis and exocytosis of a battery of bioactive molecules are

the roles of the circulating haemocytes. Basically, these cells perform inflammatory type reactions to remove foreign particles in the crustacean haemocoel by phagocytosis or encapsulating (haemocytes clumping), production of reactive oxygen metabolites, the release of microbiocidal proteins (Soderhall & Cerenius, 1992; Smith *et al.*, 2003) or antibacterial activity (Chisholm & Smith, 1995).

From the haemocytes of *Penaeus monodon,* immune related genes have been identified that consisted of those coding for enzymes and proteins in the clotting system and the prophenoloxidase activating system, antioxidative enzymes, antimicrobial peptides (antilipopolysaccharide and penaeidin homologs), serine proteinase inhibitors and a heat shock protein (Supungul *et al.*, 2002). In normal conditions, most immunoreactive factors such as peroxinectin, antibacterial peptides and clotting components are stored within the haemocytes in an inactive state. They are activated when stimulated by the existence of foreign substances through regulated exocytosis (Smith *et al.*, 2003).

A haemogram for penaeids was proposed to contain the total haemocyte count (THC) and differentiated haemocyte count (DHC) (Rodriguez & Le Moullac, 2000). The haemocyte types in crustaceans can be differentiated by morphological criteria (TEM) and by applying different cytological staining techniques (Soderhall & Cerenius, 1992). For DHC, most authors believed that there were three types of haemocytes in crustaceans (Table 2.1) based on the presence of cytoplasmic granules (Rodriguez & Le Moullac, 2000; Bachere *et al.*, 2004). Unfortunately, no classification of haemocytes in penaeids has been universally accepted, especially when nongranular haemocytes were examined under a light microscope, none of the terms hyaline, nongranular, agranular, nor undifferentiated were anatomically or functionally correct (Owens & O'Neill, 1997). However, the terms large granular haemocytes (LGH), small granular haemocytes (SGH), and hyaline haemocytes (HH) seem to be more in usage and will be used throughout the rest of this thesis.

**Table 2.1**. Differentiation of haemocyte types in crustaceans (adapted from Van de Braak *et al.,* 1996)

| Species | Method | Haemocytes type | Author |
|---|---|---|---|
| **Saltwater crustacean** | | | |
| *Homarus americanus*<br>*Panulirus interuptus*<br>*Loxorhynchus grandis* | LM | • Large granular cells<br>• Small granular cells<br>• Hyaline cells | (Hose *et al.*, 1990) |
| *Carcinus maenas* | Percoll centr. | • Hyaline cell<br>• Semigranular cell<br>• Granular cell | (Soderhall & Smith, 1983) |
| *Penaeus californiensis* | TEM and PC | • Granulocyte<br>• Small granulocyte<br>• Large granulocyte | (Martin & Graves, 1985) |
| *Penaeus monodon* | EM | • Hyaline cell<br>• Semigranular cell<br>• Granular cell | (Van de Braak *et al.*, 1996) |
| *Penaeus monodon* | FC | • Nongranular haemocyte<br>• Small granular haemocyte<br>• Large granular haemocyte | (Owens & O'Neill, 1997) |
| *Penaeus paulensis* | PC | • Hyaline haemocytes<br>• Small granule haemocytes<br>• Large granule haemocytes | (Gargioni & Barracco, 1998) |
| *Penaeus merguiensis* | TEM | • Hyaline cell<br>• Semigranular cell<br>• Granulocyte | (Lee *et al.*, 2001) |
| *Sicyonia ingentis* | TEM and PC | • Agranulocyte<br>• Small granulocyte<br>• Large granulocyte | (Martin & Graves, 1985) |
| **Freshwater crustaceans** | | | |
| *Macrobrachium rosenbergii*<br>*Macrobrachium achanturus* | PC | • Hyaline haemocytes<br>• Small granule haemocytes<br>• Large granule haemocytes | (Gargioni & Barracco, 1998) |
| *Procambarus clarkii* | LM and EM | • Hyaline cell<br>• Semigranular type<br>• Granular type | (Lanz *et al.*, 1993b) |
| *Procambarus zonangulus* | FC and PC | • Hyaline<br>• Semigranular<br>• Granular | (Cardenas *et al.*, 2000) |

LM : light microscope ; PC : phase contrast microscope ; Percoll centr. : centrifugation on Percoll gradients; TEM: transmission electron microscope; EM: electron microscope and FC : flow cytometry

The percentage of different types of haemocytes varied between species. In *P. japonicus*, circulating haemocytes consisted of 10% HH (Soderhall & Cerenius, 1992). The differential count of haemocytes of *S. ingentis* contained approximately 5.2% HH, 74.2% SGH, and 20.6% LGH. In *P. californiensis*, DHC demonstrated 12.1% HH, 78.6% SGH, and 9.3% LGH (Martin & Graves, 1985). In addition, in *Macrobrachium acanthurus* and *M. rosenbergii*, SGH were higher than the others

(Gargioni & Barracco, 1998). However, there was approximately 76.7% HH, 20.4% SGH, and 2.9% LGH in *P. monodon* (Owens & O'Neill, 1997). Furthermore, in *Procambarus clarkii* there was around 75% HH in males and 77% in females, 9% SGH in males and 8% in females, and 16% LGH in males and 15% in females (Lanz *et al.*, 1993b). In *P. paulensis* (Gargioni & Barracco, 1998) and *Procambarus zonangulus* (Cardenas *et al.*, 2000), HH count was also higher than other cell types. Clearly, there is no pattern to the percentage of cell types across crustaceans.

The role of haemocytes in defence in crustaceans appeared to be distinct between different types of haemocytes and species (Table 2.2). For instance, in crab species (Smith & Soderhall, 1983; Bell & Smith, 1993), hyaline cells were the main phagocytic haemocytes. Whereas, in the ridgeback prawn (Martin *et al.*, 1996), phagocytosis was mainly by the small granular haemocytes. SGH and LGH in the palaeomonids *M. rosenbergii* and *M. acanthurus* actively displayed phagocytic activity (Gargioni & Barracco, 1998). However, there has been no study differentiating between agranular cells and exocytosed granular cells that would therefore look nongranular. This might contribute to the different results recorded with DHC.

It has been suggested that the total count of haemocytes in crustaceans is associated with the immune competence and pathogen resistance. *P. monodon* injected with white spot syndrome virus (WSSV) isolated from infected *P. monodon* and then propagated in the healthy crayfish (*Procambarus clarkii*) showed a significant decrease in THC (Van de Braak *et al.*, 2002a). However, this claim is questionable because the data arose from small, possibly insufficient samples (n = 9). *S. ingentis* injected with five different species of bacteria at a concentration of $5 \times 10^6$ cells/ml showed a rapid decline in THC. It seemed that the ridgeback prawn eliminated all three Gram positive bacteria (*Bacillus cereus, B. subtilis*, and *Aerococcus viridans* var. *homari*) from circulation. The concentration of both Gram negative bacteria (*Pseudomonas fluorescens* and *Vibrio alginolyticus*) was rapidly reduced, but not eliminated (Martin *et al.*, 1993). A decrease in THC was also found when the bacteria (*Vibrio anguillarum*) were injected into *P. monodon* at a concentration of $1 - 5 \times 10^7$ cells/g body weight (Van de Braak *et al.*, 2002c). The authors proposed that haemocytes migrated to the injection site and accounted for the reduction in cell

concentration in the haemolymph. However, there was no significant difference compared to the control group, suggesting the effect was not real.

**Table 2.2.** Differentiation of haemocyte types involved in immune reactivity in crustaceans.

| Immuno-reactivities | Species | DHC | | | Pathogens/Parasites | Authors |
|---|---|---|---|---|---|---|
| | | HH | SGH | LGH | | |
| **Clotting system** | *H. americanus* | √ | | | | (Hose *et al.*, 1990) |
| | *P. interruptus* | √ | | | | |
| | *L. grandis* | √ | | | | |
| | *S. ingentis* | √ | | | | (Martin *et al.*, 1991) |
| | *P. clarkii* | √ | | | | |
| | *P. leniusculus* | | √ | √ | | (Wang *et al.*, 2001b) |
| **Prophenoloxidase (ProPO) activating system** | *S. ingentis* | | √ | √ | | (Hose *et al.*, 1987) |
| | *P. japonicus* | | √ | √ | | (Sequeira *et al.*, 1995) |
| | *P. monodon* | | | √ | | (Sung *et al.*, 1998) |
| | *M. rosenbergii* | | | √ | | |
| **Penaeidins** | *P. vannamei* | | √ | √ | *Aerococcus viridans, Vibrio alginolyticus, Fusarium oxysporum* | (Destoumieux *et al.*, 2000b; Munoz *et al.*, 2002) |
| **Phagocytosis** | *C. maenas* | √ | | | *Moraxella sp.* | (Smith & Soderhall, 1983; Bell & Smith, 1993) |
| | *S. ingentis* | | √ | | *Bacillus subtilis* | (Martin *et al.*, 1996) |
| | *P. merguiensis* | √ | √ | | *Escherichia coli* | (Lee *et al.*, 2001) |
| | *P. monodon* | | √ | | *Vibrio anguillarum* | (Van de Braak *et al.*, 2002b) |
| | *M. resenbergii* | | √ | √ | *Saccharomyces cerevisiae* | (Gargioni & Barracco, 1998) |
| | *M. acanthurus* | | √ | √ | | |
| | *P. clarkii* | | √ | | | (Lanz *et al.*, 1993b) |
| | *A. astacus* | | √ | √ | *Moraxella sp* | (Smith & Soderhall, 1983) |
| **Encapsulation** | *H. americanus* | √ | √ | √ | *Fusarium solani* | (Hose *et al.*, 1990) |
| | *P. interruptus* | √ | √ | √ | | |
| | *L grandis* | | √ | √ | | |
| | *A. leptodactylus* | | √ | | | (Soderhall & Cerenius, 1992) |
| **Cytotoxicity** | *A. astacus* | | √ | √ | | (Soderhall & Cerenius, 1992; Johansson *et al.*, 2000) |

DHC: differentiated haemocyte count; HH: hyaline haemocytes; SGH: small granular haemocytes; LGC: large granular haemocytes.

The changes in THC were reportedly related to timing and environmental factors. *M. rosenbergii* injected with *Enterococcus* displayed a significant increase in THC during the first 6 h, which declined to the lowest level at 42 h, increased again after 54 h and then returned to its normal level after 66 h (Cheng & Chen, 2001). Cheng and Chen (2000) revealed that THC of *M. rosenbergii* was also affected by pH, temperature, and salinity. THC was significantly higher at pH 7.5 – 7.7, at temperature 30 – 31 ºC, and increasing salinity. Conversely, THC significantly decreased at pH 4.6 – 5.0 and 9.0 – 9.5, temperature 33 – 34 ºC and salinity at 0 ppt. However these findings were doubtful because the sample size (n = 2) for each variable were too small to validate with statistical analysis.

In summary, differing results in haemogram composition and the determination of cell types are still matters of debate. The contradictions appear when analysis of total haemocyte count, differential haemocyte count/classification of haemocytes (morphological criteria such as shape and size), behaviour and the role of types of haemocytes in immune defence of crustaceans are made (Bachere, 2000). Different methods, different species, and different interpretations from the researchers, also contributed to variation in results. Therefore, standardization of methods to analyse crustacean haemocytes is one of solutions necessary to eliminate these contradictory results. In addition, the fluctuation of THC when prawns were injected with pathogens still remains unclear because of the lack of sufficient sample size to validate with statistical analysis.

### 2.2.2. Haematopoiesis

Whether haemocytes in crustaceans originate from one or several tissues is still open to question. Most researchers believe that circulating haemocytes of crustaceans do not divide, therefore old cells should be replaced by new cells released into the haemolymph (Soderhall & Cerenius, 1992). In the ridgeback prawn *S. ingentis,* haematopoiesis was described to occur in paired epigastric haematopoietic nodules that consist of an extensive network of vessels (Martin *et al.*, 1987; Hose *et al.*, 1992). However, this organ is actually the lymphoid organ which has a filtering function for the haemolymph in penaeid prawns (Anggraeni & Owens, 2000; Van de Braak *et al.*, 2002b).

In many crustaceans, on the dorsal and dorsolateral surfaces of the stomach, sheet-like haematopoietic tissue was found, surrounded by connective tissue. Cells with differing morphology were organized and densely packed in small lobules and some in the interlobular spaces (Johansson *et al.*, 2000). In the penaeid prawn, the haematopoietic tissue (HPT) was generally located at the dorsal side of the stomach and in the proximal areas of maxillipeds and, to a lesser extent, towards the antennal gland (Bell & Lightner, 1988; Van de Braak *et al.*, 2002b; Bachere *et al.*, 2004).

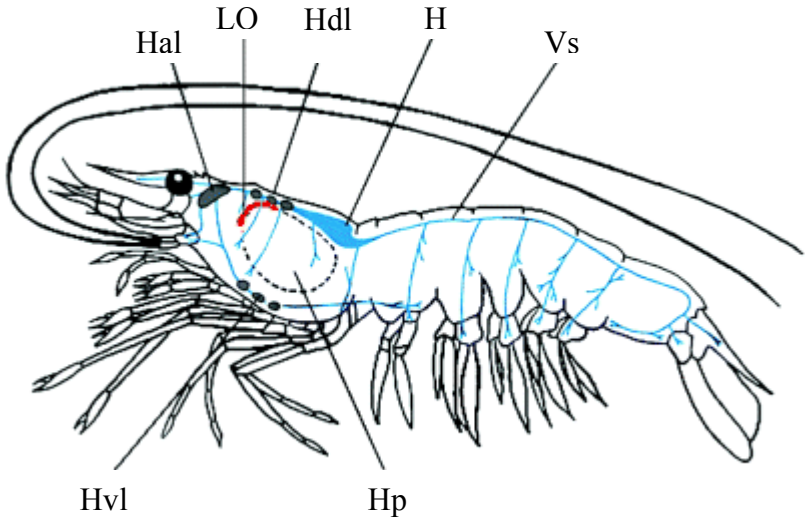



**Figure 2.1.** Open circulatory system of prawn. The vascular system (blue) was continuous with the haemolymph sinuses and irrigated the body cavity and the sites of haematopoiesis. The hematopoietic tissue consisted of densely packed lobules (grey): haematopoietic antennal lobules (Hal), haematopoietic dorsal lobules (Hdl), haematopoietic ventral lobules (Hvl) throughout the prawn's anterior region. LO, lymphoid organ; H, heart; Vs, vascular system; Hp, hepatopancreas (Bachere *et al.*, 2004).

It was proposed that the precursors of the large and small granular haemocytes of *P. monodon* were produced in haematopoietic tissue as hyaline haemocytes and those were released into the haemolymph. The small granular haemocytes, and to lesser extent, the large granular haemocytes migrated to and mature in the connective tissue (Van de Braak *et al.*, 2002b). However, some previous researchers (Martin *et al.*, 1987; Hose *et al.*, 1992) incorrectly indicated that in the ridgeback prawn the maturation of small granular haemocytes to large granular haemocytes occurred within the lymphoid organ (which they identified as haematopoietic nodules).

### 2.2.3. Clotting and wound healing

The clotting system is an important reaction to prevent blood loss and to hinder pathogenic microorganisms from entering through wounds and causing infection (Soderhall & Cerenius, 1992; Lee, 2001). This coagulation system is rapid and powerful reaction in crustaceans (Bachere, 2000). Clotting is mediated by clottable proteins (coagulagens) presented in the plasma (Yeh *et al.*, 1999). Cell coagulation in decapods is depended on the concentration of $Ca^{2+}$ ions, which may be regulated by cyclic nucleotide levels (Durliat, 1985). During the coagulation process, haemocytes release $Ca^{2+}$-dependent transglutaminase (TGase) to convert the clottable protein to covalently linked polymers. In the wound area, coagulagens are oligomerized to prevent haemolymph loss through breaks in the exoskeleton and dissemination of bacteria throughout the body (Yeh *et al.*, 1999). This process and its clottable protein were found in some crustaceans including the crab, *Macropipus puber* (Ghidalia *et al.*, 1982), the lobsters, *Homarus americanus* and *Panulirus interuptus*, and the sheep crab *Loxorhynchus grandis* (Hose *et al.*, 1990), the crayfish, *Pacifastacus leniusculus* (Kopacek *et al.*, 1993; Hall *et al.*, 1995; Hall *et al.*, 1999; Wang *et al.*, 2001b), *Procambarus clarkii*, the ridgeback prawn, *S. ingentis* (Martin *et al.*, 1991), the white prawn, *P. vannamei* (Reyes-Izquierdo & Vargas-Albores, 2001), and *P. monodon* (Yeh *et al.*, 1999).

The trypan blue test (Hose *et al.*, 1990) showed that the initiating step of the clotting process was by haemocyte lyses and the coagulation patterns related to the proportion of hyaline cells in circulating haemocytes. The higher the percentage of hyaline cells, the higher the proportion of clotting cells, because the hyaline haemocyte contained the TGase that was produced to activate coagulation of the haemolymph (Martin *et al.*, 1991). However, Wang *et al.* (2001b) stated either granular cells or semigranular cells contained calcium-dependent TGase activity essential for clot formation. TGase activity was also discovered in muscle, but not in the hepatopancreas or the plasma. Nevertheless, it was clear that haemolymph coagulation in crustaceans depended on the TGase-mediated cross linking of a specific plasma-clotting proteins (Kopacek *et al.*, 1993).

Using potassium bromide (KBr) density gradient ultracentrifugation and N-terminal amino acid sequencing, two major lipoproteins were identified from the freshwater crayfish, namely high density lipoprotein (HDL) that was identical to the β-1,3-glucan binding protein and very high density lipoprotein (VHDL) which was identical to the clotting protein (CP). The VHDL/CP was a TGase substrate, and the clotting reaction was initiated when a TGase was produced from the haemocytes (Hall *et al.*, 1995). Sequence analysis showed that CP was similar to the female specific vitellogenins (VTGs). CP from female and male crayfish was VHDL, had the same molecular mass in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions, and had the same N-terminal sequence. CP from females also formed a stabilized clot in the presence of active, endogenous TGase activity from the haemocytes. This confirmed that the CP had similar function in both sexes (Hall *et al.*, 1999). The CP isolated from plasma *Pacifastacus leniusculus* had molecular mass 400 kDa and consisted of disulfide bounded subunit. In the presence of haemocyte lysate supernatant (HLS) and calcium ions, the crayfish CP had the ability to polymerize and form clots (Kopacek *et al.*, 1993).

The clottable protein of penaeids has also been characterized. The purified *P. vannamei* CP was a 400-kDa protein and formed by 2 identical subunits when reduced with 2-mercaptoethanol (ME). The N-terminus sequence of the 179 and 125 kDa fragments were identical to the whole CP N-terminal sequence (Reyes-Izquierdo & Vargas-Albores, 2001). In *P. monodon*, the 380 kDa clottable protein was predicted to contain 44% α helix, 26% β sheet, and 16% β turn. It also contained 2.6% mannose and 1.2% N-acetylglucosamine. Northern analysis showed that the highest levels of the clottable protein was displayed in the gill and heart of the prawn, while the lower proportions were found in hepatopancreas, lymphoid organ and muscle, but not in the mature haemocytes (Yeh *et al.*, 1999). The clottable proteins in crustaceans were suggested to be homodimeric glycoprotein of around 380 – 400 kDa and to have identical amino acid composition and N-terminal sequence (Sritunyalucksana & Soderhall, 2000).

### 2.2.4. Prophenoloxidase (proPO) activating system

Phenoloxidase (PO) is an enzyme resulting from the activation of the prophenoloxidase (proPO) enzyme. The PO is responsible for the melanization process as a response to parasites in the body cavity of arthropods, crustaceans and insects (Soderhall & Cerenius, 1992; Rodriguez & Le Moullac, 2000). The proPO is activated in a stepwise process involving a prophenoloxidase activating enzyme (ppA), a serine proteinase, triggered by microbial cell wall components, such as lipopolysaccharides (LPS) of Gram negative bacteria, peptidoglycan (PG) of Gram positive bacteria or β-1,3-glucans of fungi (Johansson & Soderhall, 1989; Bachere *et al.*, 1995; Sritunyalucksana *et al.*, 1999; Lee, 2001) or by other molecules such as endogenous factors produced during tissue damage (Cerenius & Soderhall, 2004).

The proPO activating system has been studied in crustaceans, especially in the freshwater crayfish *Pasifastacus leniusculus* (Aspan & Soderhall, 1991; Wang *et al.*, 2001a), and the red swamp crayfish *Procambarus clarkii* (Lanz *et al.*, 1993a; Cardenas & Dankert, 1997). This was also found in the prawns such as in the black tiger prawn *P. monodon* (Sung *et al.*, 1998; Sritunyalucksana *et al.*, 1999), *P. stylirostris* (Le Moullac *et al.*, 1997; Le Moullac *et al.*, 1998), *P. californiensis* (Vargas-Albores *et al.*, 1998), the ridgeback prawn *S. ingentis* (Hose *et al.*, 1987) and the freshwater prawn *M. rosenbergii* (Sung *et al.*, 1998). Recently it was discovered in the spiny lobster *Panulirus interruptus* (Hernandez-Lopez *et al.*, 2003).

The 36 kDa ppA of freshwater crayfish (*P. leniusculus*) was purified from haemocytes and had the ability to cleave proPO to an active PO without any additional factors (Aspan *et al.*, 1990b; Lee, 2001). Recently, this protein was cloned from a blood cell cDNA library and its corresponding cDNA of 1,736 base pair encodes a zymogenic 468 amino acids protein, the pro-form of the prophenoloxidase-activating enzyme (proppA). Using Northern blot analysis of a mRNA preparation from the crayfish haemocytes, a transcript of 1.8 kilobase was found (Lee, 2001). The mRNA of the proppA was a single band that was synthesized in the haemocytes, but not in the hepatopancreas. The N-terminal amino acid sequence of proppA was highly hydrophobic and it had a putative signal sequence of 23 amino acids. The second residue of the mature proppA N-terminus

was determined to be N-glycosylation. The mature proppA consisted of 445 amino acids with a calculated molecular mass of 48,107 Da and an estimated isoelectric point (pI) of 9.04 (Lee, 2001; Wang *et al.*, 2001a).

The purified proteinase (ppA) cleaved chromogenic peptide substrates that were specifically hydrolysed by trypsin, and the enzyme was mainly inhibited by a commercial serine protease inhibitor (Aspan *et al.*, 1990b). Two high molecular mass proteinase inhibitors have been isolated from the haemolymph of crayfish. First, a 155 kDa trypsin inhibitor from crayfish plasma could inhibit the purified prophenoloxidase activating enzyme, ppA from crayfish haemocytes. Second, a $\alpha_2$-macroglobulin also isolated from crayfish plasma with a dimer of 190 kDa-subunit was a less effective inhibitor of ppA. This indicated that these proteinase inhibitors were involved in the regulation of proPO activating system in the crayfish (Aspan *et al.*, 1990a).

Similar to proppA, the proPO of crayfish (*P. leniusculus*) was purified from haemocytes (Aspan & Soderhall, 1991) and no proPO activity was detected in the hepatopancreas (Aspan *et al.*, 1995). Either under reducing or non-reducing conditions as determined by SDS-PAGE, the proPO had a molecular mass of 76 kDa, showing that the proenzyme contained a single polypeptide chain (Aspan & Soderhall, 1991). The crayfish proPO contained putative Cu(A) and Cu(B) sites. Therefore, the crayfish PO was more similar to haemocyanins than the tyrosinases hitherto analysed. Except for the copper-binding sites, however there was very little sequence similarity between proPO and the haemocyanins or other copper-containing proteins. There were seven putative N-glycosylation sites found in the deduced amino acid sequence suggesting that proPO was a glycoprotein (Aspan *et al.*, 1995).

The prophenoloxidase system in the red swamp crayfish (*P. clarkii*) could be activated by calcium, zymosan, trypsin and LPS (Lanz *et al.*, 1993a; Cardenas & Dankert, 1997). The effect of calcium ions was associated with the activation of an endogenous serine protease, but other calcium ion-dependent factors could also affect activation of proPO. Activation by zymosan revealed the capability of this system to recognise non-self molecules of microbial origin (Cardenas & Dankert, 1997). PO activity resulting from zymosan or LPS activators could be inhibited by

the serine protease inhibitors (leupeptine, phenylmethyl-sulphonylfluoride, and soybean trypsin inhibitor). However, the PO activity was increased by the cysteine protease inhibitors (N-ethylmaleimide, N-methylmaleimide, and iodoacet-amide) (Lanz *et al.*, 1993a).

Environmental factors such as temperature, salinity, and hypoxia (Le Moullac *et al.*, 1998; Sung *et al.*, 1998; Vargas-Albores *et al.*, 1998) induced alteration of proPO activity. The highest PO activity of *P. monodon* and *M. rosenbergii* was found at 37 ºC (Sung *et al.*, 1998). However, in *P. californiensis*, proPO activity was maintained in the physiological optimal limits up to 28 $^{o}$C (Vargas-Albores *et al.*, 1998). Salinity also had an increasing effect on proPO activity which was independent of the plasma total protein in the yellowleg prawn. The proPO activity increased with the increase of salinity up to 44 ‰. The metabolic rate of yellowleg prawn went up at high salinity as a physiological response to stress (Vargas-Albores *et al.*, 1998). Hypoxia stimulated PO activity in *P. stylirostris* that seemed to be related to a decrease of plasma inhibitor regulating the proPO system (Le Moullac *et al.*, 1998).

In the ridgeback prawn (Hose *et al.*, 1987) the proPO activity was determined to be in granules of small granule haemocytes lacking glycoprotein deposits and large granule haemocytes. The proPO activity in *P. japonicus* was also discovered both in small granular haemocytes and large granular haemocytes (Sequeira *et al.*, 1995). Cytochemical analysis of both *P. monodon* and *M. rosenbergii* revealed that the proPO system was primarily detected in granular haemocytes. It was also found that PO activity in giant freshwater prawns was significantly higher than that in tiger prawn. This might be caused by a large number or a higher level of granular haemocytes in the giant freshwater prawn (Sung *et al.*, 1998). However, in the spiny lobster (*P. interruptus*), the proPO activity was located in plasma (Hernandez-Lopez *et al.*, 2003), not in haemocytes as most other crustacea. This indicated that the proPO activity in crustaceans was variable in different species.

### 2.2.5. Beta-glucan binding protein (BGBP)

Beta-glucan binding protein (BGBP) has been described as one of the immunodefence molecules in crustaceans. This system has been studied in the freshwater crayfish *P. leniusculus* and *Astacus astacus* (Smith & Soderhall, 1983; Duvic & Soderhall, 1990; Cerenius *et al.*, 1994; Thornqvist *et al.*, 1994; Lee *et al.*, 2000), the shore crab *C. maenas* (Smith & Soderhall, 1983; Thornqvist *et al.*, 1994), and *P. vannamei* (Jimenez-Vega *et al.*, 2002). The purified β-1,3-glucan binding protein from *P. leniusculus* was a single glycoprotein that had a molecular mass of 100 kDa with 5.0 pI (Duvic & Soderhall, 1990; Cerenius *et al.*, 1994). In the white prawn *P. vannamei*, it was also found that the BGBP was a homogeneous 100 kDa protein which appeared in SDS-PAGE (Jimenez-Vega *et al.*, 2002). However, BGBP in the shore crab, *C. maenas* had a molecular mass of 110 kDa (Thornqvist *et al.*, 1994). These studies indicated a similarity but yet a variability of the molecular size of BGBP in each species. In the crayfish, the hepatopancreas was the primary organ that was responsible for biosynthesis of BGBP (Cerenius *et al.*, 1994).

The major sugar of the crayfish BGBP was mannose and also contained glucose, xylose, fucose, galactose, N-acetylglucosamine (Cerenius *et al.*, 1994). The amino acid content of BGBP from prawn was similar with the freshwater crayfish BGBP. Generally, the amino acids in the highest proportion were Asn and Gln. Ala and Gly were found in moderate concentration, whilst Met and His were the lowest. High homology was detected when the N-terminal amino acid sequences of crustacean BGBPs (*P. californiensis, P. vannamei* and *P. leniusculus*) were compared (Vargas-Albores & Yepiz-Plascencia, 2000).

A receptor of BGBP has been purified and it contained two non-covalently associated subunits of 230 kDa and 90 kDa. The receptor would only bind BGBP if this protein had been treated with a β-1,3 glucan prior to the assay for binding activity. This indicated that the activating BGBP by β-1,3 glucans induced a conformational change in the molecule and that this is an absolute requirement for the binding of this protein to its haemocyte membrane receptor (Duvic & Soderhall, 1992).

The BGBP enhanced activation of peptidase and phenoloxidase from haemocyte lysate of crayfish in the presence of laminarin (β-1,3 glucan). Besides being involved in the proPO activating system, this protein might also be involved in other processes in crayfish such as binding to foreign particles carrying laminarin in their cell walls (i.e. fungi) and function as an opsonic protein in the removal of these microorganisms from the circulation (Duvic & Soderhall, 1990; Cerenius *et al.*, 1994). The BGBP from the plasma of *C. maenas* could also function as opsonin for crab hyaline cells. The phagocytic rate could be increased four times when yeast particles were opsonized by BGBP from crayfish or crab (Thornqvist *et al.*, 1994). It was suggested that BGBP activated by β-1,3-glucan could induce haemocyte degranulation releasing proteins including proPO, ppA, peroxinectin, tranglutaminase, proteinase inhibitors, antibacterial proteins, lipopolysaccharide and β-1,3-glucan binding protein (LGBP), and masquerade-like protein (Lee & Soderhall, 2002). The BGBP of prawn was a constitutive plasma protein that, after binding to β-glucans, reacted with haemocyte surface and stimulated the release of haemocytic granules. The activation of the contents of the granules was enhanced by the presence of plasma $Ca^{2+}$ which in turn activated the PO activity (Vargas-Albores & Yepiz-Plascencia, 2000).

### 2.2.6. Penaeidins

Synthesis of antimicrobial peptides (AMPs) represents the innate, first-line host defence mechanism (Bachere, 2000; Bachere *et al.*, 2000; Destoumieux *et al.*, 2000a). Three antimicrobial peptides have been isolated from the plasma and haemocytes of the penaeid prawn *P. vannamei* and have been called penaeidin -1,-2, and -3, after genus *Penaeus* (Destoumieux *et al.*, 1997; Bachere *et al.*, 2000). It was proposed that these molecules were synthesized from mRNA to the active compound in the haemocytes (Destoumieux *et al.*, 1997). Recently, members of penaeidin family have been cloned and sequenced from the Chinese prawn, *P. chinensis* and the black tiger prawn *P. monodon* and named *Ch*-penaeidin and penaeidin-5 cDNA, respectively (Chen *et al.*, 2004; Kang *et al.*, 2004). The penaeidin family can be classified into three classes. Firstly, penaeidin 3 was from *P. vannamei, P. setiferus,* Ch-penaeidin from *P. chinensis* and penaeidin from *P. monodon*. Secondly, penaeidin 2 was from *P. vannamei* and *P. setiferus*. Finally, penaeidin 4 was found

in *P. vannamei* and *P. setiferus*. This classification corresponded to groups 1, 2 and 3 respectively (Kang *et al.*, 2004).

Penaeidins-1, -2, and -3 were highly cationic peptides with positive net charge of 7 (pen-1 and -2) and 8 (pen-3) with isoelectric point that ranged from 9.34 to 9.84 for pen-1 and -2, and pen-3, respectively. They consisted of 50 (pen-1 and -2) and 62 (pen-3) amino acid residues (Destoumieux *et al.*, 1997; Bachere *et al.*, 2000). These antimicrobial peptides (pen-2 and pen-3) had a molecular mass of 5.5 to 6.6 kDa in an acid extract of prawn plasma and haemocyte organelle-rich fractions (Destoumieux *et al.*, 1999; Destoumieux *et al.*, 2000a). The molecular mass of Ch-penaeidin was also 5.6 kDa, highly cationic with a pI of 9.77, and had eight positively charged residues (Arg + Lys) without negative residues (Kang *et al.*, 2004). These molecules were different from the other proline rich peptides that displayed activity against Gram-negative bacteria (Destoumieux *et al.*, 1999). They consisted of an $NH_2$-terminal proline-rich region and a COOH-terminal domain containing six cysteine residues stabilized by three intramolecular disulfide crosslinks (Destoumieux *et al.*, 1999; Destoumieux *et al.*, 2000a; Bachere, 2003; Kang *et al.*, 2004). However, the $NH_2$ terminal of Ch-penaeidin from *P. chinensis* was rich in glycine residues (six), not proline (Kang *et al.*, 2004). Translational of the penaeidin-5 cDNA from *P. monodon* demonstrated eight cysteine residues (Chen *et al.*, 2004).

The northern blot analysis (Destoumieux *et al.*, 2000b), *in situ* hybridization analysis (ISH) and immunocytochemistry (ICC) (Munoz *et al.*, 2002) revealed that the haemocytes were abundant with penaeidin mRNAs. These were located in circulating haemocytes in blood vessels, sinuses and infiltrating haemocytes (Munoz *et al.*, 2002). Penaeidin mRNAs were detected in the heart, gills, epigastric lymphoid organ, testis, and intestine but no penaeidins were detected in the brain, hepatopancreas, eyes, subcuticular epithelia, and stomach (Destoumieux *et al.*, 2000b; Kang *et al.*, 2004). However, Munoz *et al.* (2002) stated that these peptides were also found in subcuticular epithelium, midgut, midgut caecum, muscle, and brain. Immunolabelling indicated that penaeidins seemed to be stored in granular haemocytes. Ultrastructure localization of penaeidins showed that these molecules mainly expressed in large granule haemocytes and to a lesser extent in small granule

haemocytes and no penaeidins were detected in hyaline cells (Destoumieux *et al.*, 2000b; Munoz *et al.*, 2002). This confirmed that after synthesising, penaeidins were kept in a mature form in granules before being secreted into the plasma (Destoumieux *et al.*, 2000b).

The experimental challenge of *P. vannamei* with heat-killed microorganisms (*Aerococcus viridans*, *V. alginolyticus*, and *Fusarium oxysporum*) revealed that the penaeidin level decreased at 3 hpi in circulating haemocytes, returned to non-challenged levels after 12 hpi and then slightly elevated and remained stable after 24 hpi. This indicated that microbial injection did not enhance synthesis of penaeidins in haemocytes (Destoumieux *et al.*, 2000b). In contrast, the concentration of penaeidins went up at 3 hpi and reached a peak at 6 hpi in the plasma. Furthermore, there was also an increase in the penaeidin immunoreactivity patterns in the cuticle at 24 hpi when the concentration of penaeidins fell in the haemocytes of the efferent blood vessels. This suggested that the antimicrobial peptides which attached to the prawn cuticular surface might protect the prawn from foreign material by their chitin binding property after haemocytes released them into the plasma (Destoumieux *et al.*, 2000b).

Subsequently, it was found that although there was a significant decrease in penaeidin levels at 12 hpi and return to normal levels at 48 hpi, the penaeidin levels increased three times after 72 hpi. Based on this finding, two different phases of prawn immune response to microbial infection could be distinguished. In the first phase (12 hpi), antimicrobial peptide and protein was produced in the haemocytes. Penaeidins migrated from the haemocytes in circulation and the tissues to injured tissues. As a result, penaeidins decreased in the total circulating population. In the second phase (48 – 72 hpi), in the injection site and subcuticular surfaces, high levels of penaeidin-labelling was detected. In addition, in the blood circulation, haemocytes showed high transcriptional activity from increased 18S rRNA concentration and penaeidin activity (Munoz *et al.*, 2002).

Penaeidin antibacterial activity appeared as a strain-specific inhibition mechanism. These peptides showed a rapid killing activity or bacteriostatic properties. This confirmed that they had multiple modes of action. Furthermore, penaeidin antifungal

activity seemed to be associated with inhibition of spore germination. The molecules inhibited the fungal hyphae and caused abnormal morphology at low concentration (Destoumieux *et al.*, 1999; Destoumieux *et al.*, 2000a). In short, the function of penaeidins in antimicrobial activity and chitin binding might play an important role in chitin assembly, wound healing, and protection in developmental and moulting stages of prawns (Bachere, 2003).

### 2.2.7. Lectins/agglutinins

Lectins are glycoproteins, generally without catalytic activity and are at least bivalent binding carbohydrates promoting agglutination of different cell surfaces. Due to their functional capacity in agglutinating, they are also called agglutinin. Lectins may be expressed in almost all living creatures (Marques & Barracco, 2000). These molecules have a potential role in invertebrate nonself recognition. They agglutinate microorganisms and mediate binding between haemocyte surfaces and foreign particles to enhance their phagocytosis, thus they have an opsonic function (Soderhall & Cerenius, 1992; Marques & Barracco, 2000).

Interaction between lectins and carbohydrate was also involved in some biological activities, for example the cellular and tissue transport of carbohydrates, glycoproteins and calcium, cytolytic and cytotoxic factors, migration, cell adhesion, apoptosis and nodule formation (Marques & Barracco, 2000; Lee & Soderhall, 2002). In addition, there might be three different methods for agglutinin-mediated recognition of foreign materials. Firstly, cell-surface agglutinins reacted with carbohydrate determinants of membrane molecules of microorganisms. Secondly, agglutinins bound to the surface glycoconjungates of microorganisms opsonising for receptors in immunocompetence invertebrate cells. Lastly, surface agglutinin or microorganisms bound to sugar moieties of membrane molecules of invertebrate cells (Renwrantz, 1983).

Lectins/agglutinins have been characterized from either the serum or the haemolymph of some prawns including: the freshwater prawn *M. rosenbergii* (Vasta *et al.*, 1983), the sea prawn *P. longirostris* (Fragkiadakis & Stratakis, 1995), the Indian white prawn *P. indicus* (Maheswari *et al.*, 1997; Jayasree, 2001b, 2001a;

Maheswari *et al.*, 2002), and the black tiger prawn *P. monodon* (Sritunyalucksana *et al.*, 1999). In prawns, all these lectins were specific for sialic acid or its derivatives (Vasta *et al.*, 1983; Bachere *et al.*, 1995).

Using affinity chromatography and adsorption on formalinized erythrocytes (Fragkiadakis & Stratakis, 1995), two lectins were purified from haemocytes of *P. longirostris.* One lectin had molecular mass of 440 kDa (27-kDa subunit) and was specific for N-acetylneuraminic acid. The other had a molecular mass of 210 kDa (36-kDa subunit) and was specific for *N*-acetylgalactosamine. However, they required divalent cations ($Ca^{2+}$) and recognized *N*-acylaminosugars, suggesting that both lectins had the same physiological functions. These lectins strongly agglutinated *Pseudomonas aeruginosa* and *Escherichia coli*, but not *Bacillus subtilis* and *Saccharomyces cerevisiae*.

Haemagglutinin activity was also detected in the serum and the haemolymph of *P. indicus*. The serum agglutinin was stable in a wide range of pH (pH 3 – 9), but labile by heating at 80 $^{o}$C or above (Maheswari *et al.*, 1997). Gel filtration in high performance liquid chromatography (HPLC) revealed that it had a molecular mass of 200 kDa and under reducing conditions in SDS-PAGE, it was a homo-oligomer of 27-kDa subunit (Maheswari *et al.*, 2002). Agglutinin from the haemolymph of the Indian white prawn was stable at pH 7 – 9 and temperature between 30 $^{o}$C and 40 $^{o}$C (Jayasree, 2001b). Using gel filtration chromatography, this prawn haemolymph agglutinin had molecular mass of 181 kDa that could be separated in two monomers (97 kDa and 84 kDa) under SDS-PAGE (Jayasree, 2001b). Both agglutinins could react with human red blood cells (RBC) and some mammalian (rat and rabbit) RBC types (Maheswari *et al.*, 1997; Jayasree, 2001b) and they were also cation ($Ca^{2+}$ and $Mg^{2+}$) independent (Maheswari *et al.*, 1997; Jayasree, 2001a). It was found that the natural agglutinin functioned as a defence protein with simultaneous agglutinating, haemolytic, and antibacterial properties against bacteria *Vibrio alginolyticus* (v-5) (Jayasree, 2001b, 2001a), *V. damsela, V. parahaemolyticus, V. anguillarum, V. fluvialis,* and *Aeromonas hydrophyla* (Maheswari *et al.*, 2002).

Agglutinin activity was also detected in plasma haemocyte fraction, haemocyte lysate fraction and haemolymph with cell lysate fraction (HLF) of *P. monodon*

(Sritunyalucksana *et al.*, 1999). HLF had the highest agglutinin activity against human RBC and *E. coli.* Like *P. longirostris* agglutinin, *P. monodon* agglutinin was calcium-ion dependent. In dialysed HLF, the agglutinin activity significantly decreased and its activity returned to the normal level after additional $CaCl_2$ (Fragkiadakis & Stratakis, 1995; Sritunyalucksana *et al.*, 1999). All the findings above confirmed that similarities and heterogeneity of agglutinins existed between species and within species (Cooper & Lemmi, 1981).

### 2.2.8. Phagocytosis

Phagocytosis is a cellular defence mechanism and together with humoral components, constitutes the first line of immune response once a foreign substance has overcome the physicochemical barrier of the cuticle (Soderhall & Cerenius, 1992). During phagocytosis, invaders are internalised into the cell into a digestive vacuole, the phagosome. The clearance of phagocytosed material involves the release of enzymes into the phagosome and the generation of reactive oxygen intermediates (ROIs) (Rodriguez & Le Moullac, 2000). It seems that haemocytes are responsible for phagocytic clearance of foreign particles suspended in the haemolymph (Johnson, 1987).

Most research concerning phagocytosis in crustaceans has been conducted through the observation of the clearance of injected bacteria, particulate substances or enhancement with immunostimulants. In addition, the phagocytic activity seems to be undertaken by different haemocyte types in different crustacea. In the freshwater crayfish, *P. clarki,* it was suggested that the granular and especially the small granular haemocytes phagocytosed foreign particles. Using acid phosphatase and nonspecific esterase staining, these haemocytes displayed high levels of lysosomal enzyme activity (Lanz *et al.*, 1993b). *In vitro*, phagocytosis activity against *Moraxella* sp. by haemocytes of *A. astacus* was carried out by the large granular and small granular haemocytes (Smith & Soderhall, 1983). In the palaemonids, *M. rosenbergii* and *M. acanthurus,* the SGH and LGH also showed phagocytic activity to the yeast *S. cerevisiae* when examined on blood cell monolayers mounted on glass coverslips (Gargioni & Barracco, 1998).

However, using flow cytometry to examine the blood cells of *P. merguiensis*, it was found that the hyaline haemocytes and small granular haemocytes were involved in phagocytosis of *E. coli* (Lee *et al.*, 2001). In *P. monodon* injected with *V. anguillarum*, the small granular cells were the primary phagocytic cells in the lymphoid organ (Van de Braak *et al.*, 2002b). Furthermore, hyaline cells of *C. maenas* were responsible for phagocytosis of *Moraxella* sp. (Smith & Soderhall, 1983; Bell & Smith, 1993). Therefore in crustaceans, clearance of microorganisms through phagocytosis can be carried out by one or two types of haemocytes and vary for different species.

Phagocytic activity was found to be affected by factors such as temperature and stimulants. In haemocytes from *H. americanus* tested for *in vitro* phagocytosis using *A. viridans*, the mean percentage of phagocytosis was almost the same at 16, 18 and 20 ºC ranged between 13.0 and 13.8%. However, the phagocytic activity significantly decreased to 5.8 and 5.4% at 22 and 24 ºC, respectively (Steenbergen *et al.*, 1978). Stimulated with phorbol myristate acetate (PMA), the shore crab hyaline cells produced superoxidase anions that could enhance phagocytosis of these cells (Bell & Smith, 1993). It was also found that peroxinectin, a cell adhesion molecule, enhanced phagocytosis and encapsulation depending on the size of the foreign particle (Holmblad & Soderhall, 1999).

### 2.2.9. Encapsulation

Encapsulation occurs when the size of foreign particles is too big to be engulfed by phagocytosis, then several haemocytes will seal off (encapsulate) the pathogens from circulating haemocytes (Soderhall & Cerenius, 1992; Jones, 1999). Parasites, such as trematodes, nematodes, cestodes, parasitoids, large protozoa, and fungi, that are too large to ingested by a single blood cell, can be surrounded by multicellular sheaths (Ratcliffe *et al.*, 1985). This mechanism also contributes to elimination of agglutinated bacteria from the prawn tissues (Bachere *et al.*, 2004).

The encapsulation process has been described by using haemocyte preparations from *A. astacus* injected with fungal blastospores of *Beauveria bassiana* (Soderhall *et al.*, 1984). Histological sections of the gill revealed that haemocyte capsules were

formed around the haemocyte lysate supernatant (HLS) coated blastospores. The capsules consisted of many non-flattening cells. Generally, between four and six layers of cells composed the cellular envelope. In the centre of the granulomas, a huge brownish deposit of melanin developed.

Undoubtedly, the haemocytes are the major cells that are responsible for encapsulation of particulates. The types of haemocytes involved in encapsulation vary in different species. Light microscope (LM) observation of haemocytes *H. americanus*, *P. interruptus*, and *L. grandis* revealed that the large granular haemocytes were the primary cells which were responsible for encapsulation of fungal hyphae (*F. solani*), followed by the small granule haemocytes, and to lesser extent the hyaline haemocytes. However, in *H. americanus* there was no indication on the activity of hyaline cells in encapsulation (Hose *et al.*, 1990). In *A. leptodactylus*, the small granular cells were the first cells that reacted and encapsulated foreign particles (Soderhall & Cerenius, 1992).

Other important contributing factors might be involved in promoting encapsulation in crustaceans. Encapsulation processes could be stimulated by peroxinectin, the 76 kDa cell-adhesion factors in *P. leniusculus* and *A. leptodactylus* (Johansson & Soderhall, 1989; Johansson *et al.*, 2000). Haemocyte lysate protein in *A. astacus* could function as opsonin in encapsulation activity to fungal blastospores of *B. bassiana* (Soderhall *et al.*, 1984). Integrin-mediated adhesion might also play a role in encapsulation of foreign particles (Holmblad & Soderhall, 1999). Furthermore, temperature, site of parasitization, the presence of super and multiparasitism, age, sex, and nutritional state of the host influenced the encapsulation process (Ratcliffe *et al.*, 1985).

### 2.2.10. Nodule formation

Nodule formation occurs when the number of invading microorganisms is too numerous to be cleared by phagocytosis alone (Ratcliffe *et al.*, 1985; Holmblad & Soderhall, 1999; Lee, 2001). This mechanism occurs together with other immune responses such as phagocytosis to improve the efficiency of clearance of massive foreign particles. Nodules are also called as cell aggregates, cell clumps, brown

bodies, or granulomata depending on size, melanization, species, and author (Ratcliffe *et al.*, 1985). The result of nodule formation is that the microorganisms are clumped and isolated in several layers of haemocytes. Then the nodule becomes heavily melanized because of the host's phenoloxidase activity (Soderhall & Cerenius, 1992).

The formation of nodules in *C. maenas* was initiated when bacteria appeared to bind to the outside of haemocytes, which then aggregated to form cell clumps. The haemocytes involved discharging substances and initiated haemolymph coagulation. Additional haemocytes made the cell clumps become large, forming concentric flattened layers around a central, compact, often necrotic, and sometimes melanized core. In this crab, most of the nodules were formed in the gills and sinuses between the hepatopancreatic tubules (Ratcliffe *et al.*, 1985). However, information on this mechanism is very limited in prawns and still awaits further studies.

### 2.2.11. Cytotoxicity

Cytotoxicity is cell-killing of foreign materials by natural killer cells. This activity may appear in all multicellular organisms (Leys, 1999). Cytotoxic processes were mediated by blood cells and have been studied in several invertebrate species (Ratcliffe *et al.*, 1985). However, in crustaceans, cytotoxic reactions are not well known and still remain to be analysed (Soderhall & Cerenius, 1992). It was demonstrated that large granular and small granular haemocytes of *A. astacus* could function as cytotoxins and lysed tumour and non-tumour cell lines as target cells, including erythrocytes (Soderhall & Cerenius, 1992; Johansson *et al.*, 2000).

### 2.2.12. Apoptosis

Apoptosis, programmed cell death, is a mechanism that naturally occurrs to develop and maintain homeostasis in multicellular organisms (Thomson, 2001). Apoptosis plays important roles in sculpting the developing organisms, precise regulation of cell numbers, functioning as a defence mechanism to remove unwanted and potentially dangerous cells like cells infected with viruses, and tumour cells (Steller, 1995). This process can be differentiated from necrosis by morphological changes of

cell, including shrinkage, blebbing of plasma membrane, chromatin condensation and DNA fragmentation (Thomson, 2001). Apoptosis affects scattered single cells rather than tracts of contiguous cells (Wyllie *et al.*, 1980).

Mechanisms of apoptosis have been observed in penaeid prawns with viral diseases. It was discovered in the black tiger prawn (*P. monodon*) infected with midcrop mortality syndrome, MCMS (Anggraeni & Owens, 2000). This process also appeared in the cultured black tiger prawn, kuruma prawn (*P. japonicus*), and *P. vannamei* infected with WSSV (Sahtout *et al.*, 2001; Rojtinnakorn *et al.*, 2002; Granja *et al.*, 2003; Rodriguez *et al.*, 2003; Wongprasert *et al.*, 2003), in *P. monodon* injected with yellow head virus (YHV) (Khanobdee *et al.*, 2002) and virus-associated peripheral neuropathy and retinopathy (PNR) (Callinan *et al.*, 2003).

Using Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick-End Labelling (TUNEL) assay for DNA fragmentation, it was found that apoptosis was generally expressed in the lymphoid organ (LO) of black tiger prawns suffering MCMS (Anggraeni & Owens, 2000) and kuruma prawn exposed to WSSV (Wu & Muroga, 2004). The TUNEL assay revealed that abdominal epithelium, stomach epithelium, hepatopancreatic interstitial cells, gill and muscle of *P. monodon* infected with WSSV were the main tissues demonstrating apoptosis (Sahtout *et al.*, 2001). Pathological observation of H & E stained sections showed that apoptosis occurred in subcuticular epithelium, gills, haematopoietic tissue, lymphoid organ, haemocytes, and heart of the black tiger prawn infected with WSSV (Wongprasert *et al.*, 2003). In addition, in the black tiger prawn injected with YHV, this process was found in LO, haemocytes and gills (Khanobdee *et al.*, 2002).

Furthermore, using TEM, Wongprasert *et al.* (2003) illustrated the pathological changes of apoptosis in black tiger prawn tissues infected with WSSV. Aggregation and fragmentation of chromatin into dense masses of granules surrounded by a membrane occurred in the nuclei of several epidermal cells in the first 6 hpi. In intracellular spaces, the cells bulged and budded-off fragments that developed into membraned nuclear fragments at this time. At 24 hpi, most apoptotic cells appeared. Haemocytes and neighbouring epithelial cells engulfed some of these cells. Interestingly, WSSV virions were never detected in apoptotic cells. In YHV-

infected prawn cells, the cytoplasm was markedly condensed. The nuclear envelope and cytoplasmic organelles such as mitochondria, ribosomes, and rough endoplasmic reticulum (rER) were preserved (Khanobdee *et al.*, 2002). Glial cells in prawn infected with PNR also showed apoptosis with nuclei condensed into a characteristic narrow rim of cytoplasm surrounded by basophilic spheres (Callinan *et al.*, 2003).

Using an expressed sequence tag (EST) approach to determine gene expression in haemocytes of the kuruma prawn challenged with WSSV, a high proportion of apoptotic and tumour related proteins were detected in the library of viral infected animals. Proteasome 26S subunit, a multicatalytic protease involving in apoptosis, was found only in the WSSV-infected library. Expression of an apoptotic factor, the programmed cell death 6-interacting protein (Alix), was higher in the viral infected library than in the uninfected library indicating that apoptosis played a role in the defence mechanism (Rojtinnakorn *et al.*, 2002).

Whether apoptosis gives advantages or disadvantages to prawns infected with viral diseases remains debatable. On one hand, Flegel & Pasharawipas (1998) who developed a new concept on active viral accommodation for the crustacean response to viral infections suggested that prawns and other crustaceans would die due to viral diseases if those infections provoked the innate cellular mechanism of apoptosis. Sahtout *et al.* (2001) and Khanobdee *et al.* (2002) who supported this hypothesis, suggested that cell death resulting from apoptosis due to WSSV and YHV infection, might be the primary cause of death in infected prawns. Furthermore, a recent study revealed that apoptosis was not an effective mechanism for kuruma prawn infected WSSV. It was also found that a high incidence of apoptosis in the LO was always correlated with high mortalities (Wu & Muroga, 2004). It has been proposed that tolerance might occur in an early viral binding step resulting in specific memory that suppresses suicide cells during or after the viral infections (Flegel & Pasharawipas, 1998). In addition, if prawns or viral pathogens possessed genes which inhibited apoptosis, prawns would survive from YHV (Khanobdee *et al.*, 2002).

On the other hand, Hasson *et al.* (1999b) and Anggraeni & Owens (2000) considered the possibility of apoptosis to eliminate virus-infected cells in both TSV infection in *P. vannamei* and MCMS infected *P. monodon*. Moreover, Granja *et al.* (2003) who

studied the effect of hyperthermia on apoptosis in *P. vannamei* injected WSSV believed that viral replication and infection could be reduced by the apoptotic process. This mechanism was responsible for increasing survival rate of *P. vannamei*-infected WSSV. The difference in these points of views might have resulted from different species, viruses, and methods that they used. Stage of infection and dose of viruses might also contribute to the discrepancy.

### 2.2.13. Conclusion

Most authors agreed to classify differentiated haemocytes into three types, large granular haemocytes, small granular haemocytes, and hyaline haemocytes. However, the roles of haemocytes as primary line in defence mechanisms of prawns seem to be distinct between different types of haemocytes and species. The differences also came up when total haemocytes count (THC) was taken into account. These divergences appeared due to the differences of methods and species that were used in experiments. Although, it was commonly accepted that haemocytes were produced in haematopoietic tissue, where the actual maturation of haemocytes occurred is still unclear or variable.

In crustaceans, immunodefence such as the clotting systems, phagocytosis, encapsulation and cytotoxicity were executed by different types of haemocytes which differed between species. A similar pattern was observed in prophenoloxidase systems and the production of specific antimicrobial peptides of penaeids, the penaeidins. Similarity and heterogeneity also appeared between species when non-self recognition reaction, lectins/agglutinins and another bioactive protein, beta-glucan binding protein were examined. Furthermore, the advantages and the disadvantages of the mechanism of cell death, apoptosis, in infected prawns still remain controversial. However, it is suggested that there might be cellular communication between different types of immune cells to deal with foreign substances.

## 2.3. Lymphoid Organ as Part of the Immune System

### 2.3.1. Structure of the lymphoid organ

The lymphoid organ (LO) was first found in korai prawn *Penaeus orientalis* Kishinouye. It was also discovered in ginger prawn *P. japonica* Bate, *P. carinatus* Dana, white prawn *P. indicus* de Man, and *P. merguensis* de Man (Oka, 1969). The structure of the lymphoid organ was incorrectly described by Martin *et al.* (1987) as haematopoietic nodules in the ridgeback prawn, *Sicyonia ingentis* (Anggraeni & Owens, 2000; Van de Braak *et al.*, 2002c). Later, many studies of the structure and the role of the lymphoid organ in penaeids were conducted (Bell & Lightner, 1988; Kondo *et al.*, 1994; Anggraeni & Owens, 2000; Van de Braak *et al.*, 2002c; Shao *et al.*, 2004). The view that this organ might belong to the vascular system, because of a pair of afferent and many efferent vessels (Oka, 1969) is largely discredited.

The normal structure of lymphoid organ in penaeids has two lobes which are located just ventro-lateral to the junction of the anterior stomach chambers (Figure 2.2). The lymphoid lobes are apposed slightly dorso-anterior to the ventral hepatopancreatic lobe. They are similar and contain a generally basophilic mass of anastomosing tubules which connect via the subgastric artery, to the anterior aorta (Bell & Lightner, 1988). Although the lumen of LO duct has generally a larger diameter, the cells in the LO duct and tubules have identical morphology (Van de Braak *et al.*, 2002c).

In a tubule, the lumen often has haemocytes. It might be open or nearly occluded with them. Haemal sinuses and a thin fibrous material occupied spaces between tubules (Bell & Lightner, 1988) (Figure 2.3b). The central haemal space of the LO tubules can contain large granular haemocytes and, to a much lesser extent, small granular haemocytes. The central lumen is lined with a flattened layer of elongated endothelial cells, followed by a densely packed layer of cells. These cells often contain many vacuoles and up to 30 granules per cell section, varying in size and electron density, and strongly look like small granular haemocytes. The small granular cells exhibit many pseudopods that surround foreign material and lead into the outer layer of the tubule wall. The cells toward the outer wall are more dispersed

and contain numerous bleb like extensions of the cytoplasm and seem to have a folded membrane (Van de Braak *et al.*, 2002c).

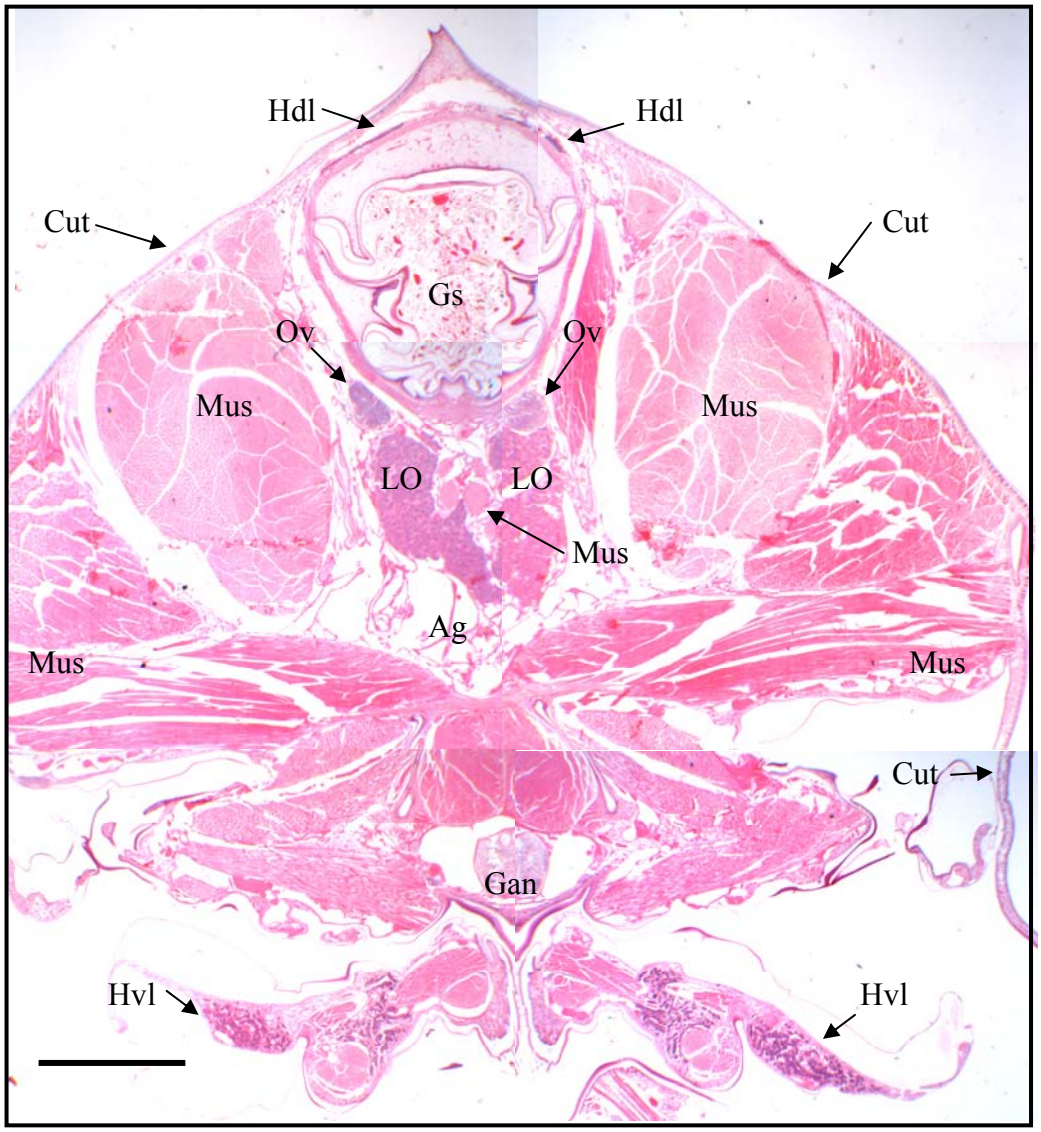



**Figure 2.2.** Cross-sectional view of the lymphoid organ (LO) and surrounding tissue of *P. monodon*. The LO consists of two lobes located ventro-lateral of the gastric sieve and dorsal of antennal gland. H & E stain. Scale bar = 100 µm. Ag, antennal gland; Cut, cuticle; Gan, ganglion; Gs, gastric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

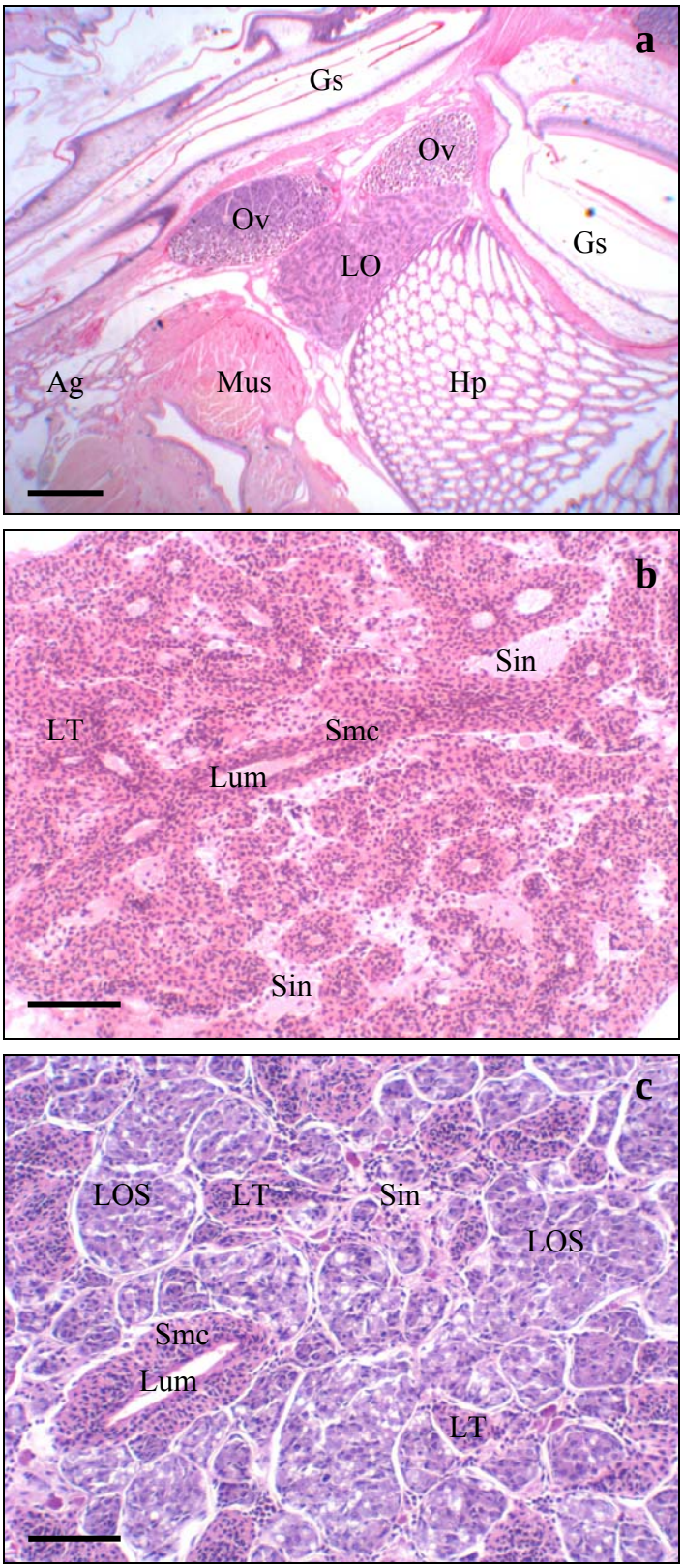



**Figure 2.3.** Light micrograph of H & E stained tissue section of the LO of *P. monodon*. (a) Overall longitudinal view of the lymphoid organ and surrounding tissue; (b) normal lymphoid organ tubules without LOS cells; (c) lymphoid organ with LOS cells. Scale bar = 200 µm (a), 100 µm (b and c). Ag, antennal gland; Gs, gastric sieve; Hp: hepatopancreas; LT: lymphoid tubule (Lum, lumen; Smc, stromal matrix cells); Mus, muscle; Ov, ovary; Sin, haemal sinuses.

The lymphoid organ's position in females is slightly different to the males. This organ lies between the hepatopancreas and the first branch of the ovary in the female. The ovary closely presses the organ onto the upper anterior part of the hepatopancreas. Whereas, the lymphoid organ of the male lies between the hepatopancreas and the stomach, loosely connected to both (Oka, 1969). In the prawn larvae, the position and the structure of the lymphoid organ has been described as slightly different to adult prawns suggesting developmental changes to this organ (Nakamura, 1987).

### 2.3.2. The Structure of lymphoid organ spheroid cells

The most remarkable feature of histopathological changes of the lymphoid organ at the cellular level is the presence of spheroid cells. Previous studies described these cells as multinucleate giant cells (Owens *et al.*, 1991), proliferative cells (Nadala *et al.*, 1992), nodular structures (Kondo *et al.*, 1994) or lobular hyperplastic proliferation and degeneration of LO cells (Turnbull *et al.*, 1994). However, following most authors, (Lightner *et al.*, 1987; Owens & Hall-Mendellin, 1989; Hasson *et al.*, 1999b; Anggraeni & Owens, 2000; Van de Braak *et al.*, 2002c; Shao *et al.*, 2004), the term lymphoid organ spheroid (LOS) cells or spheroid cells or spheroids will be used throughout this thesis. The characteristics of LOS cells vary slightly between the descriptions published. This might due to the differences in pathogens, dose of infections, and species. However, there are similarities in pathological features illustrated by different authors.

The most prominent characteristics of spheroid cells compared to the normal tubules (stromal matrix) of the lymphoid organ were that the spheroids lacked a lumen, had a more basophilic cytoplasm with H & E stain (Lightner *et al.*, 1987; Owens & Hall-Mendellin, 1989; Owens *et al.*, 1991; Bonami *et al.*, 1992; Turnbull *et al.*, 1994; Anggraeni, 1998; Van de Braak *et al.*, 2002c) (Figure 2.3c) and an increase in the cytoplasm to nuclear ratio (Owens *et al.*, 1991). These spheroid cells appeared hypertrophic, spherical or irregular (Lightner *et al.*, 1987; Hasson *et al.*, 1999b) and surrounded by fibrous connective tissue or elongated flattened cells (Owens *et al.*, 1991; Anggraeni, 1998; Van de Braak *et al.*, 2002c; Shao *et al.*, 2004). Furthermore, these cells had undergone a noticeable transformation tending toward anaplasia,

almost neoplasia (Owens & Hall-Mendellin, 1989; Owens *et al.*, 1991). The LOS cells sometimes exhibited cytoplasmic vacuoles (Owens & Hall-Mendellin, 1989; Owens *et al.*, 1991; Bonami *et al.*, 1992; Anggraeni, 1998; Hasson *et al.*, 1999b; Van de Braak *et al.*, 2002c; Shao *et al.*, 2004)

The nuclei of spheroid cells appeared hypertrophic and had marginated chromatin (Owens *et al.*, 1991; Boonyaratpalin *et al.*, 1993; Van de Braak *et al.*, 2002c). Pyknotic or karyorhectic nuclei were often observed within the spheroids (Van de Braak *et al.*, 2002c) (Figure 2.4a). Basophilic or eosinophilic cytoplasmic inclusions were common within the LOS cells (Owens & Hall-Mendellin, 1989; Bonami *et al.*, 1992) and magenta intranuclear inclusions could also be observed (Owens *et al.*, 1991) (Figure 2.4b) depended on the infectious agents. Furthermore, these inclusion bodies could be differentiated with Feulgen stain. For instance, Owens *et al.* (1991)found that the inclusions of prawns infected with LPV demonstrated Feulgen-positive reaction suggesting DNA viruses as the causative agent. Meanwhile, Bonami *et al.* (1992) reported Feulgen-negative reaction in the inclusion bodies of the prawns infected with LOVV revealing the presence of RNA virus. This confirmed that the production of spheroid cells could be from either DNA or RNA viruses.

According to Hasson *et al.* (1999b) the size of LOS masses ranged from 50 and 150 μm in diameter. These authors revealed that in TSV-challenged prawns, the LOS underwent morphological changes during the experiment. Therefore, they categorized the LOS cells into three distinct morphotypes referred to as Types A, B, and C (Figure 2.4c). Similarly, Owens (unpubl. data) classified the development of LOS into three stages: formation, encapsulation and degeneration. Later, Littik (2003) modified Owens categories with an intermediary phase between each phase. Furthermore, Type A appeared a slightly basophilic, homogeneous cell mass with few or no necrotic cells. The cytoplasm to nuclear volumetric ratio varied from 2:1 to 3:1. Type B spheroids exhibited an increase of necrotic cells/debris and few to a moderate number of vacuoles. Type C characterised by an overall increased basophilia, highly basophilic nuclei about 33 to 50% smaller than in the Type A and B, a decrease cytoplasm to nuclear volumetric ratio (ranging between 0.5:1 and 1:1) and few to numerous cytoplasmic vacuoles (Hasson *et al.*, 1999b).

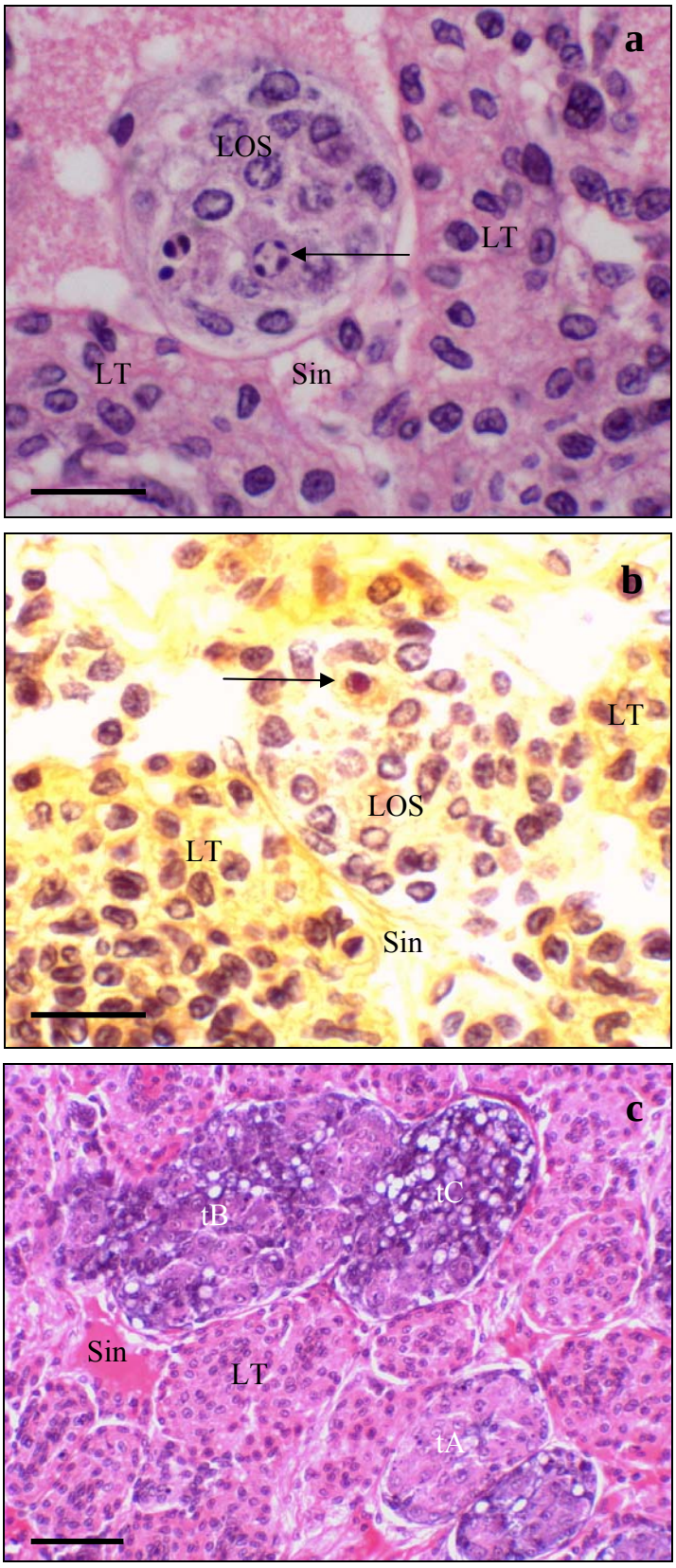



**Figure 2.4.** Longitudinal section of the LO of *P. monodon*. (a) Lymphoid organ spheroid (LOS) cells with karyolitic nuclei (arrow), H & E stain, scale bar = 50 µm; (b) phloxine & tartrazine stain of the LO showing intranuclear eosinophilic inclusion body resembling LPV inclusion body of Owens *et al.* (1991), scale bar = 50 µm; (c) three distinct morphotypes of LOS cells of *P. merguiensis* representing a developmental series: Type A (tA), Type B (tB) and Type C (tC) of Hasson *et al.* (1999b), H & E stain, scale bar = 20 µm. LT, lymphoid tubule; Sin, haemal sinuses.

The spheroids were formed in haemal sinus of the lymphoid organ and were separated from the stromal matrix of cells (Anggraeni & Owens, 2000). Spheroid cells as a result of staining for phenoloxidase (PO) and peroxidase activity, cytochemically had similar characteristics to small granular haemocytes and large granular haemocytes (Anggraeni & Owens, 2000; Shao *et al.*, 2004). However, morphologically by light microscopy, spheroid cells were not granulated. It seemed that LOS cells were exocytosed granular haemocytes. It was supposed that LOS cells initiated as haemocytes migrating (diapedesis) from the LO tubule lumen, through stromal matrix as phagocytic cells, contributing to the phagocytic nature of the stromal matrix. This was part of the normal haemocyte migratory route from the heart and dorsal aorta through the stromal matrix area into the haemal sinuses behind the stromal matrix and thence to the main haemocoel (Anggraeni & Owens, 2000). Both in the spheroids (Owens *et al.*, 1991; Van de Braak *et al.*, 2002c) and the matrix (Anggraeni & Owens, 2000) mitotic activity was never observed. This confirmed that the lymphoid organ was not a haematopoietic tissue as described by Martin *et al.* (1987) and Hose *et al*. (1992).

### 2.3.3. The role of the lymphoid organ

It is commonly accepted that the lymphoid organ may play important roles in the immunodefence mechanism of penaeids (Kondo *et al.*, 1994; Anggraeni, 1998; Hasson *et al.*, 1999b). The lymphoid organ had trapping ability when foreign substances were injected. The injected foreign substances were firstly found in the arteriolar tubules and then in the nodular structures between 1 and 3 months after the injection (Kondo *et al.*, 1994). This organ could immobilise foreign substances from the haemolymph, before they went from the arterial system into the open circulatory system. The LO was supplied directly from the heart, as a result it had a specialised type of arterial ending where sufficient pressure was maintained (Van de Braak *et al.*, 2002c).

The lymphoid organ (mistakenly identified as haematopoietic nodules of *S. ingenstis*) also eliminated bacteria from circulation more efficiently than other phagocytic organs such as the gills and digestive gland. Once bacteria were within this organ, they adhered and were phagocytosed by small granular haemocytes. Bacteria were

not observed in either hyaline or large granular haemocytes (Martin *et al.*, 1996). The findings were also supported by Van de Braak *et al.* (2002c) who found that small granular cells lining the endothelium were the major phagocytic cells in the LO. Furthermore, Anggraeni and Owens (2000) claimed that the spheroid cells had the characteristics of exocytosed, granular haemocytes that had phagocytosed foreign material, especially viruses, and might be the major defence mechanism to viral diseases.

Oka (1969) used the term of 'lymphoid organ' based on the location and structure of this organ. Since the 'lymphoid organ' of penaeids has no similarity with mammalian lymphoid tissue and crustaceans lack lymphoid cells, Van de Braak *et al.* (2002c) proposed to reconsider this terminology as 'phagocytic organ', 'haemolymph filtering organ', or 'antigen clearance organ' based on its function. However, this terminology (lymphoid organ) was generally accepted in studies of the immunology of penaeid prawns.

### 2.3.4. Diseases associated with the lymphoid organ

The lymphoid organ is only found in penaeids. In addition, the spheroid cells due to viral infections, have been observed in some penaeid prawn species i.e. *P. monodon, P. penicillatus* (Lightner *et al.*, 1987), *P. esculentus,* and *P. merguensis* (Owens & Hall-Mendellin, 1989; Owens *et al.*, 1991). Spheroids-associated lymphoid change was also discovered in *P. stylirostris* (Nadala *et al.*, 1992), *P. vannamei* (Bonami *et al.*, 1992; Hasson *et al.*, 1995), *P. japonicus* (Kondo *et al.*, 1994) and *P. chinensis* (Shao *et al.*, 2004).

The spheroid cells were firstly described as hypertrophic and metastasis of the lymphoid organ (Lightner *et al.*, 1987) and later on these spheroids were considered to be associated with lymphoidal parvovirus, LPV (Owens *et al.*, 1991), lymphoid organ vacuolization viruses, LOVV (Bonami *et al.*, 1992), rhabdovirus of penaeid prawn, RPS (Nadala *et al.*, 1992), yellow head virus, YHV (Boonyaratpalin *et al.*, 1993), and lymphoid organ virus, LOV later named gill associated virus, GAV (Spann *et al.*, 1995; Spann *et al.*, 1997). The presence of LOS cells was also found in association with Taura syndrome virus, TSV (Hasson *et al.*, 1995), spawner-

isolated mortality virus, SMV (Fraser & Owens, 1996), and recently detected in monodon slow growth syndrome, MSGS (Anantasomboon *et al.*, 2006) and Laem-Singh virus, LSNV (Sritunyalucksana *et al.*, 2006). Furthermore, these pathological changes were observed in white spot syndrome associated bacilliform virus, WSBV (Chang *et al.*, 1996; Guoxing *et al.*, 1997), and infectious viral diseases in *P. orientalis* in Korea (Park *et al.*, 1998).

A natural bacterial infection associated with the lymphoid organ has yet to be reported. However, some experimental studies revealed that changes of lymphoid organ were not only caused by viral infections, but also resulted from bacterial infections, as described by Martin *et al.* (1996) in the ridgeback prawn, *S. ingentis* injected with *Bacillus subtilis* bacteria, in *P. monodon* infected with *V. anguillarum* (Van de Braak *et al.*, 2002c) and *V. vulnificus* biotype I (Alday-Sanz *et al.*, 2002). However, LOS changes in the lymphoid organ due to bacterial infection (Alday-Sanz *et al.*, 2002; Van de Braak *et al.*, 2002c) are still doubtful because of the lack of statistical analysis; there were no comparative studies in histology between the challenged group and the control group, and there was no proof that the animals that they used were free from viral infection. Furthermore, these studies clearly show that bacterial antigens were primarily trapped in the stromal matrix of the LO whilst viruses were found in the areas of spheroidal change.

### 2.3.5. Conclusions

Since it was first discovered in *P. orientalis*, many researchers have studied the structure and the roles of the lymphoid organ in penaeid prawns. It is commonly accepted that the LO plays an important role in the defence mechanisms against foreign invaders. This organ has trapping ability for foreign material, immobilises foreign substances from the haemolymph, phagocytoses invaders, and contributes in elimination or degradation of pathogens. LPV, LOVV, LOV/GAV, TSV, YHV, WSBV, MSGS and LSNV are viruses associated with the LO. Generally, diseases associated with these viruses show spheroid cells as a result of LO changes at the cellular level. The spheroids display hypertrophy of nuclei, margination of chromatin and more basophilic cytoplasm. The publications that claim bacteria can

change the lymphoid organ of penaeids by producing spheroids are still unsubstantiated.

## 2.4. Moulting as It Interacts with the Immune System

### 2.4.1. Moult staging

In crustaceans, moulting is a cyclic phenomenon relating to growth. During this process, many structural, biochemical and behavioural changes are experienced by the organism (Peebles, 1977; Longmuir, 1983). There are two main methods to determine the moulting cycle in crustaceans. First, indication of actual time periods and second, observation of morphological changes of the skeleton and tissues (Travis, 1955). The last method was first developed by Drach who divided the moult stages of brachyurans *Cancer pagurus* and *Maia squinado* into four main stages A, B, C, D and some substages (Travis, 1955; Longmuir, 1983).

Later, the moult staging was developed further based on the modification of Drach's criteria. In the spiny lobster *Panulirus argus* (Travis, 1955, 1957), the moult cycle was investigated based on histological and histochemical changes to the skeleton. The moult stages of carideans *Processa edulis-edulis*, *P. acutirostris* and *Leander xiphias* were observed relating to colour changes, carbohydrate metabolism, and reproductive activity (Scheer, 1960). The structure and metabolism of the integumentary tissue were used to described the moult stages of the land crab *Gecarcinus lateralis* (Skinner, 1962). Furthermore, Stevenson *et al.* (1968) and Stevenson (1972) gave details of the moult stages in the crayfish *Orconectes sanborni* and *O. obscurus* relating to setae development, the epidermis changes, epidermal DNA content and protein content. Moreover, setal development was used to predict the moult cycle of *Astacus leptodactilus* (Van Herp & Bellon-Humbert, 1978), *Panulirus marginatus* (Lyle & MacDonald, 1983) and *Macrobrachium rosenbergii* (Peebles, 1977).

In penaeid prawn this physiological mechanism has been described in *P. merguiensis* (Longmuir, 1983), *Penaeus esculentus* (Wassenberg & Hill, 1984; Smith & Dall, 1985), *P. setiferus*, *P. stylirostris* (Robertson *et al.*, 1987), *P. indicus* (Vijayan *et al.*,

1997) and *P. monodon* (Promwikorn *et al.*, 2004). Most of the authors agreed to classify the moult staging schemes of penaeids into 5 major stages and substages. However, they had slight differences in defining the moult stages of penaeid species. These discrepancies might arise because of different methods and different species that were used to examine the stage of moult.

**Table 2.3.** Criteria for the moult staging of *P. esculentus* based on setal development (Smith & Dall, 1985)

| Moult staging | Criteria | Duration |
|---|---|---|
| A1 | Begins immediately after ecdysis.<br>Body is very soft and slippery and has membranous consistency.<br>Setae and setal bases are filled with cellular matrix. | 1h |
| A2 | Cuticle no longer slippery, but still soft and membranous.<br>Retraction of cellular matrix starts from proximal ends of setae.<br>Secretion of endocuticle begins. | 5h – 8h |
| B | Cuticle becomes more rigid with a parchment-like consistency.<br>In setal lumen, constriction begins and develops into cones. | 2d |
| C | Rigidity of exoskeleton reachs maximum.<br>Most setae show setal cones.<br>Epidermis fills setal base with a narrow translucent fringe. | 1d – 2d |
| D0 | Retraction of epidermis from setal bases until a straight line below setal base is formed. | 4d |
| D1` | In uropod, further retraction of epidermis from setal bases. Epidermal line is wavy.<br>In pleopod, invagination of epidermis begins. | 3d |
| D1`` | Epidermal line is moderately scalloped in uropod.<br>Invagination of epidermis becomes deeper in pleopod. | 3d |
| D1``` | Scalloping of epidermal is uniform and reaches maximum depth in uropod.<br>In pleopod, invagination at maximum depth. | 3d |
| D2 | Tip of new setae disrupt uniform edge of scalloping in uropod.<br>Setal shafts develop a distinct orange red colour.<br>Proximal ends of setae are forked and alter to become dull. | 2.5d |
| D3 | Exoskeleton becomes brittle and delicate.<br>Pinpoints of light appear in new setal nodes of uropod. | 9h |
| D4 | Exoskeleton becomes fragile. | 1h |
| E | Ecdysis. Ejection from old exoskeleton and everts the setae on the new exoskeleton. | 60s |

Using microscopical examination of setal development (setogenesis) in an excised whole pleopod of the banana prawn *P. merguiensis* (Longmuir, 1983) and the setogenesis of the posterior median part of the inner uropod of Indian white prawn *P. indicus* (Vijayan et al., 1997) , the moult cycle could be divided into stage A (early postmoult), stage B (late postmoult), stage C (intermoult), stage D (premoult) and stage E (ecdysis). In *P. merguiensis* stage D consisted of substage D0 (early premoult), substage D1 (new setal development) which composed of D1` (setal tip formation), D1`` (epidermal invagination), D1``` (epidermal invagination completion), and substage D2 (exoskeleton synthesis), and in *P. indicus* stage D ended at substage D2 - 3.

In *P. esculentus* and *P. monodon* (Smith & Dall, 1985; Promwikorn *et al.*, 2004), the edge of the inner uropod near the telson tip was observed for moult staging. This was supported with either histological sections of the abdomen or observation of setogenesis in the exopodite of the pleopods of *P. esculentus* (Smith & Dall, 1985). These criteria allowed defining five main moult stages, the premoult and postmoult substages and the relative duration of moult stages (Table 2.3). *P. setiferus* and *P. stylirostris* were staged using the development of cellular matrix in the setal bases and the degree of epidermal retraction coupled with the development of new setae (Robertson *et al.*, 1987).

Substages D2 and D3 could not be differentiated by morphological observation in *P. indicus*. Thus, these two substages were expressed as a single substage (Vijayan *et al.*, 1997). Although moult stage D of *P. merguiensis* was divided until substage D3 – D4, Longmuir (1983) was not able to describe these two substages by means of setogenesis. Therefore, substage D2 was the final substage that could be determined before ecdysis. However, Smith and Dall (1985) could categorize stage A into substages A1 and A2 and stage D from substage D0 to D4 in *P. esculentus*.

### 2.4.2. Moult cycle duration

The duration of the moult stages also differs between species. As the growth rate depends on size and temperature, the comparison of moult cycle duration between species is difficult (Robertson *et al.*, 1987). It was found that the duration of the

moult cycle of adult *P. setiferus* (males = 43 ± 7.1 g; females = 57 ± 5.8 g) and *P. stylirostris* (52 ± 6.9 g) was 13 -14 days and 11 – 12 days, respectively at temperature 27 – 29 $^{o}$C, salinity 34 – 41 ppt, and photoperiod 13 h light and 11 h dark. In *P. setiferus*, postmoult (A and B) occupied approximately 22%, intermoult (C) 19%, and premoult (D) 59% of the cycle. For *P. sytilirostris*, stages A and B occupied around 27%, stage C 17% and stage D 56% of the cycle (Robertson *et al.*, 1987).

Adult *P. indicus* with total length (TL) of 100 – 120 mm were held at temperature 30 ± 1 ºC and salinity 25 ppt had a moult cycle of between 7.5 d to 11.2 d. The premoult took 70.9% of the moult cycle. Intermoult and postmoult accounted for the remaining stages of the cycle; 10.45% and 18.35%, respectively (Vijayan *et al.*, 1997). *P. esculentus* with carapace length (CL) of 25.5 – 28.5 mm that were maintained at 25 ± 1 ºC and salinity 32 – 36 ppt had a moult cycle of approximately 20 d. Postmoult occupied approximately 10% of total period, intermoult constituted 5 to 10% of the moult cycle, while premoult had 80% of the moult period (Smith & Dall, 1985).

The size of animals also contributed to differences in the moult cycle duration. In *P. indicus* there was a significant difference ($p < 0.05$) between the size of animals and the duration of moult staging. It was found that the bigger the size of the prawn, the greater the moult cycle duration. Moult cycle duration of prawns sized 30 – 40 mm was approximately 4 d (premoult period of 3.2 d), whilst in young adult of size 60 – 80 mm was 7.5 d with premoult period of 5.4 d. The moult cycle duration increased to 10 d (premoult of 6.9 d) when the prawn gained the adult size of 80 – 120 mm (Vijayan *et al.*, 1997).

The process of ecdysis also varied between species. The fastest ecdysis was found in *P. esculentus*, 18 s (Wassenberg & Hill, 1984). Ecdysis of *P. indicus* was often more rapid than *P. merguiensis*, lasting between 30 and 50 s, and 40 s, respectively (Longmuir, 1983; Vijayan *et al.*, 1997). In *P. esculentus, P. setiferus, and P. stylirostris* the process of ecdysis lasted longer but less than 60 s (Smith & Dall, 1985; Robertson *et al.*, 1987). This discrepancy might be caused by the difference in determining ecdysis (Wassenberg & Hill, 1984; Vijayan *et al.*, 1997). It was

generally found that moulting in penaeids occurred at night (Longmuir, 1983; Wassenberg & Hill, 1984; Vijayan *et al.*, 1997).

### 2.4.3. Moulting and prawn immunity

The physiological functions of prawn are affected by moulting and it may also affect the immunological state of prawns. Currently, just a few studies have been done to investigate the association between defence mechanisms and moult staging in prawns (Le Moullac *et al.*, 1997). During the moult cycle, haematological variation appears in individuals and between species. For example, total haemocytes count (THC) of *P. stylirostris* was found significantly lower ($P < 0.1\%$) in intermoult (stage C) than in postmoult (stage B) and premoult (stages D0, D1, D2) (Le Moullac *et al.*, 1997). A different pattern was observed in *P. vannamei* in which the THC was significantly higher at C stage than at A and B stages (Liu *et al.*, 2003). In *M. rosenbergii*, the THC was significantly lower in premoult (D3) than in intermoult (C) (Cheng & Chen, 2001).

Using flow cytometry to analyse the relationship between moult staging and haemocyte types of *P. japonicus*, Sequeira *et al.* (1995) revealed that during moult stages B and D1, the highest number of cells was hyaline haemocytes, followed by small granular haemocytes and large granular haemocytes had the lowest number of cells. However, in intermoult (stage C) the percentage of hyaline haemocytes decreased while the number of small granular and large granular haemocytes increased. In contrast, the number of hyaline cells of *P. vannamei* was significantly higher at C stage, than at A, B, and D2/D3 stages. Large granular cells were significantly higher at stage D0/D1 than A and B stages (Liu *et al.*, 2003). In *P. stylirostris*, hyaline and small granular cells showed no significant differences at different moult stages. However, large granular cells were significantly higher in the intermoult stage (Le Moullac *et al.*, 1997). A similar pattern for large granular cells was also observed in *S. ingentis* (Hose *et al.*, 1992).

In *S. ingentis* nearly 100% of small granule haemocytes lacking glycoprotein deposits and large granule haemocytes showed strong prophenoloxidase activity in moult stage D. Whilst in the intermoult stage, fewer than 1% of these cell types

displayed proPO activity (Hose *et al.*, 1987). In contrast, phenoloxidase activity in *P. stylirostris* and *P. vannamei* was significantly higher ($P < 0.02$) in intermoult when large granular cells reached the highest number (Le Moullac *et al.*, 1997; Liu *et al.*, 2003). Phagocytic activity of *P. vannamei* against *V. alginolyticus* was also significantly higher at stage C. The clearance efficiency of the prawn showed no significant differences between stage C and B, D0/D1 and D2/D3 stages. However, clearance efficiency was significantly lower at stage A (Liu *et al.*, 2003).

The sensitivity of prawn to pathogens was supposed to be related to the stage of the moult. Mortality of *P. stylirostris* immersed in *Vibrio* AM23 ($2 \times 10^5$ cfu/ml) was significantly lower at intermoult (21%) than at premoult (48%) (Le Moullac *et al.*, 1997). In *P. vannamei* injected with *V. algonolyticus*, Liu *et al.* (2003) noticed that the cumulative mortality was also significantly lower at intermoult stage than that at the postmoult stage. These studies indicated that during the moult cycle, resistance to vibriosis might be correlated to the phenoloxidase activity (Le Moullac *et al.*, 1997; Liu *et al.*, 2003). However, there is no report comparing the immunological state between infected and uninfected prawn that might differ during the moult cycle. This is a gap that still needs investigation.

Although it is generally believed that the LO is involved in defence reactions of penaeid prawns, there is no paper revealing the relationship between the LO and moult cycle. Anggraeni & Owens (2000) predicted that area of spheroid cells might increase with increased animal size, if the spheroid cells accumulated in the haemal sinuses of the LO during the life of a prawn. However, there was no correlation between the area of spheroid cells and the prawn size. Therefore, they hypothesised that the spheroid cells were disposed of, possibly during ecdysis. This is the basis for further investigation of the relationships between the LO and moult cycle of the penaeid prawn.

### 2.4.4. Conclusion

Many writers described moult staging in different species of crustaceans. In prawns, most researchers categorized this physiological process into 5 major stages and various substages, although they had different approaches to define this process and

used different species. It was also found that the duration of moulting and ecdysis varied between species and size of animals. In terms of duration of a moult cycle, the premoult stage occupied the longest period, followed by post-moult, and the shortest was intermoult. Moult related changes in haemocytes and defence reactions seemed to be different within individuals and between species of prawns. Infected and noninfected prawns (health status) might also contribute to individual variability.

## 2.5. General Conclusion

Most authors claim a uniform theory concerning the immune response of crustaceans but most research actually only comes from the study of crayfish, crabs, or lobsters. It is doubtful whether there is a uniting theory because of the variability of immuno-reactivity between species of crustaceans. The variety of experimental methods can also contribute to different results by these researchers. Put simply, all aspects of immunoprotection in crustaceans are still a puzzle that needs advanced investigation.

# CHAPTER 3
# GENERAL MATERIALS AND METHODS

## 3.1. Experimental Animals

*Penaeus monodon* were used as experimental animals, unless otherwise stated. Prawns were caught with a cast net from commercial farms in northern Queensland, Australia. Prawns were reared and maintained in a 1,000 L plastic bin with recirculating system and an aerator. Salinity was maintained at 35 ‰ and temperature ranged from 28 – 30 ºC. During the experiment, prawns were kept individually in an aquarium to determine the moulting stages. Each aquarium was equipped with recirculating pump and an air lift corner filter. Prawns were fed at a rate of 10% of the biomass divided between 2 daily feedings (in the morning and the afternoon).

**Figure 3.1.** Experimental *P. monodon* were kept individually in recirculation aquaria to determine their moult stages. There were two modules which consisted of 8 aquaria and 1 recirculation tank with filter and each aquarium had an air lift corner filter. Salinity was maintained at 35 ‰ and temperature around 28 – 30 ºC.

### 3.2. Weight and Total Length of Prawn

For weight and total length measurements, each prawn was put in iced water as an anaesthetic, blotted dry with a paper towel, and weighed with a scale accurate to 0.001 g (ADAM AFP210L). The total length of each prawn was measured as the distance from the rostrum tip to the tip of the telson using a ruler with scale accurate 1 mm.

### 3.3. Moult Staging

Prawns were held individually in aquaria to study the moult stages. Determination of moult stage was done by microscopical examination of setal development and epidermal withdrawal in the inner uropod near the telson tip (Smith & Dall, 1985; Promwikorn *et al.*, 2004). Moult stages were categorised into stage A, B, C, D0, D1, D2 and D3/D4 and E (ecdysis). When the moult stages were simplified into three main categories, stage A and B were postmoult, stage C was intermoult and D was premoult. Photographs were taken under a light microscope (Olympus BH-2) connected to a digital camera Olympus Camedia (C-5050Z00M) or Q Imaging 32-0109A-443 Micropublisher 5.0 RTV.

### 3.4. Histology

#### 3.4.1. Tissue fixation

Prior to histological examination, prawns were anaesthetised by placing in iced water for a few minutes. The prawns were immediately fixed in Davidson's fixative by injecting 0.5 mL of the fixative into the hepatopancreas and adjacent area of the cephalothorax. Each prawn was immersed in Davidson's fixative at a ratio of tissue to fixative 1:10 for 48 hours. Then the cephalothorax was cut in half longitudinally, placed in a histocassette, stored in 70% ethanol and then processed for routine histological examination using standard paraffin embedded procedure (Bell & Lightner, 1988) and stained with H & E (see below).

### 3.4.2. Tissue embedding and cutting

Graded ethanol (70%, 80%, 90%, 95%, and 100%) was used to dehydrate the tissues before clearing with xylene. This procedure was run in a Shandon Elliot processor. Using a Tissue-Tek II embedding centre, the tissues were embedded in paraffin wax. A rotary microtome cut sections at 5 μm which were placed on slides. These tissue sections were dried out in an oven for at least 30 minutes at 60 ºC.

### 3.4.3. Haematoxylin and eosin staining

Haematoxylin and eosin was applied to stain the dried tissues as follows:

- xylene (2 minutes)
- xylene (2 minutes)
- ethanol (2 minute)
- ethanol (1 minute)
- ethanol (1 minute)
- tap water (1 minute)
- Mayer's haematoxylin (Appendix) (8 minutes)
- tap water (1 minute)
- Scott's tap water substitute (Appendix) (30 seconds)
- Young's eosin (Appendix) (4 minutes)
- tap water (1 minute)
- ethanol (10 dips)
- ethanol (1 minute)
- xylene (2 minute)
- xylene (1 minute)
- mounted in DPX (dibuthylphthalate-polystyrene-xylene)
- slides were dried in an incubator for a minimum one hour at 37 ºC.

## 3.5. Analysis of the Lymphoid Organ

Quantification of histopathological changes in the lymphoid organ was conducted by modifying a transect method that was developed by Littik (2003). This method was based on the abundance of LOS as assessed by using light microscopy and a

micrometer eyepiece with magnification 100X (10 x 10). Along a transect, the perpendicular bars of the eyepiece were used to count (sample) the number of the normal tubules and the LOS. Only the normal stromal matrix tubules and the LOS cells that come into contact with the bands at 10 divisions (unless otherwise stated) on either side of the scale line were counted. The sampling was carried out along the longest diagonal of the LO. Care was exercised to keep the sampling process on the right track and direction.

The ratio of the lymphoid organ spheroid cells to the total tissue (spheroid to total tissue) was determined as follows:

$$\text{STT ratio} = \frac{\text{The count of intersections of the eyepiece on the LOS}}{\text{The count of intersections of the eyepiece on the all the tissue}} \times 100\%$$

### 3.6. Data Analysis

Generally, to examine the effect of moulting stages, sex, weight, and total length on the spheroids to total tissue ratio, data was analysed using univariate analysis of variance (ANOVA) on SPPS Program version 11 after the data was determined to be normally distributed by using Q-Q plots. Post-hoc analysis was performed using least significant difference (LSD) to examine the mean differences in independent variables. Differences between means were considered significant at the $P < 0.05$ level.

# CHAPTER 4
# VALIDATION OF QUANTITATIVE ANALYSIS OF THE LOS CELLS IN PRAWNS

## 4.1. Introduction

Since it was discovered by Oka (1969), the lymphoid organ has received much attention directed towards discovering the roles of this organ in penaeid prawns. It is commonly accepted that the lymphoid organ plays important roles in immunodefence against foreign substances. This organ has trapping ability, immobilizes foreign substances and phagocytoses invaders (Kondo *et al.*, 1994; Martin *et al.*, 1996; Anggraeni & Owens, 2000; Van de Braak *et al.*, 2002c). It was proposed that the LO might be the major defence mechanisms to viral diseases in penaeid prawns (Anggraeni & Owens, 2000).

The changes of the lymphoid organ at the cellular level have been studied to some extent. Most studies of the LOS were conducted by applying routine histological, cytological and histochemical techniques (Owens *et al.*, 1991; Hasson *et al.*, 1999b; Anggraeni & Owens, 2000; Van de Braak *et al.*, 2002c; Shao *et al.*, 2004). In chronic TSV infections in *Penaeus vannamei*, the lymphoid organ spheroid cells were divided into three distinct morphotypes representing a developmental series (Hasson *et al.*, 1999a). The series began with type A which was characterized by homogeneous cell masses containing no or few necrotic cells. Type B LOS displayed an increased number of necrotic cells and nuclear pyknosis with few to moderate vacuole formation. Type C was typified by increasing nuclear condensation and vacuoles. By applying routine histological techniques with *in situ* hybridization, Hasson *et al.* (1999a) used gene probes to quantify the prevalence and the severity index for the LOS in TSV infection.

Similarly, Owens (unpubl. data) classified the changes of LOS cells into 3 phases: formation, encapsulation and degeneration. The formation phase was characterized by basophilic homogenous cell masses. When the lymphoid organ spheroid cells were bounded by a layer elongated fibrocytes, the LOS entered the encapsulation phase. The degeneration phase occurred when vacuolative degeneration of spheroids

appeared. Furthermore, Littik (2003) modified Owens' classification with an intermediary phase between each phase. In addition, Shao *et al.* (2004) differentiated two cell types within the lymphoid organ spheroids. In the first type, the cytoplasm to nuclear volumetric ratio was low, and cells developed marginated chromatin with 1 – 2 nucleoli. The second type had a large cytoplasmic to nuclear ratio, homogeneous chromatin, and underdeveloped marginated chromatin with one or no nucleoli.

In analysing the LOS, Anggraeni and Owens (2000) used an *in situ* hybridization probe for 18S rRNA, TUNEL assay, and Jandel Scientific Software (Sigma Scan and Sigma Scan pro) to analyse the area of spheroid cells and stromal matrix. To make the study cheap, simple and speedy, Littik (2003) introduced a quantitative measurement using a transect technique. This simple technique was reliable to quantify the histopathological changes in the lymphoid organ by using a light microscope and graduated eyepiece. However, in that study the researcher made three transect lines along the diagonal planes of the LO. As a result, there was an overlapping sampling in measuring the LOS. The present study modifies the transect method to provide a simple, rapid, and accurate method and to remove the problem of double counting to determine histopathological changes in the lymphoid organ of penaeids. This chapter validates, as far as possible, the likelihood that the sampling methods used were robust enough to represent whole lymphoid tissue.

## 4.2. Materials and Methods

### 4.2.1. Experimental animals

Slides of longitudinal sections of *P. merguiensis* (generously provided by Kerry Claydon, James Cook University) with H & E staining were observed for comparing a transect line at 1 and 10 divisions of the graduated eyepiece. These slides were also used to compare between three transect lines across a diagonal of the LO and one transect along the longest diagonal of the LO. Furthermore, to assess whether a transect was representative of the whole LO i.e. did it matter where the section was made relative to the whole organ, 10 *P. monodon* were examined from a commercial farm in northern Queensland, Australia.

### 4.2.2. Analysis of the lymphoid organ

A transect method developed by Littik (2003) was modified. Ten slides of longitudinal section of *P. merguiensis* were observed to compare the spheroids to total tissue ratio between 1 division and 10 divisions in one transect across the longest diagonal of the LO. As there was no difference between the two methods (see below 4.3), then sampling of spheroids was carried out by using 10 divisions for the other 20 slides and throughout other experiments. Therefore, there were 30 slides that were observed in this experiment to compare between the three transect lines and one transects lines (Figure 4.1). Only the normal tubules and the LOS cells that came into contact with the 1 or 10 divisions in either side of the scale line in the eyepiece were counted. All these measurements were performed in a magnification of 100X of the light microscopy. The abundance of the spheroid cells in the lymphoid organ was determined as the STT ratio (see Section 3.5).

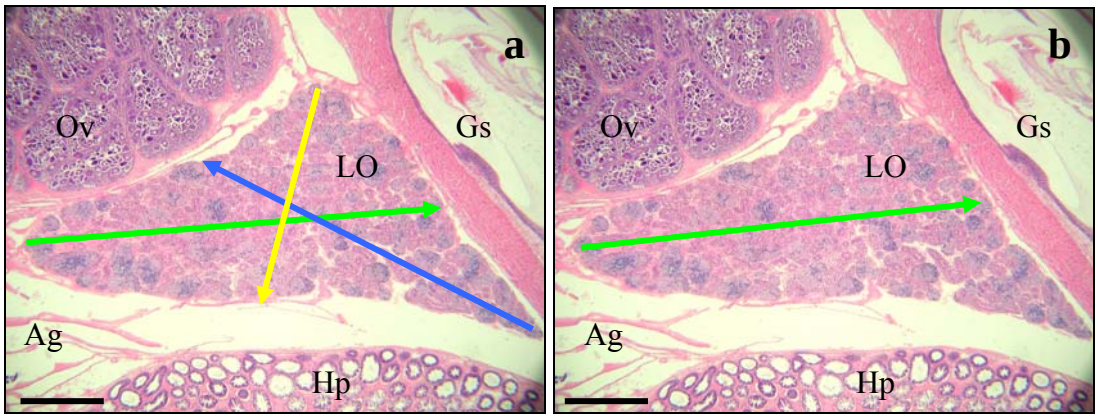



**Figure 4.1.** Transect lines for measurement of the LOS cells of *P. merguiensis*. (a) Three transect lines as previously developed by Littik (2003); (b) one transect along the longest diagonal of the lymphoid organ in the present study. H & E stain. Scale bar = 500 µm. Ag: antennal gland; Gs: gastric sieve; Hp: hepatopancreas; LO: lymphoid organ; and Ov: ovary.

In assessing the reliability of the sagittal section of the lymphoid organ, each histological block of 10 prawns were cut as a serial section 5 µm thick. Slides were made from each tenth section and 10 slides were made up from each block. This meant that there was about 500 µm (0.5 mm) of the sagittal section of the lymphoid organ was examined. This ensured that between one quarter and a half of the organ was examined, depending on the size of the lymphoid organ in the prawn.

### 4.2.3. Data analysis

Data was analysed by using a paired sample t-test in SPSS version 11 to compare between 1 division and 10 divisions of the graduated eyepiece. The difference was considered significant at P (two-tailed) < 0.05 level. Chi-squared test was performed to determine the difference between three transect lines and one transect lines at P < 0.05 level in Excel. The mean of STT ratio, standard deviation (SD), and coefficient of variation (CV) of one transect line in 10 divisions was also calculated so as to know at least how many slides should be assessed using this method.

The repeatability of a given longitudinal section representing the whole of the lymphoid organ was assessed by measuring the abundance of the lymphoid organ spheroid cells in every tenth slide in each block or prawn. A Chi-squared test was used to determine the spheroid to total tissue (STT) ratio with the mean of the ten slides from each block as *expected value* and the STT of randomly selected slides each block as an *observed value*. Randomization was carried out by using the random number generation in Excel.

## 4.3. Results

The lymphoid organ spheroid cells could be easily differentiated from the normal tubule by H & E staining. The LOS appeared to have a more basophilic cytoplasm, lacked the central lumen and had hypertrophic nuclei. Analysing the lymphoid organ spheroid with the quantitative method (transect method) by graduated eyepiece showed that there was no significant differences ($t = -0.727$; $df = 9$; $P > 0.05$) on the ratio of LOS to total tissue (STT ratio) when using 1 division or 10 divisions (Table 4.1).

The percentage of spheroid to total tissue from 30 prawns was very variable, ranging from 11.54% – 69.09% with a mean (± SD) 42.89 ± 13.98% (Figure 4.2). The mean of STT ratio and the standard deviation became stable when the number of prawns sampled reached 20 (Figure 4.2). Furthremore, it was found that the CV value of the data varied from 19 – 38%. The high variability of this CV value might occur due to the high variability of STT ratio between individul prawns that might have resulted

from differences of infection dose. Infected and uninfected animals might also have effect on the variability.

**Table 4.1.** The pheroid to total tissue (STT) ratio of *P. merguiensis* (n = 10), using every division and every tenth division of the scale of the graduated eyepiece.

| Number of animals | STT ratio (%) | |
|---|---|---|
| | Every division | Every tenth division |
| 1 | 47.50 | 45.00 |
| 2 | 32.46 | 31.58 |
| 3 | 32.06 | 29.41 |
| 4 | 37.78 | 37.04 |
| 5 | 61.90 | 69.05 |
| 6 | 59.19 | 59.46 |
| 7 | 35.93 | 37.04 |
| 8 | 41.27 | 41.03 |
| 9 | 50.00 | 55.56 |
| 10 | 48.18 | 48.48 |
| Mean | 44.63 | 45.37 |

Chi-squared tests of the two transect methods showed that the three transects and one transect were probably giving the same result ($\chi^2 = 24.533$; df = 29; $P > 0.05$). This means that using a single transect does not result in loss of sensitivity of the STT result compared to the three transect method.

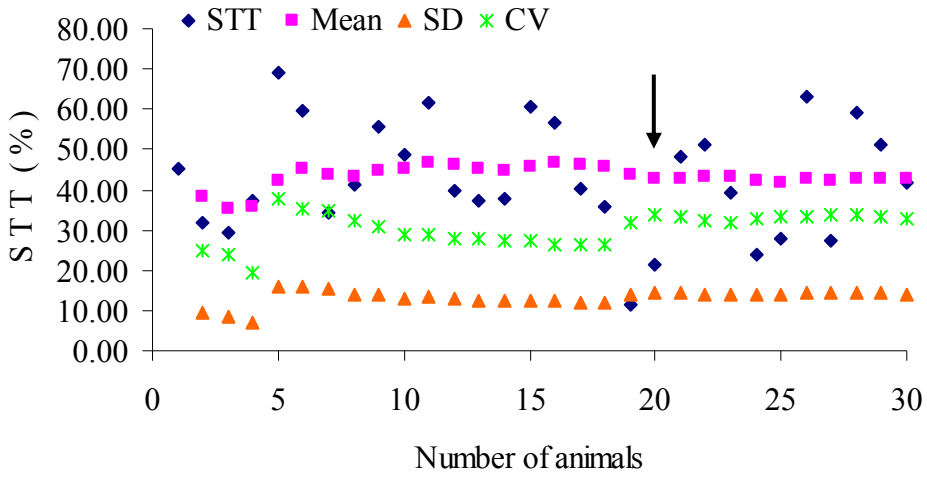



**Figure 4.2.** The spheroid to total tissue (STT) ratio of *P. merguiensis* (n = 30) by using one transect line in 10 divisions. SD, standard deviation; CV, coefficient of variation.

In assessing the repeatability and the representational nature of a single slide compared with the total tissue in the sagittal section of the lymphoid organ, it was found that in only two of the 50 trials was the Chi-squared test significant ($P < 0.05$) (Table 4.2). That meant that the chosen slide was not representative of the tissue 2 times out of 50. The chosen slide would represent the whole tissue 96% of the time. Therefore, the methodology was likely to be sound.

**Table 4.2.** Chi-square test of randomly selected slides ($\chi^2_{0.05,9} = 16.919$) of *P. merguiensis*. The red values represent when the Chi-squared was significantly different.

| Number | Sum of the Chi-squared ($\chi^2$) | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| 1 | 6.73734 | 7.74736 | 8.56792 | 16.34915 | 13.55885 |
| 2 | 13.10596 | 16.93989 | 8.32690 | 7.62206 | 13.86490 |
| 3 | 10.67365 | 8.02425 | 11.84960 | 6.38363 | 3.88428 |
| 4 | 4.84594 | 4.56094 | 16.86792 | 5.60284 | 14.17446 |
| 5 | 15.86414 | 1.23377 | 9.76098 | 9.79530 | 2.26715 |
| 6 | 5.41486 | 15.78923 | 10.57922 | *20.78334* | 9.18159 |
| 7 | 9.19821 | 5.67153 | *17.65637* | 11.37502 | 12.78347 |
| 8 | 5.89369 | 8.28438 | 5.30072 | 10.38974 | 12.97129 |
| 9 | 12.27661 | 3.93459 | 4.11652 | 4.32422 | 5.88471 |
| 10 | 9.86171 | 6.48430 | 6.20218 | 7.66106 | 5.20260 |

## 4.4. Discussion

Quantitative methods have been used to analyse the area of spheroid cells and stromal matrix cells in MCMS infected in laboratory-held and cultured *P. monodon*. (Anggraeni & Owens, 2000). To asses the total LO area, these authors utilized a computer with image measurement and analysis softwares (Jandel Scientific Software). It was apparent that this technique required skilled labour, was expensive (for the software) and time consuming.

In assessing the abundance and the size of the lymphoid organ spheroid, Littik (2003) utilized a simple quantitative technique. Since the histological sections of the lymphoid organ were cut randomly in a three dimensional tissue, it was likely that the two dimensional slide section chosen for the measurement of spheroids was not identical in each prawn. Furthermore, the size of the prawn influenced the size of the lymphoid organ. Therefore, to circumvent the positioning of the section and the size

bias, Littik (2003) applied a ratio for the spheroid size and called it spheroid-total length (STL) ratio.

However, in measuring the length of the spheroids and tubules in the LO, Littik (2003) used three transects along diagonals of the LO. As a result, there was an overlapping area enumerated. To overcome the problem, the present study only used one transect along the longest diagonal of the lymphoid organ and called it spheroid to total tissue (STT) ratio. The Chi-squared test revealed that the three transects and one transect were likely to give the same result in measuring the abundance of the lymphoid organ spheroid cells. Therefore, these two methods can equally be used to determine the lymphoid organ spheroids. However, the one transect method would be utilized throughout this thesis because it was much easier, simpler, rapid and had no bias.

The assessment of which divisions in the graduated eyepiece to use along the transect showed that there was no significant difference ($P > 0.05$) between 1 and 10 divisions in determining the abundance of the lymphoid organ spheroids. This confirmed that these two measurement methods could be performed to determine the abundance of spheroids in the lymphoid organ of penaeids. However, practically speaking, using every tenth division was simpler than using every division with limited loss of accuracy. Therefore, 10 divisions were utilized throughout the thesis.

Analysis with the Chi-squared test showed that only two (4 %) from 50 simulations were not representative of the whole tissue. Conversely, 96 % longitudinal sections would represent the whole tissue of the lymphoid organ. This confirmed that it did not matter where the longitudinal section was made relative to the lymphoid organ; it would give the same result or would be representative of the organ. This also revealed that one slide of the midsagittal section of the LO was accurate enough to determine the whole tissue of the LO. Therefore, the methodology was accurate to determine the changes of the lymphoid organ in penaeid prawns.

These findings suggested that the modified transect method was likely to be valid in assessing the abundance of the lymphoid organ spheroid cells within the lymphoid organ of penaeid prawns. It also confirmed that longitudinal section of the lymphoid

organ was likely to represent the abundance of the LOS within the three dimensional LO.

# CHAPTER 5
# THE RELATIONSHIP BETWEEN THE LOS CELLS AND MOULTING STAGES IN PRAWNS IN AQUARIA

## 5.1. Introduction

Crustaceans and other arthropods have a cyclic phenomenon for development and growth that is not encountered in most other groups of animals. Moulting is a process involving morphology, physiology, biochemistry, and behavioural changes in the life cycle of the animal. It may also affect the immunological status of the crustaceans. Moulting can be staged in two different ways based on actual time and morphological changes of the skeleton or tissues (Travis, 1957). The second method was first developed by P. Drach in 1939 (Travis, 1955) and later adapted by most researchers to determine the moult cycle of crustaceans.

The external changes of the exoskeleton and microscopical examination of setal development and epidermal withdrawal of the uropod and pleopod were commonly used to stage the moulting in penaeid prawns (Peebles, 1977; Longmuir, 1983; Smith & Dall, 1985; Robertson *et al.*, 1987; Vijayan *et al.*, 1997; Promwikorn *et al.*, 2004). Smith & Dall (1985) also verified their finding with histological examination of the second abdominal segment. This physiological mechanism has been observed in *M. rosenbergii* (Peebles, 1977), *P. merguiensis* (Longmuir, 1983), *P. esculentus* (Smith & Dall, 1985), *P. setiferus*, *P. stylirostris* (Robertson *et al.*, 1987), *P. indicus* (Vijayan *et al.*, 1997) and *P. monodon* (Promwikorn *et al.*, 2004). Most of the authors agreed in dividing the moult cycles into 5 stages (A, B, C, D, and E) and substages. In addition, Wassenberg and Hill (1984) have also described the moulting behaviour of the tiger prawn *P. esculentus.*

Since the growth rate depended on size and temperature, it was difficult to compare the moult cycle duration between species (Robertson *et al.*, 1987). The greater the size, the longer the moult cycle duration of the prawn (Vijayan *et al.*, 1997). Salinity and photoperiod also contributed to moult cycle variation. Premoult (D) occupied the longest period of the moult followed by intermoult (C) and postmoult (A and B) (Smith & Dall, 1985; Robertson *et al.*, 1987; Vijayan *et al.*, 1997). Ecdysis, the

actual act of flicking out of the old exoskeleton, generally was less than 1 minute in penaeid prawns (Longmuir, 1983; Wassenberg & Hill, 1984; Smith & Dall, 1985; Robertson *et al.*, 1987; Vijayan *et al.*, 1997).

Biochemical and physiological changes in various tissues of crustaceans occurred during the moult cycle. Glycogen, phosphatase and calcium, colour change, reproductive activity, and carbohydrate, protein, and lipid content varied during the cycle of the moult. The concentration of haemocyanin, the major component of the haemolymph also fluctuated (Travis, 1955, 1957; Scheer, 1960; Stevenson, 1972; Sarda *et al.*, 1989; Chen & Cheng, 1993; Chang, 1995). Haemolymph protein, oxyhaemocyanin, osmolality, $Cl^-$, $Na^+$, $K^+$, and $Ca^{2+}$ levels differed with the moult cycle (Cheng *et al.*, 2002). Changes to crustacean cardioactive peptide were also associated with the moult cycle (Phlippen *et al.*, 2000).

The immune components of prawns in association with moulting stages have been studied to a lesser extent. Haematological variation in both THC and DHC appeared in individuals and between species during the cycle of the moult (Hose *et al.*, 1992; Sequeira *et al.*, 1995; Le Moullac *et al.*, 1997; Cheng & Chen, 2001; Liu *et al.*, 2004). The stage of the moult also contributed to the differences in prophenoloxidase activity, phagocytic activity, and clearance efficiency of penaeid prawns (Hose *et al.*, 1987; Le Moullac *et al.*, 1997; Liu *et al.*, 2004). Furthermore, the resistance of prawn to pathogens was suggested to relate to this physiological mechanism (Le Moullac *et al.*, 1997; Liu *et al.*, 2004).

However, there is no study revealing the relationship between the lymphoid organ spheroid cells and the cyclic physiological process of the prawn, moulting. A previous study predicted that if the lymphoid organ spheroid cells accumulated in the haemal sinus of the LO, then the area of the spheroid cells might increase with increased animal size (Anggraeni & Owens, 2000). However, they did not find any correlation between spheroid cell area and the prawn size. Therefore, they hypothesised that the spheroid cells were disposed of possibly during moulting (ecdysis). The present study investigated the association between the moult stages and the abundance of the lymphoid organ spheroid cells within the lymphoid organ of penaeid prawns.

## 5.2. Materials and Methods

### 5.2.1. Experimental animals

Prawns (*Penaeus monodon*) were caught with a cast net from commercial farms in northern Queensland and transported to aquaria at the School of Veterinary and Biomedical Sciences, James Cook University on October 29th 2004. The prawns were immediately acclimated and kept in two 1,000 L plastic bins with a recirculating system and two aerators for each bin. The prawns were transferred and kept individually in glass aquaria (60 x 60 x 30 cm) with recirculation throughout the experiment.

Prior to use, the filter system and experimental aquaria were chlorinated with liquid chlorine (100 g/l) at 30 ppm overnight. The next day, the equipment was rinsed with fresh water overnight and once again the following day. During the experiment, salinity was maintained at 35 ‰ both in the bins and aquaria and temperature ranged from 28 – 30 ºC. The experimental animals were fed with commercial prawn pellet of 10% total body weight divided into 2 daily feedings. The waste was siphoned from the aquaria once every two days.

Fifty-four prawns, with a mean body weight of 5.28 ± 2.38 g (1.94 – 12.93 g) and with a mean total length of 8.8 ± 1.8 cm (6.7 – 11.8 cm) were used for the first experiment. The first experiment was conducted from 29 October 2004 until 08 January 2005. In the second experiment (08 January – 15 April 2005), fifty prawns, with a mean body weight of 15.17 ± 3.28 g (9.07 – 23.20 g) and with a mean total length of 12.2 ± 0.8 cm (10.4 – 14.0 cm) were used.

### 5.2.2. Moult staging

Prawns were held individually in aquaria to study the moult stages. Moult was staged by microscopical examination of setal development and epidermal withdrawal in the inner uropod near the telson tip (Smith & Dall, 1985; Promwikorn *et al.*, 2004). Prior to staging the moult, prawns were anaesthetised by placing in ice water for a few minutes. Sampling was done soon after moulting, at 1 h, 2 h, 4 h, 8 h, 16h,

and 1 day until 12 days after moulting for the first experiment. For the second experiment sampling was carried out soon after moulting, the day of moulting, and 1 day to 13 days after moulting. Three animals were sampled at every time point for each experiment. Three prawns were kept alive as controls to observe the cycles of the moulting (ecdysis) during the experiments. The lunar cycle was categorized into four cycles by using a lunar calendar, i.e. new moon phase, first quarter moon phase, full moon phase, and last quarter moon phase in which day of sampling was included into 3 – 4 days prior to and 3 – 4 days following the apex of the lunar phases.

### 5.2.3. Histology

Prior to histological examination, prawns were fixed in Davidson's fixative by injecting 0.5 mL of the fixative into the hepatopancreas and adjacent area of the cephalothorax and then immersed in the fixative at a ratio of tissue to fixative 1:10 for 48 hours. The cephalothorax was cut in half longitudinally, placed in a histocassette, stored in 70% ethanol and then processed for routine histological examination using standard paraffin embedded procedure (Bell & Lightner, 1988). Sections were cut at 5 μm and stained with H & E.

### 5.2.4. Analysis of the lymphoid organ

Histopathological changes in the lymphoid organ were quantified by using the transect method (Littik, 2003) which was modified (Chapter 4). Sampling of lymphoid organ spheroid cells was carried out along the longest diagonal of the lymphoid organ. Only the normal tubules and the LOS cells that come into contact with the crossbars at 10 divisions on either side of the scale line were counted. Care was exercised to keep the sampling process on the right tract and direction.

### 5.2.5. Data analysis

Fifty-four slides from the first experiment and fifty slides from the second experiment were assessed to examine the correlation between time of sampling, moult stages, lunar cycle, sex, weight, total length and the measurement of the LOS cells (spheroids to total tissue ratio, prevalence of vacuolated spheroid and number of

vacuoles in spheroids). Data was analysed using univariate analysis of variance (ANOVA) on SPPS Program version 11. ANOVA was run twice in each LOS measurement in two different moult categories, one with moult stages (A, B, C, D0, D1, D2, D3/D4) and another one with three main moult stages (postmoult, intermoult and premoult). Differences between means were tested using least significant differences (LSD) multiple comparison and considered significant at the $P < 0.05$ level.

## 5.3. Results

### 5.3.1. Moult staging

The setal development (setogenesis) and retraction of epidermis from the setal bases (apolysis) of the inner uropod near the telson tip gave a clear indicator of the moult cycle of *P. monodon*. Based on these criteria, the moult cycle of *P. monodon* could be categorized into five stages: postmoult (stages A and B), intermoult (stage C), premoult (stage D consist of sub-stages D0, D1, D2, D3/D4) and ecdysis (stage E).

#### 5.3.1.1. Stage A

Stage A (early postmoult) (Figure 5.1) could be divided into two substages, A1 and A2. Substage A1 began soon after the prawn had flicked clear of the old cuticle. The whole body and exoskeleton were very soft and slippery. Setae were also very delicate. The setae and setal bases were filled with cellular matrix. The setal cone could not be seen. The setal node was poorly developed. Substage A1 lasted 1 hour after moulting (first experiment).

When the cellular matrix started to retract from the distal end of the setae, the prawn entered the substage A2. Constriction of cellular matrix continued until only the distal half of setae was filled. Setal bases were still filled with cellular matrix. Setal node more developed. The cuticle was still soft to touch but not slippery. However, hardening of the exoskeleton had begun especially in the carapace and the sixth abdominal. Stage A2 lasted between 4 and 8 hours after moulting (first experiment).

5.3.1.2. Stage B

Eight hours after moulting, stage B (late postmoult) (Figure 5.1) started. This stage was marked by the retraction of the cellular matrix until the proximal half of the setae was emptied and was easily recognized by the clear space present in the base. The constriction of cellular matrix continued at the proximal end of the setae to form setal cones. Setal cones began to develop in some setae. Well developed setal nodes could be observed in most setae. Hardening of the exoskeleton continued especially in the sixth abdominal segment and the carapace. This stage lasted around 16 – 24 h after moulting (first experiment).

5.3.1.3. Stage C

Stage C (Figure 5.1) started 24 hours or day 1 after moulting (both experiments). Cellular matrix was absent from the setal lumen. The secretion and hardening of the exoskeleton reached a maximum in this stage and was marked by complete development of setal cones. The setal bases were filled by the epidermis as well as around the setal nodes. Stage C ended when the epidermis retracted from the setal bases.

5.3.1.4. Stage D

When the separation of cuticle at the setal bases began due to the retraction of epidermis and the development of new setae, the prawn entered the premoult, stage D (Figure 5.1). Most researchers agreed in dividing the premoult stages into 5 substages from D0 to D4. Stage D0 was marked by the withdrawal of epidermis (apolysis) from the setal bases of the uropod. The retraction of epidermis from the setal bases continued until it formed a straight line at the bases of the setae. This was the end of the substage D0. This substage was observed between 2 and 3 days after moulting in the first trial or between 1 and 4 days after moulting in the second trial.

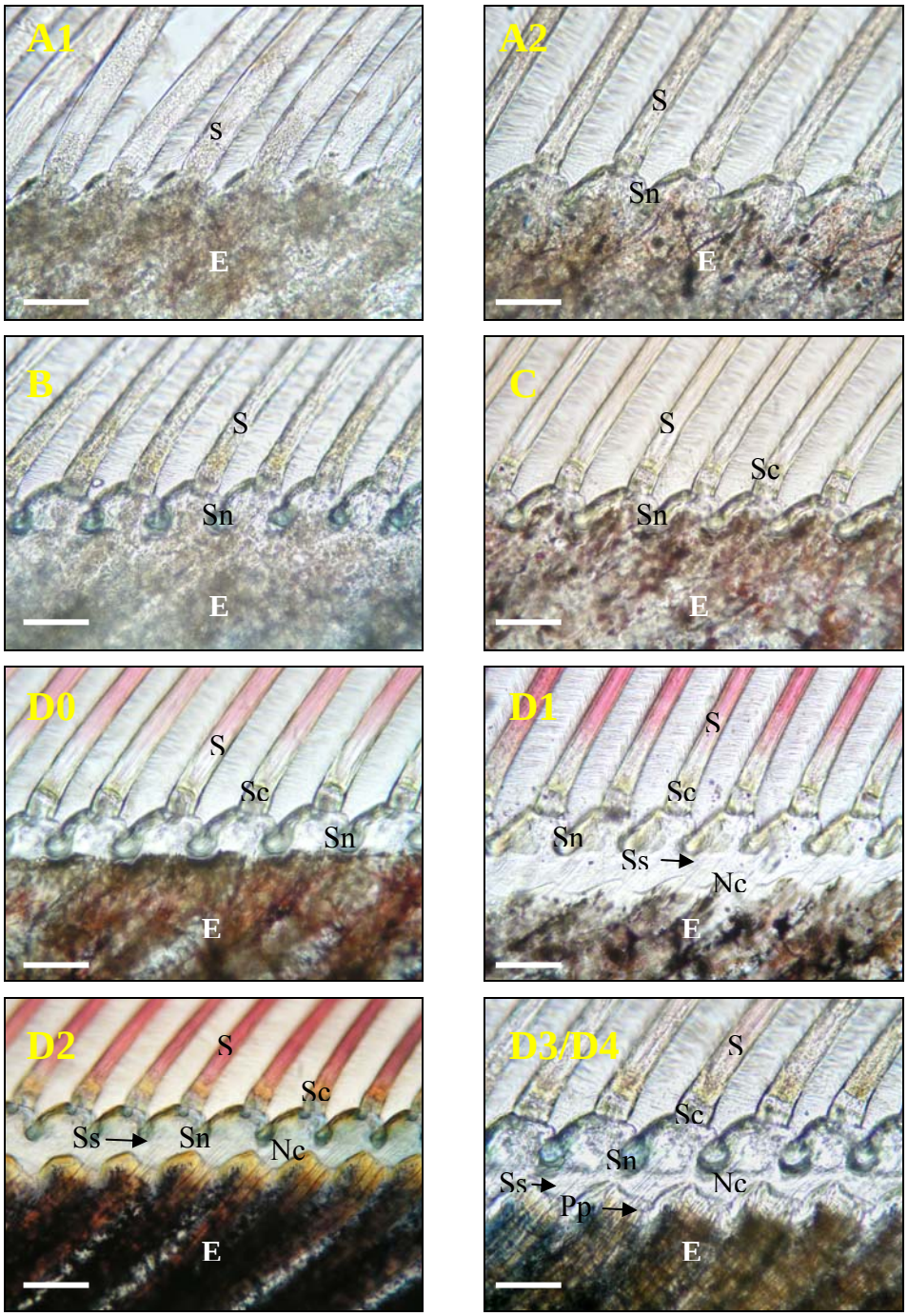



**Figure 5.1.** Moult staging of *P. monodon.* Median part of inner uropod near the telson tip was examined to stage the moult and photographed under a light microscope (Olympus BH-2) connected to digital camera (Olympus Camedia C-5050 ZOOM). Scale bar = 100 µm. E, epidermis; S, setal shaft; Sc, setal cone; Sn, setal node; Ss, new setal shaft, Nc, new cuticle; Pp, pinpoints of light where new setal node will develop.

In stage D1 (Figure 5.1), further withdrawal of epidermis from the setal bases was recognised by a clear zone between the setal bases and epidermis. The clear zone marked the formation of new cuticle. The setal node had swollen like a translucent

membrane. Setal invagination appeared like a tube for the first time and setal tips could be observed. Retraction of epidermis from the setal bases continued and formed a wavy like pattern. Sometimes pigment was observed to form straight line under the wavy epidermis. New setal shafts of the developing setae appeared as translucent fibre from the wavy edge of the epidermis. At the end of stage D1, the scalloping of the epidermal edge became uniform and reached a maximum. This stage extended for a period of 3 to 8 days after moulting in the first experiment and around 3 to 13 days in the second experiment.

At the next premoult, stage D2 (Figure 5.1), setal nodes started to deform. Later, the setal shaft changed its colour to red orange. This was the most obvious feature that could be observed in stage D2. This stage could be observed from 7 to 12 days after moulting in the first experiment and 6 to 12 days after moulting in the second experiment. The present study did not differentiate between moult stage D3 and D4 due to the need for scarifying the animals at every sampling time. Therefore, these two stages were expressed as a single stage observed before ecdysis. Stage D3/D4 was characterized by the appearance of light pinpoint in the new setal nodes when examined under the light microscope. This stage was observed 10 – 12 days after moulting in the first experiment and day 13 after moulting in the second experiment. This stage lasted for a few hours before moulting.

5.3.1.5. Stage E

Stage E was the last process observed and was called ecdysis. The prawns flicked out of the old exoskeleton and everted the setae of the new exoskeleton. The exuviation process began in the carapace followed by the abdomen while the animal was lying on it side on the bottom of the aquarium. This extraction process took less than 1 minute.

Staging the moult of the prawns was sometimes difficult. It was commonly found that the setogenesis was completed before the withdrawal of epidermis in the setal bases (apolysis). However, in some prawns the epidermis retracted before the setal cones in the setae were formed (Figure 5.2). In staging the moult, if this occurred in

the median part of the inner uropod of the prawn, then the withdrawal of epidermis was used to determine the moult stage rather than the development of setae.

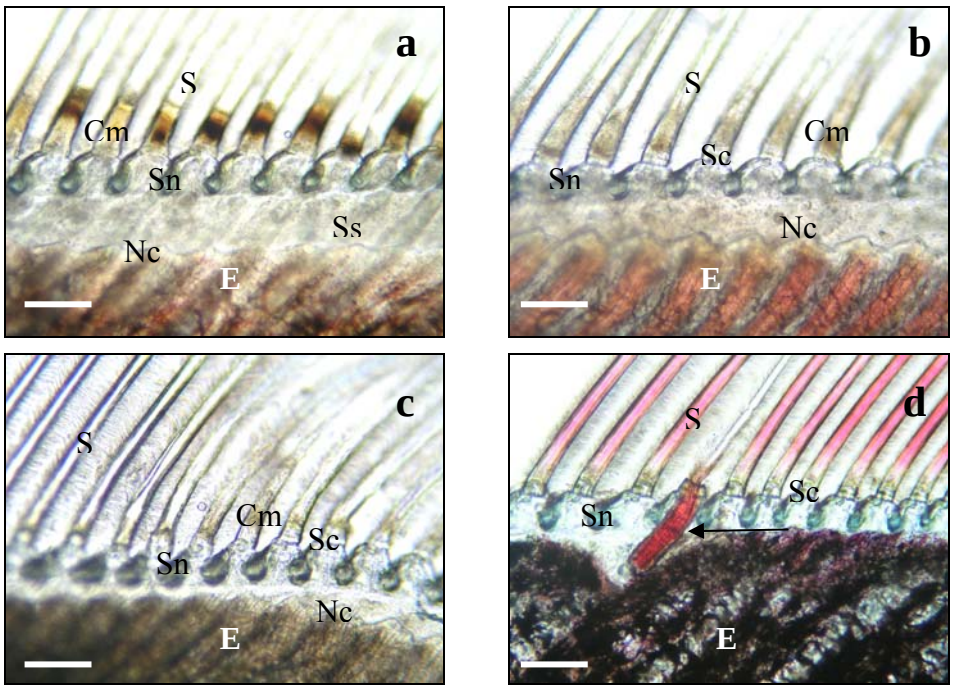



**Figure 5.2.** Abnormal setal development in the median part of the inner uropod near the telson tip of *P. monodon*. (a) Further retraction of epidermis from the setal bases suggested stage D1 while the constriction of cellular matrix in the proximal end of setae where the setal cones would be formed pointed to stage B; (b) late stage D2 was marked by the colour change of the setal shaft to red orange while setal cones have not yet formed in the setae (stage B); (c) apolysis in stage D2 with some setae already having setal cones (stage B), some setae were not symmetrical (swollen like) in deformity of uropod; (d) one setum (arrow) just grew like the final stage before moulting (stage D4) while the epidermis had already retracted from the setal bases (stage D1). Scale bar = 200 µm. E, epidermis; S, setal shaft; Ss, new setal shaft, Sc, setal cone; Sn, setal node; Nc, new cuticle; Cm, cellular matrix.

Observations on the cycle of the moult from 3 control prawns revealed that one moult cycle took around 8 to 12 days in the first experiment or between 8 and 22 days in the second experiment (Table 5.1). It was also found the premoult occupied the longest period of the moult (2 – 12 days after moulting in the first experiment or 1 – 13 days after moulting in the second experiment), followed by intermoult (1 day after moulting) and postmoult (1 – 16 hours after moulting). Some mortality occurred during the experiment. In the first experiment there were 5 mortalities, 4 prawns jumped out of the tanks and 1 prawn got stuck in a corner filter. In the second experiment 5 mortalities occurred, 3 prawns jumped out, 1 prawn died during moulting, and another 1 died for no known reason.

**Table 5.1.** Moulting (ecdysis) of three control *P. monodon* at four consecutive lunar phases throughout the experiments. Different colours represent the time of the ecdysis of different animals and the number in the same column is the moulting period of prawn from one period to another period. There were three prawns in the third control in the first experiment due to replacement of prawns that jumped out of the tanks. FM, full moon; LQM, last quarter moon; NM, new moon; FQM, first quarter moon.

| Lunar phases | Control animals | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | First study | | | | | Second study | | |
| | 1 | 2 | 3 | | | 1 | 2 | 3 |
| | | | a | b | c | | | |
| FM | | | | | | | | |
| LQM | | | | | | | | |
| NM | 8 | | 9 | | | | | |
| FQM | 10 | | 9 | | | | | |
| FM | | | | | | | | |
| LQM | 9 | | | | | | | |
| NM | 9 | 8 | | | | | | |
| FQM | 9 | 8 | | 10 | | | | |
| FM | 10 | 10 | | | | | | |
| LQM | | 9 | | | 12 | | | |
| NM | 10 | | | | | | | |
| FQM | | | | | | 12 | | 13 |
| FM | | | | | | | | |
| LQM | | | | | | 12 | | 13 |
| NM | | | | | | 11 | | |
| FQM | | | | | | | 8 | 15 |
| FM | | | | | | 12 | 10 | |
| LQM | | | | | | | | 17 |
| NM | | | | | | 13 | 15 | |
| FQM | | | | | | | | |
| FM | | | | | | 16 | 14 | 21 |
| LQM | | | | | | | | |
| NM | | | | | | 14 | | |
| FQM | | | | | | | 22 | 19 |
| Moulting period (days) | 8-10 | 8-10 | 9 | 10 | 12 | 12-16 | 8-22 | 13-21 |
| Average (days) | 9.3 | 8.8 | 9.0 | 10.0 | 12.0 | 12.9 | 16.3 | 13.8 |

### 5.3.2. Moult stages and lunar phases

It was found that most prawns (50.00%) in the first experiment moulted around last quarter moon (Table 5.2), followed by first quarter moon (18.52%), full moon (16.67%) and the lowest was at new moon (14.82%). The lunar pattern of moulting

changed for the second experiment. The data (Table 5.2) showed that ecdysis peaked around full moon (38.00%), followed by first quarter moon (24.00%), last quarter moon (22.00%) and then new moon (16.00%).

**Table 5.2.** The percentage of moulting (ecdysis) of *P. monodon* in the first and second experiments at four lunar phases. NM, new moon; FQM, First quarter moon; FM, full moon; LQM, last quarter moon.

| | Number of animals | | Ecdysis (%) | |
|---|---|---|---|---|
| Lunar phases | First study | Second study | First study | Second study |
| NM | 8 | 8 | 14.81 | 16.00 |
| FQM | 10 | 12 | 18.52 | 24.00 |
| FM | 9 | 19 | 16.67 | 38.00 |
| LQM | 27 | 11 | 50.00 | 22.00 |
| Total | 54 | 50 | 100.00 | 100.00 |

In the control animals (Table 5.1), it seemed that ecdysis occurred in any phase of the moon or every week in the first experiment. While in the second experiment, moulting occurred every two weeks or within one interval of the lunar phase. Later on, the animal moulted in two intervals of the moon phases (every three weeks). It seemed that prawns in the second trial moulted in synchrony with the moon phase. The discrepancy might have occurred due to the differences in age and size of the prawns in the two experiments. It was found that prawns in the second experiment were older and larger than those in the first experiment.

The moult stages of sampled prawns also varied within the lunar rhythm. In the first experiment (Table 5.3), stage D2 was more abundant (5.56%) than the other stages at full moon. Stage A was the highest (14.81%) at last quarter moon. Stage D1 was the largest at new moon and accounted for 12.96% of samples. In the first quarter moon, sampled prawns were equally distributed within the cycles of moult. In the second experiment (Table 5.4), stage D1 was most abundant around new moon, first quarter moon, and last quarter moon. In the first quarter moon, only stage A was found to account for 8.00% of samples.

In the first experiment, 40.74% of experimental animals were sampled around the last quarter moon. There were 29.62% of prawns sampled at new moon. Sampling at full and first quarter moon accounted for 14.96% and 14.80% of animals, respectively (Table 5.3). In the second experiment, most prawns were sampled

around full moon (42.00%), followed by last quarter and new moon accounted for 26.00% and 24.00% of animals respectively. First quarter moon produced the lowest percentage of sampling accounting for 8.00% of experimental animals (Table 5.4).

**Table 5.3.** The percentage of *P. monodon* sampled at different moult stages at four lunar phases in the first experiment. NM, new moon; FQM, first quarter moon; FM, full moon; LQM, last quarter moon.

| Lunar phases | Moult stages (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | D0 | D1 | D2 | D3/D4 | Total |
| NM | 3.70 | 0.00 | 1.85 | 1.85 | 12.96 | 9.26 | 0.00 | 29.62 |
| FQM | 3.70 | 1.85 | 1.85 | 3.70 | 0.00 | 3.70 | 0.00 | 14.80 |
| FM | 0.00 | 1.85 | 1.85 | 0.00 | 3.70 | 5.56 | 2.00 | 14.96 |
| LQM | 14.81 | 7.41 | 0.00 | 1.85 | 7.41 | 1.85 | 7.41 | 40.74 |
| Total | 22.21 | 11.11 | 5.55 | 7.40 | 24.08 | 20.36 | 9.41 | 100.13 |

**Table 5.4.** The percentage of *P. monodon* sampled at different moult stages at four lunar phases in the second experiment. NM, new moon; FQM, first quarter moon; FM, full moon; LQM, last quarter moon.

| Lunar phases | Moult stages (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | D0 | D1 | D2 | D3/D4 | Total |
| NM | 0.00 | 2.00 | 0.00 | 8.00 | 12.00 | 2.00 | 0.00 | 24.00 |
| FQM | 8.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 8.00 |
| FM | 6.00 | 4.00 | 4.00 | 4.00 | 14.00 | 10.00 | 0.00 | 42.00 |
| LQM | 0.00 | 0.00 | 2.00 | 2.00 | 12.00 | 6.00 | 4.00 | 26.00 |
| Total | 14.00 | 6.00 | 6.00 | 14.00 | 38.00 | 18.00 | 4.00 | 100.00 |

### 5.3.3. The abundance of the LOS cells

With H & E staining, the spheroid cells (Figure 5.3) appeared to have a more basophilic cytoplasm and spheroids lacked a central lumen. Some spheroids were surrounded by flattened epithelial cells and some were vacuolated. There were 64.84% of prawns from the first experiment exhibited spheroid cells within the lymphoid organ. The abundance of the spheroids from all samples varied from 0.00 – 75.00% with a mean (± SD) of 17.46 ± 20.80%. It was found that 57.41% of samples showed vacuoles within the spheroid cells. The number of vacuoles ranged between 0.00 – 10.50 with a mean of 1.03 ± 1.93 per spheroid. The prevalence of vacuolated spheroids varied from 0.00 – 100.00% with a mean of 36.02 ± 38.57%.

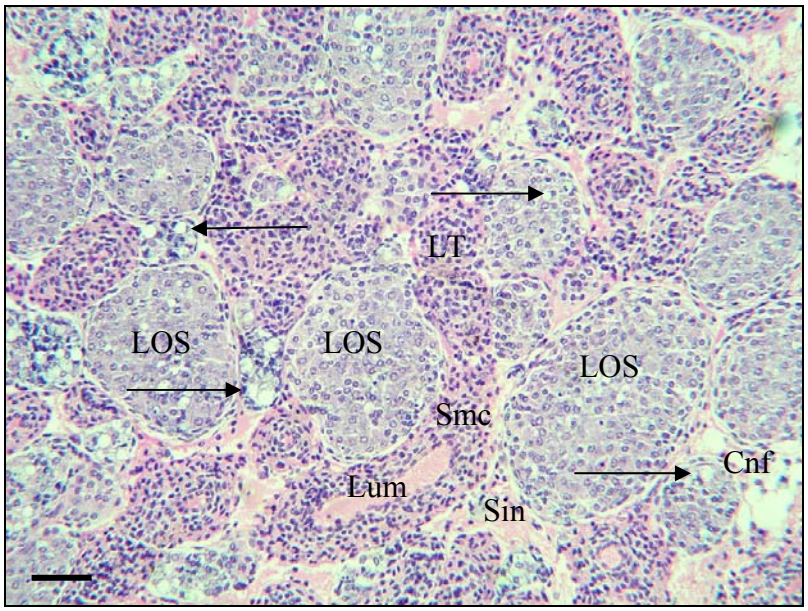



**Figure 5.3.** Light micrograph of longitudinal section of the LO of *P. monodon*. LOS cells accumulated in the haemal sinuses (Sin) and appeared to have a more basophilic cytoplasm and lack of a central lumen (Lum) compare to the normal lymphoid tubule (LT). Some spheroids demonstrated cytoplasmic vacuoles (arrow). H & E stain. Scale bar: 50 µm. Cnf, connective tissue fibre; Smc, stromal matrix cells.

In the second experiment, there was a decrease in the ratio of spheroid cells, the prevalence of vacuolated spheroids and the number of vacuoles in spheroid. There were 48.00% of animals showed spheroid cells within the lymphoid organ. The STT ratio varied between 0.00 – 55.56% (8.92 ± 13.99%). There were 30.00% of all slides examined had vacuoles within the spheroids. The number of vacuoles ranged between 0.00 – 6.00 (0.27 ± 0.89) per spheroid. The prevalence of vacuolated spheroids varied from 0.00 to 100.00% (10.70 ± 22.88%). One way analysis of variance showed that the STT ratio ($F = 5.932$; $df = 1, 103$; $P < 0.05$), the prevalence of vacuolated spheroids ($F = 16.238$; $df = 1, 103$; $P < 0.05$) and the number of vacuoles in spheroid ($F = 6.625$; $df = 1, 103$; $P < 0.05$) were significantly higher in the first experiment than in the second.

5.3.3.1. Time after moulting and the LOS cells

In the two experiments, it seemed that the LOS tended to be higher soon after moulting (Figure 5.4a), decreased at day 1 after moulting, and slightly climbed up according to the time after moulting then reached a peak at day 7 (40.92%) in the first experiment and day 11 (29.28%) in the second experiment with a fluctuation

between days. After that, the spheroid to total tissue ratio dropped dramatically in the first experiment and slightly in the second experiment until the end of sampling. However, a univariate analysis of variance revealed that there was no significant differences in the time after moulting on the spheroid to total tissue ratio (Table 5.5), either in the first experiment ($F = 2.405$; $df = 11, 53$; $P > 0.05$) or in the second experiment ($F = 3.379$; $df = 12, 49$; $P > 0.05$).

**Table 5.5.** F values of main effects on the STT ratio of *P. monodon* in the first and second experiments.

| Source | First study | | | Second study | | |
|---|---|---|---|---|---|---|
| | F | df | Sig. | F | df | Sig. |
| Weight | 0.824 | 1 | 0.394 | 0.003 | 1 | 0.956 |
| Total length | 0.773 | 1 | 0.409 | 0.213 | 1 | 0.664 |
| Sex | 0.037 | 1 | 0.852 | 2.559 | 1 | 0.171 |
| Time after moulting | 2.405 | 11 | 0.127 | 3.379 | 12 | 0.094 |
| Moult stages | 0.721 | 2 | 0.519 | 4.384 | 1 | 0.090 |
| Lunar phases | 0.674 | 3 | 0.595 | 5.945 | 2 | 0.048 |
| Error | | 7 | | | 5 | |

However, it was likely the effect of time after moulting on the ratio of STT was being masked by the interaction between time after moulting and lunar cycles. This interaction between the time after moulting and lunar cycles was significant in the second experiment ($F = 22.259$; $df = 1, 49$; $P < 0.05$) but not in the first ($F = 0.258$; $df = 3, 53$; $P > 0.05$). It was found that in the second trial, during full moon, the STT ratio peaked at 3 and 12 days after moulting. Around last quarter moon, the ratio of STT had peaks at day 8 and 11 after moulting. At the dark moon (new moon), STT ratio peaked at day 6 after moulting (Figure 5.5). At the first quarter moon, only prawns moulting soon after were sampled, therefore no trend was discernable.

Time after moulting interactions with the other independent variables (time after moulting * sex, time after moulting * moult stages, time after moulting * sex * moult stages, time after moulting * sex * lunar cycles, time after moulting * moult stages * lunar cycles and time after moulting * sex * moult stages * lunar cycles) could not be analysed in both experiments because of the loss of degrees of freedom (df).

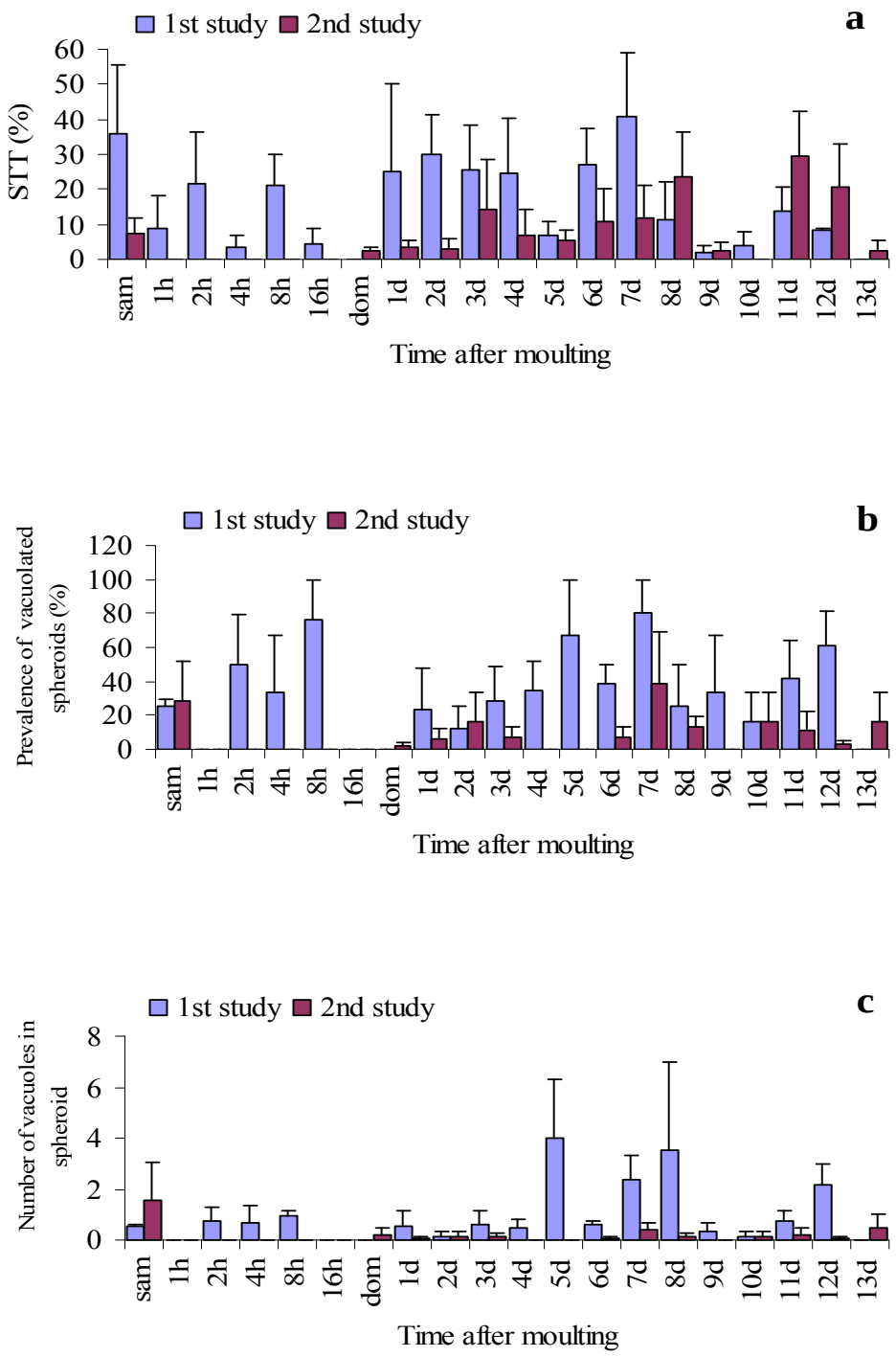



**Figure 5.4.** Mean (± SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in spheroids (c) of *P. monodon* at different time after moulting. The duration of the moult cycle was between 8 and 12 days in the first experiment and from 8 to 22 days in the second experiment. sam, soon after moulting; dom, day of moulting.

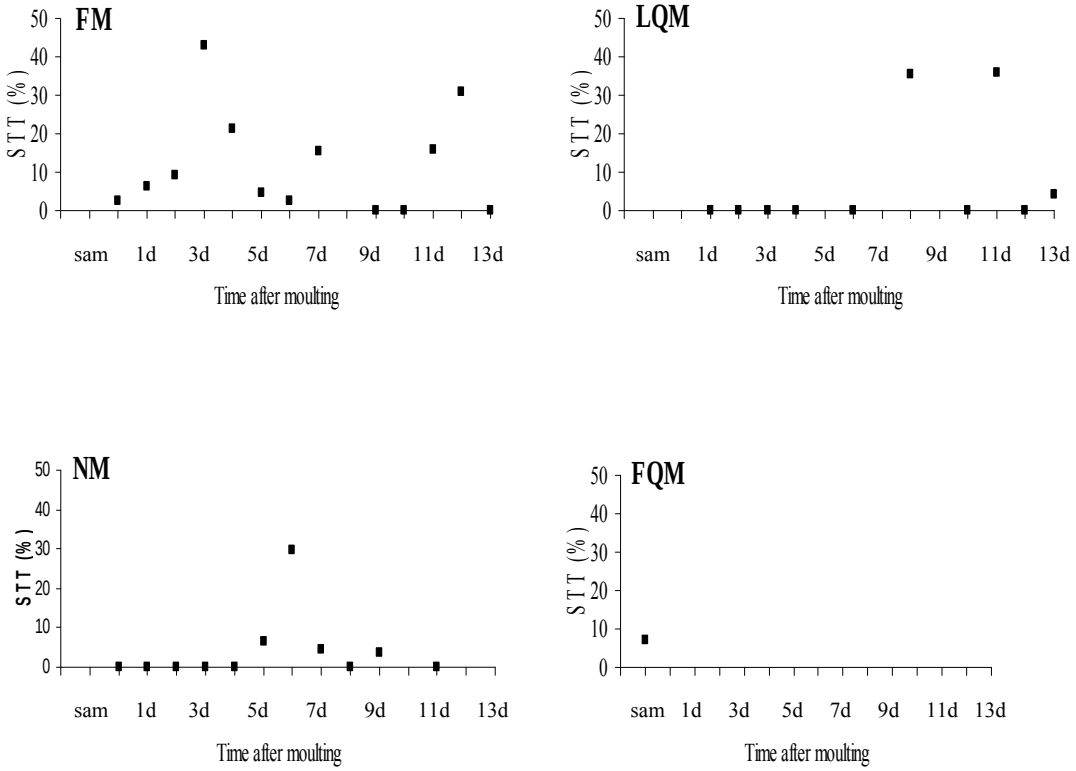



**Figure 5.5.** The spheroid to total tissue (STT) ratio of *P. monodon* at time after moulting at different lunar phases (FM, full moon; LQM, last quarter moon; NM, new moon, FQM, first quarter moon) in the second experiment.

This scenario (loss of degrees of freedom in interaction between independent variables) occurred in any measure of LOS cells throughout this chapter unless otherwise stated.

The prevalence of vacuolated spheroids varied with the time after moulting (Figure 5.4b). The highest prevalence was found at day 7 after moulting during the experiments, with prevalence of 80% in the first experiment and 38.46% in the last experiment. On the other hand, the lowest prevalence (0%) was 1 hour and 16 hours in the first experiment and at day 4, 5 and 9 after moulting in the next experiment. ANOVA showed no significant differences of the time after moulting on the prevalence of vacuolated spheroids either in the first ($F = 1.145$; $df = 11, 53$; $P > 0.05$) or in the second experiment ($F = 0.399$; $df = 12, 49$; $P > 0.05$). The interactions between the time after moulting and lunar cycles also revealed no significant effect ($P > 0.05$) on the prevalence of vacuolated spheroid cells either in

the first study ($F = 1.146$; $df = 3, 53$; $P > 0.05$) or in the second ($F = 0.283$; $df = 1, 49$; $P > 0.05$).

The number of vacuoles in the spheroid also differed with the time after moulting (Figure 5.4c). The highest number of vacuoles occurred at day 5 after moulting in the first experiment (4.00), while it was soon after moulting in the second experiment (1.53). Neither in the first experiment ($F = 0.399$; $df = 11, 53$; $P > 0.05$) nor in the second ($F = 0.024$; $df = 12, 49$; $P > 0.05$), the time after moulting had significant differences on the number of vacuoles. Furthermore, its interactions with the lunar cycles also revealed no significant effect on the number of vacuoles (ANOVA) both in the first experiment ($F = 0.817$; $df = 3, 53$; $P > 0.05$) and in the second experiment ($F = 0.001$; $df = 1, 49$; $P > 0.05$).

5.3.3.2. Moult stages and the LOS cells

The abundance of the lymphoid organ spheroid cells was different within the stage of moulting and both experiments (Figure 5.6a). In the first experiment, the STT ratio reached a peak at stage C (25.00%) and fell slightly until the last stage before moulting (stage D3/D4). While in the second experiment, the STT ratio reached a peak at stage D2 (18.53%) and dropped dramatically at stage D3/D4 before moulting. However, univariate analysis of variance demonstrated no significant differences of the moult stages on the STT ratio ($F = 0.721$; $df = 2, 53$; $P > 0.05$ and $F = 4.384$; $df = 1, 49$; $P > 0.05$ in the first and the second experiments, respectively).

The prevalence of vacuolated spheroids varied between the experiments and the stage of the moult (Figure 5.6c). The prevalence was highest at stage D1 (46.76%) in the first experiment and at stage D3/D4 (25.00%) in the second experiment. While, the lowest prevalence was found in stage D0 (9.36%) in the first experiment and in stage B (4.17%) in the second experiment. Statistically, there was no significant difference of the moult stages on the prevalence of vacuolated spheroid in the first trial ($F = 0.869$; $df = 2, 53$; $P > 0.05$), nor was significant in the second ($F = 0.229$; $df = 1, 49$; $P > 0.05$).

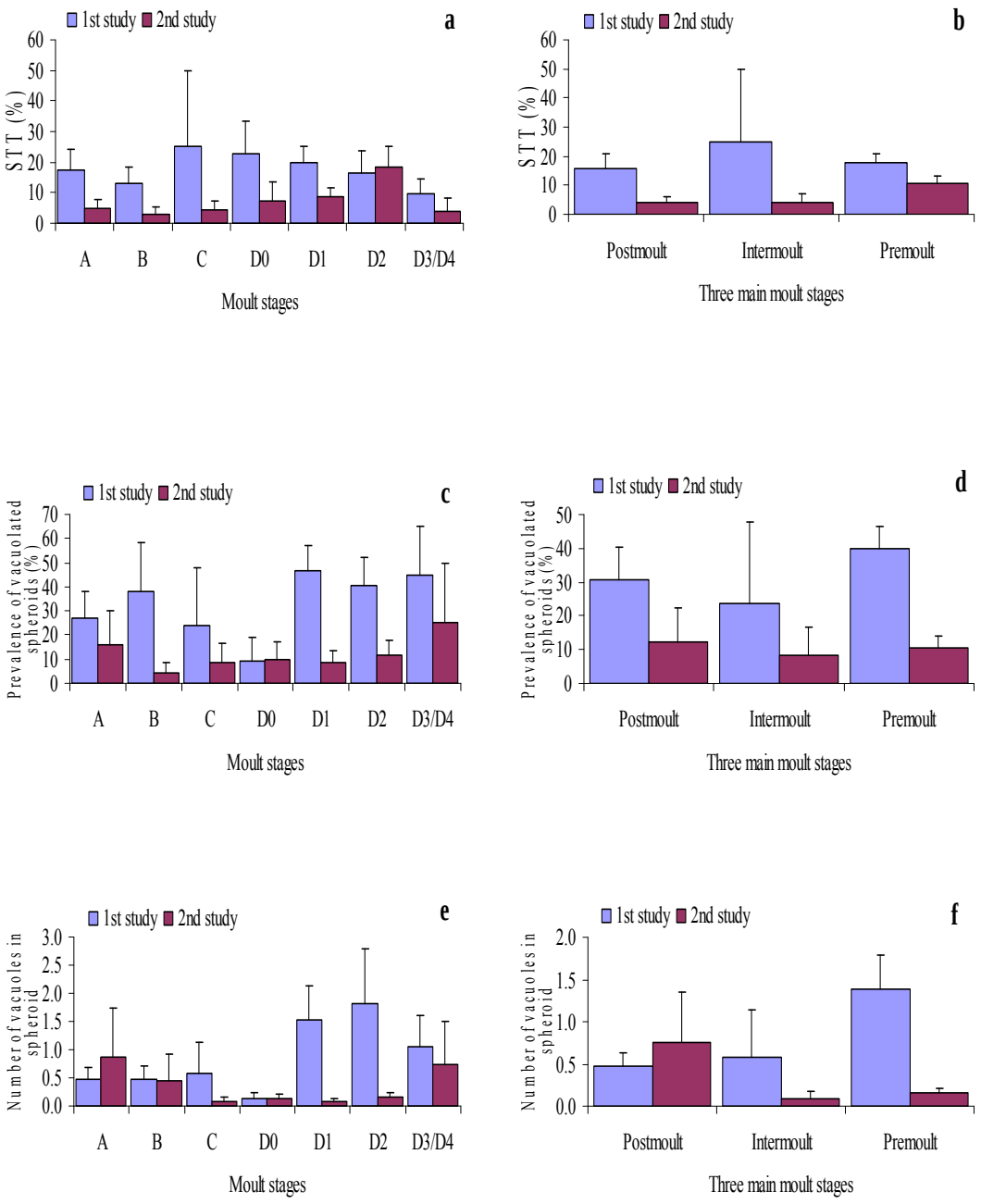



**Figure 5.6.** Mean (± SE) the spheroids to total tissue (STT) ratio (a and b), the prevalence of vacuolated spheroids (c and d) and the number of vacuoles in spheroids (e and f) of *P. monodon* at different moult stages (a, c and e) and three main moult stages (b, d, and f) in the first and second experiments.

The number of vacuoles in the spheroids also differed between moult stages in both experiments. The number of vacuoles in the spheroids was higher in stage D2 in the first experiment and in stage A in the second experiment than in the other stages and were 1.83 and 0.88, respectively (Figure 5.5e). Statistical analysis demonstrated that

neither in the first study ($F = 1.102$; $df = 2, 53$; $P > 0.05$) nor in the second study ($F = 0.098$; $df = 1, 49$; $P > 0.05$) the moult stages had significant effect on the number of vacuoles in spheroids.

When the moult stages were categorized into three main moult stages, postmoult (A and B), intermoult (C) and premoult (D), the variability of STT ratio, the prevalence of vacuolated spheroids and the number of vacuoles in spheroids also occurred in the two experiments. In the first study the highest ratio of STT was at the intermoult stage (25.00%), followed by premoult (17.62%) and postmoult (15.90%). However, in the second study, the premoult stage had the highest STT ratio (10.61%), intermoult (4.29%) was the next highest and postmoult had the lowest (4.07%) (Figure 5.6b). Furthermore, premoult had the highest prevalence of vacuolated spheroids (39.96%) followed by postmoult (30.83%) and the lowest was intermoult (23.81%) in the first experiment. In the second experiment, intermoult (8.33%) was also lower than premoult (10.41%) and postmoult (12.50%) (Figure 5.6d).

In addition, premoult had the highest number of vacuoles in spheroids, while postmoult was lower than the intermoult stage in the first experiment. In the second experiment, postmoult had the highest number of vacuoles followed by premoult and the lowest was intermoult stage (Figure 5.6f). However, the differences between the three main moult stages, alone or with its interactions with the other variables in all categories of LOS could not be analysed statistically (ANOVA) because it lost degrees of freedom in first trial and in the second.

5.3.3.3. Lunar phases and the LOS cells

The STT ratio, the prevalence of vacuolated spheroids and the number of vacuoles in spheroid varied with the lunar cycles in both experiments (Figure 5.7). In the first experiment, the STT ratio, the prevalence of vacuolated spheroids and the number of vacuoles in the spheroid were highest at the new moon, decreased at first quarter moon and became slightly elevated at the last quarter moon. However, in the second experiment, the STT ratio increased across the lunar cycle. The prevalence of vacuolated spheroids and the number of vacuoles similarly reached a peak at first quarter moon and decreased until the end of the cycle.

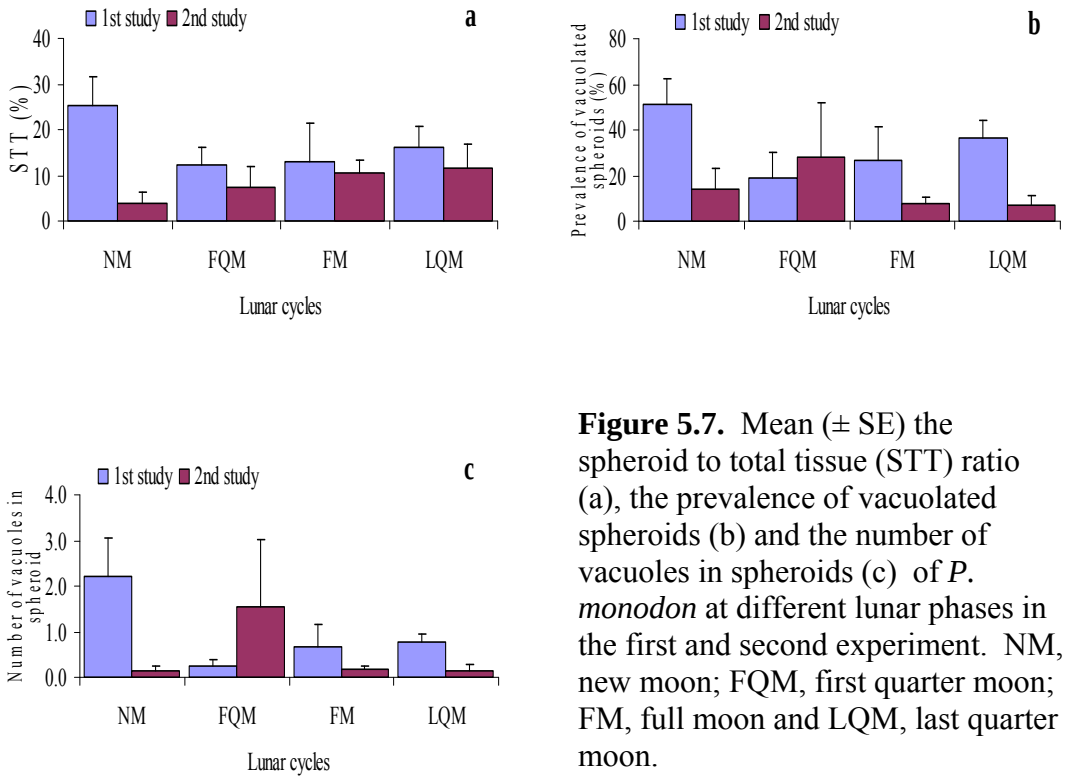



**Figure 5.7.** Mean (± SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in spheroids (c) of *P. monodon* at different lunar phases in the first and second experiment. NM, new moon; FQM, first quarter moon; FM, full moon and LQM, last quarter moon.

Univariate analysis of variance revealed that there was a significant effect of the lunar cycle on the ratio of STT in the second experiment ($F = 5.945$; $df = 2, 49$; $P < 0.05$), but not in the first ($F = 0.674$; $df = 3, 53$; $P > 0.05$). Unfortunately, post-hoc LSD comparison failed to show any differences between individual means within the lunar cycle in the second experiment. Furthermore, neither in the first experiment ($F = 0.554$; $df = 3, 53$; $P > 0.05$) nor in the second experiment ($F = 0.159$; $df = 2, 49$; $P > 0.05$), the lunar cycle showed significant differences on the prevalence of vacuolated spheroids. ANOVA also demonstrated the lack of significant effect of the lunar cycle on the number of vacuoles, either in the first study ($F = 0.290$; $df = 3, 53$; $P > 0.05$) or in the second ($F = 0.046$; $df = 2.49$; $P > 0.05$). However, except for time after moulting (see above), the lunar cycle's interactions with the other independent variables could not be analysed due to loss degrees of freedom in both experiments in terms of STT ratio, the prevalence of vacuolated spheroids or the number of vacuoles in the spheroids.

Furthermore, univariate analysis of variance revealed no significant differences of weight ($F = 0.824$; $df = 1, 53$; $P > 0.05$), total length ($F = 0.773$; $df = 1, 53$; $P > 0.05$),

and sex ($F = 0.037$; $df = 1, 53$; $P > 0.05$) on the spheroid to total tissue ratio in the first trial. Similar results were also found in the second trial. Weight ($F = 0.003$; $df = 1, 49$; $P > 0.05$), total length ($F = 0.213$; $df = 1, 49$; $P > 0.05$) and sex ($F = 2.559$; $df = 1, 49$; $P > 0.05$) had no significant differences on the STT ratio. Neither in the first experiment nor in the second experiment, did weight ($F = 0.002$; $df = 1, 53$; $P > 0.05$ and $F = 0.145$; $df = 1, 49$; $P > 0.05$), total length ($F = 0.017$; $df = 1, 53$; $P > 0.05$ and $F = 0.104$; $df = 1, 49$; $P > 0.05$) and sex ($F = 0.721$; $df = 1, 53$; $P > 0.05$ and $F = 1.250$; $df = 1, 49$; $P > 0.05$) demonstrate significant differences on the prevalence of vacuolated spheroids.

Moreover, weight ($F = 0.018$; $df = 1, 53$; $P > 0.05$), total length ($F = 0.015$; $df = 1, 53$; $P > 0.05$), and sex ($F = 0.001$; $df = 1, 53$; $P > 0.05$) had no significant effect on the number of vacuoles in the first study. Similarly, weight ($F = 0.122$; $df = 1, 49$; $P > 0.05$), total length ($F = 0.148$; $df = 1, 49$; $P > 0.05$) and sex ($F = 0.582$; $df = 1, 49$; $P > 0.05$) suggested no significant effect on the number of vacuoles in the second study. In both the first and second trials, sex interactions with the other variables in terms of STT ratio, the prevalence of vacuolated spheroids and the number of vacuoles could not be analysed due to loss degrees of freedom.

In the first experiment the correlation (Pearson correlation) between STT ratio and the prevalence of vacuolated spheroids was significant, $r = 0.334$ ($P = 0.014$). The correlation between the prevalence of vacuolated spheroids and the number of vacuoles within the spheroid cells was also significant, $r = 0.612$ ($P = 0.000$). However, the correlation between STT ratio and the number of vacuoles in the spheroid was not significant, $r = 0.192$ ($P = 0.165$). In the second experiment, there was no correlation between STT ratio and the prevalence of vacuolated spheroids, $r = 0.141$ ($P = 0.330$) or between STT ratio and the number of vacuoles in the spheroid, $r= 0.059$ ($P = 0.682$). However, between the prevalence of vacuolated spheroids and the number of vacuoles there was a strong correlation, $r = 0.755$ ($P = 0.000$).

In the second experiment, some slides demonstrated eosinophilic foci (Figure 5.8) and bacterial granulomas (Figure 5.9) within the lymphoid organ. Bacterial granulomas were also found in the hepatopancreas, the heart and antennal gland of

the prawns (Figure 5.10). All these changes were observed only in the second experiment and not in the first.

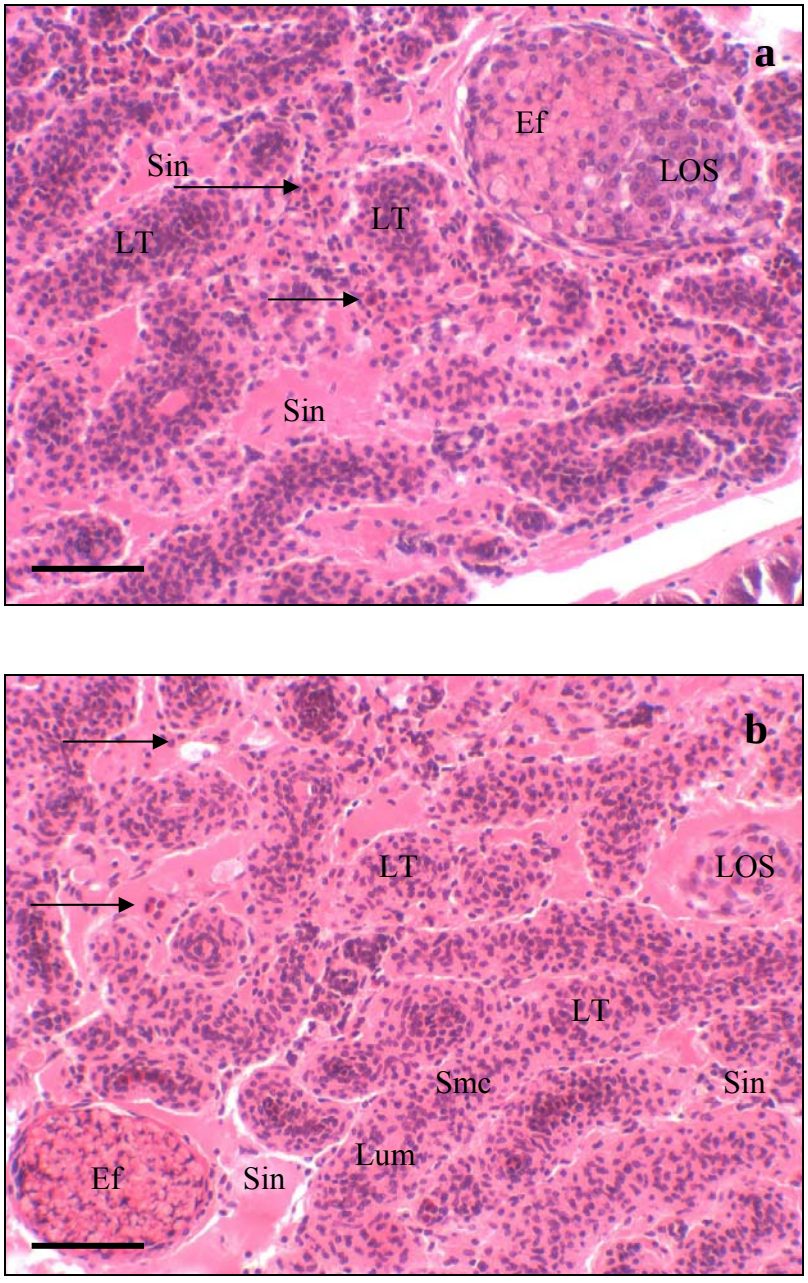



**Figure 5.8.** Light micrograph of longitudinal section of the LO of *P. monodon* from one slide demonstrated eosinophilic foci (Ef) and LOS cell in one tubule within the LO (a) suggesting eosinophilic foci (b) developed from LOS cell. Circulating haemocytes (arrow) were observed in the interstitial space between tubules (Sin). H & E stain. Scale bar = 50 µm. LT, lymphoid tubule (Smc, stromal matrix cells and Lum, lumen); Sin, haemal sinuses.

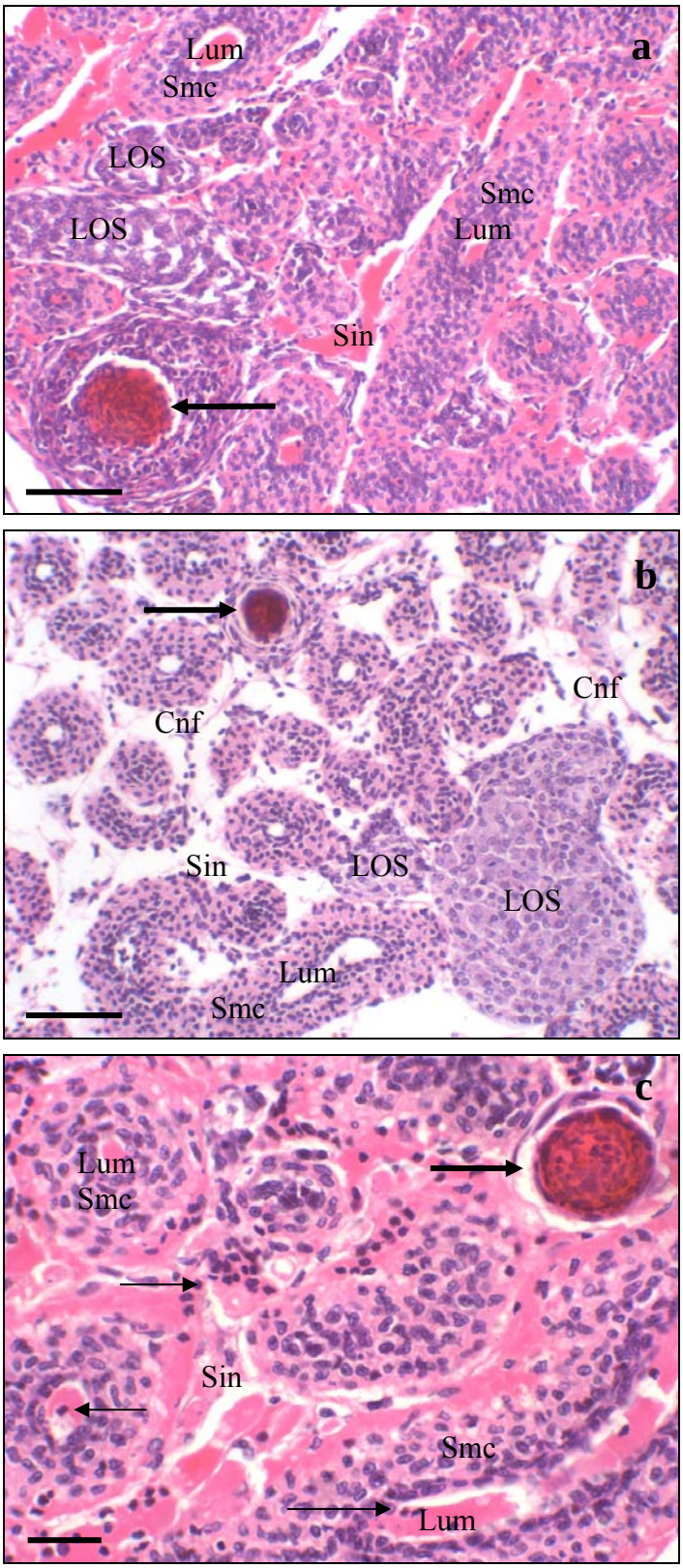



**Figure 5.9.** Light micrograph of longitudinal section of the LO of *P. monodon* showing bacterial granuloma (arrow) in the interstitial space between tubules (Sin). Multiple layers of haemocytes encapsulated the melanized nodule (a and b) or elongated flattened cells surrounded the nodule (c). H & E stain. Scale bar = 50 µm (a and b) and 20 µm (c). Cnf, fibrous connective tissue; Lum, lumen; Smc, stromal matrix cells; Sin, haemal sinuses.

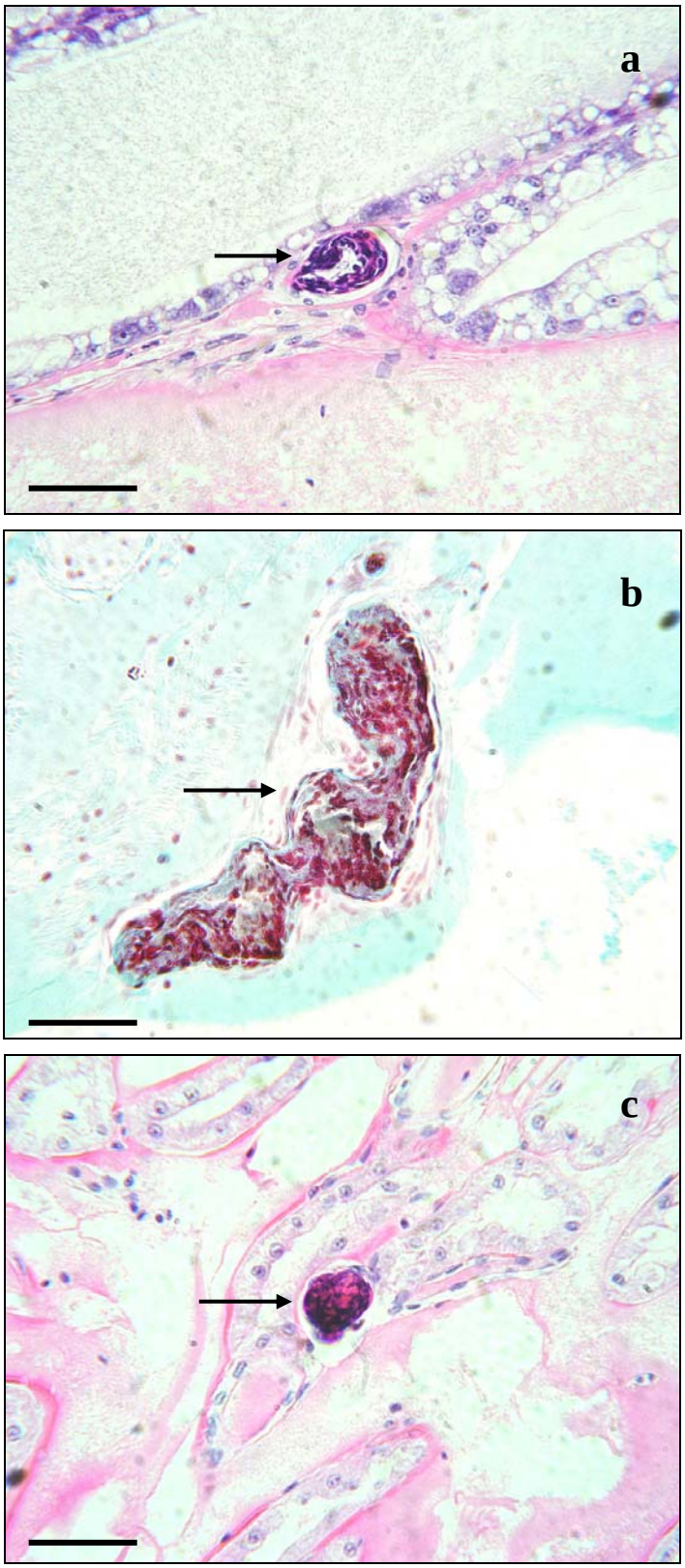



**Figure 5.10.** Light micrograph of longitudinal section of various tissues of *P. monodon* showing bacterial granulomas (arrow) in hepatopancreas (a), the heart (b) and antennal gland (c). H & E stain (a and c). Gram stain (b). Scale bar = 50 µm.

## 5.4. Discussion

### 5.4.1. Moult staging

The moulting cycle of *P. monodon* has been classified by using epidermal retraction of uropod tissue from the cuticle (Promwikorn *et al.*, 2004). However, that paper did not mention which part of the uropod that they used to stage the moult. The consistency, with which the area of the appendages is examined, is an important part in determining the moult cycle. For example, in the inner uropod of penaeid prawns, the setal development and the retraction of the epidermis from the setal bases in the proximal area developed slowly, while in the distal area, it progressed rapidly. The median part of the inner uropod could be utilized to overcome these two extremes. Similar changes of setogenesesis have been also found in the pleopod of *P. merguiensis* (Longmuir, 1983).

Therefore in the present study, the method that was previously used by Promwikorn *et al.* (2004) was applied in combination with criteria developed by Smith and Dall (1985) without the histological examination of the abdominal segment of the prawn. Setal development and epidermis withdrawal of the inner uropod near the telson tip gave clear characteristics to determine the stage of the moult in *P. monodon*. This method was simple, rapid and repeated moult staging could be performed on the experimental prawns because sacrificing the animal was not necessary to assess the stage of moult. However in this experiment, sacrificing animals was necessary to determine the ratio of lymphoid organ spheroid cells in each moult stage.

Postmoult (stage A and B) could be defined by observing the distribution of cellular matrix within the setae. Stage A1 started soon after the prawn shed the old cuticle. The new cuticle was very soft and slippery, as was the setae. The protoplasmic matrix filled the setae and setal bases. When the cellular matrix began to retract from the distal end of the setae, the prawns commenced stage A2. Even though they were still soft to touch, the hardening of the exoskeleton began, particularly in the carapace and sixth abdominal, and the slippery feel of the cuticle disappeared. Setal cones still could not be observed. According to Smith and Dall (1985) in *P.*

*esculentus*, when the secretion of the endocuticle and the retraction of the cellular matrix began, the exoskeleton lost its slippery feel.

Stage B was marked by the formation of setal cones. Formation of setal cones was indicated by the constriction of cellular matrix at the base of setae. Longmuir (1983) suggested that cellular matrix formed the setal cones. The cellular matrix might also contribute to the invagination of the setae, in muscular activity at moulting, and supported the skeleton of the setae until they hardened. In this stage, the hardening of the exoskeleton continued until it reached the necessary rigidity. In *P. esculentus*, the changing of skeletal hardness from a membranous to a parchment-like consistency occurred in stage B (Smith & Dall, 1985), while in *P. indicus* this happened in stage A (Vijayan *et al.*, 1997). This discrepancy was due to the differences in defining "parchment like" as a measure of exoskeleton rigidity (Smith & Dall, 1985).

The appearances of cones in most of the setae and the absence of cellular matrix within the setae were the most striking features that could be observed in intermoult stage (stage C). The exoskeleton achieved maximum rigidity in this stage. Most authors agreed in defining the postmoult and intermoult stage in penaeid prawns (Peebles, 1977; Longmuir, 1983; Smith & Dall, 1985; Robertson *et al.*, 1987; Vijayan *et al.*, 1997; Promwikorn *et al.*, 2004). However in the later moult stages, there was inconsistency in dividing stage D into substages due to the methods that they used.

The retraction of epidermis from the setal bases signalled the end of the intermoult stage and the beginning of the premoult stage (stage D0). This could be seen clearly from the transparent edge of the appendages below the setal bases. The withdrawal of pigment and epidermis from the setal bases occurred at the same time. Similar patterns were found in *P. esculentus* (Smith & Dall, 1985). A straight line below the setal bases as a result of epidermis withdrawal marked the end of stage D0.

Further separation of epidermis from the setal bases was marked by the appearance of a clear zone between the epidermis and the setal bases and defined the stage D1. The clear zone implied the formation of the new cuticle (Promwikorn *et al.*, 2004).

Stage D1 continued to develop until it achieved a uniform and maximum wavy edge of epidermis. This stage occupied the longest time in *P. monodon* and could be observed from 3 to 8 days after moulting.

In *P. esculentus, P. merguiensis*, and *P. indicus*, stage D1 was further substaged to D1', D1'', and D1''' according to the developmental stage of the new setae (Longmuir, 1983; Smith & Dall, 1985; Vijayan *et al.*, 1997). In previous moult staging of *P. monodon* when the epidermal line changed from straight to wavy, the moult stage was categorized as stage D2 (Promwikorn *et al.*, 2004), whilst in other penaeid prawns, it was classified into D1' (Longmuir, 1983; Smith & Dall, 1985; Vijayan *et al.*, 1997). In this study it was termed D1. It seemed that Promwikorn *et al.* (2004) used the development of scalloping epidermal tissue to define stages D1, D2, and D3. Other authors (Longmuir, 1983; Smith & Dall, 1985; Vijayan *et al.*, 1997) applied the terminology D1', D1'', and D1''', while the present study simplified these stages into one stage, D1.

The most noticeable feature that could be observed in stage D2 was the colour change of the new setal shaft to red-orange at the end. In this stage the setal cones began to deform. In the previous study, this sign commenced the stage D4, the last stage before ecdysis (Promwikorn *et al.*, 2004). When the light pinpoint appeared from the new setal nodes, the prawns entered the stage D3/D4. This stage could not be separated into two different moult stages due to the requirement for the experimental animals being sacrificed every sampling time. Maximum reabsorption of components of the old exoskeleton such as nutrients and minerals occurred at this stage and reincorporated into the new exoskeleton (Longmuir, 1983; Smith & Dall, 1985; Withers, 1992). As a result, the exoskeleton of the prawns became soft.

The stage D3/D4 was the last stage that could be seen in *P. monodon* before ecdysis. Even though the previous study (Promwikorn *et al.*, 2004) defined the moult stages until D4, this stage was defined as D2 in the present study. In *P. merguiensis* the moult stages also lasted to stage D3 - D4 (Longmuir, 1983), in *P. indicus* (Vijayan *et al.*, 1997), *P. setiferus* and *P. stylirostris* (Robertson *et al.*, 1987) lasted to early stage D2-3, while in *P. esculentus*, it could be defined until stage D4 (Smith & Dall,

1985). The inconsistency in defining stage D in penaeid prawns might be due to the different methods and species used.

The last stage observed was ecdysis (moulting). The prawns shed the old exoskeleton and everted the new setae of the new exoskeleton. At ecdysis, the plastic exoskeleton was stretched by water uptake and an expanded haemocoel (Withers, 1992). The exuviae of *P. monodon* were removed in two parts, firstly the carapace and secondly the rest of exuviae (appendages and abdomen). Similar observations were found in *P. esculentus* (Wassenberg & Hill, 1984), *P. merguiensis* (Longmuir, 1983), and *P. indicus* (Vijayan *et al.*, 1997).

The exuviation process took less than 1 minute in *P. monodon* similarly in *P. esculentus* (Smith & Dall, 1985), *P. setiferus* and *P. stylirostris* (Robertson et al., 1987). The ecdysis process of *P. indicus* lasted between 30 - 50 s, in *P. merguiensis* (Longmuir, 1983) it was about 40 s, while Wassenberg and Hill (1984) found it much shorter, approximately 18.1s in *P. esculentus*. This discrepancy was suggested as being due to the differences in defining ecdysis (Wassenberg & Hill, 1984; Vijayan *et al.*, 1997). In addition, it was commonly found that this exuviation process occurred at night.

It seemed that the prawns began to stop feeding at the end of stage D2 and started to feed at stage B - C. According to Charmantier-Daures and Vernet (2004) animals did not feed from the end of stage D2 until stage B and used the stored reserves in the hepatopancreas (also known as the midgut gland) to supply the energy required for ecdysis. When they began to feed, prawns also ate the shed exuviae to recycle nutrients and minerals (Withers, 1992).

Since stage A, B, (postmoult) and C (intermoult) were determined according to the development of setae (setogenesis) and stage D (premoult) was based on the epidermal retraction from the setal bases (apolysis), a problem arose when these two criteria did not developed synchronously. It was generally found in the normal prawns that the apolysis occurred after the setogenesis was completed. However in several prawns, the withdrawal of epidermis from the setal bases had already occurred while the cellular matrix had just retracted at the proximal end of setae

(Figure 5.2). As a result, it was difficult to differentiate between stage B, C, and D. To overcome this problem, the retraction of epidermis from the setal bases was used to stage the moult. This problem might indicate that the setogenesis developed slowly and also suggested slow growth of the animals. Furthermore, another appropriate method should be considered in staging the moult of abnormal prawns.

The stages of moult were an important factor in any stressful procedures such as handling, shipping, therapeutic treatment or harvesting (Robertson *et al.*, 1987). Also they claimed that moult staging of broodstock of penaeids was beneficial in predicting the cycles of larval production. Furthermore, because the rigidity of the prawns affected the market value, it was important to stage the moult before harvesting. All in all, determination of moult stages should be considered for pond management.

The lunar effect on moulting has been studied in some crustacean species. *Gonodactylus zacae* (Reaka, 1976), *Panulirus ornatus* (Skewes *et al.*, 1994) and *P. esculentus* (White, 1973) predominantly moult around the full moon or/and new moon. However, *Pseudosquilla ciliata* (Reaka, 1976), *Excirolana chiltoni* (Klapow, 1972) and *P. schimitti* (Nascimento *et al.*, 1991) mostly moulted around the last quarter moon or/and first quarter moon. The differences in moulting activity were suggested to be due to age differences, species, terminal moult, temperature, photoperiod, salinity, nutrition, ethology and group effect, parasitism and pollutants (Charmantier-Daures & Vernet, 2004).

In the current experiments, moulting related to lunar patterns appeared different between the two experiments. Most moulting activity (ecdysis) occurred around last quarter moon and first quarter moon in the first experiment (Table 5.1). In the second experiment, ecdysis was highest at full moon followed by first quarter moon (Table 5.1). These two patterns were also supported by highest number of postmoult stage (stage A and B) and late premoult stage (stage D2 and D3/D4) at the phases (Table 5.3 and 5.4). Postmoult and late premoult stages illustrated that moulting activity had just occurred or would occur soon, respectively (Skewes *et al.*, 1994). This disparity might be due to differences in age, animal size and moult periodicity

since the external factors such as temperature and photoperiod were similar during the experiments.

### 5.4.2. The lymphoid organ spheroid cells

Most studies on the lymphoid organ of penaeids paid particular attention to the histopathological changes of the lymphoid organ at cellular level, the lymphoid organ spheroid cells. The characteristic features of spheroid cells has been extensively described in naturally or artificially infected prawns (Owens & Hall-Mendellin, 1990; Owens *et al.*, 1991; Bonami *et al.*, 1992; Kondo *et al.*, 1994; Spann *et al.*, 1995; Fraser & Owens, 1996; Spann *et al.*, 1997; Anggraeni, 1998; Hasson *et al.*, 1999b; Anggraeni & Owens, 2000; Van de Braak *et al.*, 2002c; Shao *et al.*, 2004). However, this study presented the first attempt to investigate the interaction between the abundance of the LOS cells within the lymphoid organ and the cycle of the moult in penaeid prawns.

Since the study of Anggraeni and Owens (2000) found that the spheroid cells fluctuated with the life of penaeid prawns, it was likely that there must be a mechanism driving the fluctuation. As some of the immunological factors of the prawns were associated with the moult cycles (Chen & Cheng, 1993; Sequeira *et al.*, 1995; Le Moullac *et al.*, 1997; Cheng *et al.*, 2002; Liu *et al.*, 2004), therefore Anggraeni and Owens (2000) proposed and it was taken as the hypothesis of this study, that the lymphoid organ spheroid cells must be disposed of and possibly during ecdysis. However, in this study there was no evidence found to support the supposition. The present study strongly suggested the absence of moult effect on the abundance of spheroid cells within the lymphoid organ of *P. monodon*, in terms of STT ratio, prevalence of vacuolated spheroids and the number of vacuoles within the spheroids.

It has previously been described that immunodefence and haematological factors of penaeid prawns were related to the cycles of moult (Table 5.6). Sequeira *et al.* (1995) reported that in *P. japonicus*, hyaline haemocytes were significantly higher at stage B than C, in both females ($P < 0.01$) and males ($P < 0.05$), small granular haemocytes were significantly higher ($P < 0.05$) at stage D0 than at other stages in

females, while large granular haemocytes were significantly higher ($P < 0.05$) at stage D0 than B in both sexes. In contrast, in *P. stylirostris*, only LGH were found to be significantly higher at stage C than at stage B and D0 (Le Moullac *et al.*, 1997). Different results were also discovered in *P. vannamei*. HH were significantly higher ($P < 0.05$) at stage C than at stage A, B and D2/D3. On the other hand, granular haemocytes were significantly higher ($P < 0.05$) at stage D0/D1 than at stage A, B, and D2/D3 (Liu *et al.*, 2004).

The inconsistency between studies also appeared when the THC was taken into account. Liu *et al*. (2004) found that THC was significantly higher ($P < 0.05$) at stage C than at stage A, B, and D2/D3. Contrarily, Le Moullac (1997) observed that THC was significantly lower ($P < 0.05$) at stage C than at other stages. However, in *M. rosenbergii* THC was significantly lower at stage D3 than other stages (Cheng & Chen, 2001).

Furthermore, the phenoloxidase activity was significantly lower ($P < 0.02$) in premoult stage (D) than in intermoult stage (C) in *P. stylirostris* (Le Moullac *et al.*, 1997). In *P. vannamei*, phenoloxidase activity was significantly lower ($P < 0.05$) at stage A than stage C. Respiratory burst was significantly lower ($P < 0.05$) at stage A than the other stages examined. Phagocytic activity was higher significantly ($P < 0.05$) at stage C than at other stages. Clearance efficiency was also higher significantly ($P < 0.05$) at stage C and B than at stage A (Liu *et al.*, 2004). Haemocyanin and haemolymph protein levels of *P. japonicus* were significantly higher ($P < 0.01$) at stage D0 than at stages B and D3 (Chen & Cheng, 1993). However, in *P. vannamei*, oxyhaemocyanin and haemolymph protein were significantly lower ($P < 0.05$) at stage A and B than at stage C, D0/D1 and D2/D3 (Cheng *et al.*, 2002).

Studies on the resistance of the prawns to pathogens also resulted in discrepancy. In *P. stylirostris* injected with *Vibrio* AM23, the mortality was significantly higher ($P < 0.02$) at premoult (D) than intermoult (C) (Le Moullac *et al.*, 1997). A different result was found in *P. vannamei* injected with *V. alginolyticus* in which cumulative mortality was significantly higher ($P < 0.05$) at postmoult (A and B) than intermoult (C) (Liu *et al.*, 2004).

**Table 5.6.** Moult related changes in immunological and haematological components of prawns.

| Immunoreactivities/ Haematological Components | Species | Pathogens/ Parasites | Moult Stages | | | | | | | Authors |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Postmoult | | Intermoult | Premoult | | | | |
| | | | A | B | C | D0 | D1 | D2 | D3/D4 | |
| HH (Females) | *P. japonicus* | | | a | bc | c | ab | | | Sequeira *et al.* (1995) |
| HH (Males) | *P. japonicus* | | | a | b | ab | ab | | | Sequeira *et al.* (1995) |
| HH | *P. vannamei* | | b | b | a | ab | ab | b | b | Liu *et al.* (2004) |
| SGH (Females) | *P. japonicus* | | | c | b | a | c | | | Sequeira *et al.* (1995) |
| LGH (Females) | *P. japonicus* | | | b | a | a | b | | | Sequeira *et al.* (1995) |
| LGH (Males) | *P. japonicus* | | | b | a | a | b | | | Sequeira *et al.* (1995) |
| LGH | *P. stylirostris* | | | b | a | b | ab | ab | | Le Moullac *et al.* (1997) |
| GH | *P. vannamei* | | d | c | ab | a | a | bc | bc | Liu *et al.* (2004) |
| THC | *P. vannamei* | | c | c | a | ab | ab | bc | bc | Liu *et al.* (2004) |
| THC | *P. stylirostris* | | | a | b | a | a | a | | Le Moullac *et al.* (1997) |
| THC | *M. rosenbergii* | | bc | ab | a | | ab | ab | c | Cheng & Chen (2001) |
| STT ratio | *P. monodon* | | a | a | a | a | a | a | a | present study (1st study) |
| STT ratio | *P. monodon* | | a | a | a | a | a | a | a | present study (2nd study) |
| Phenoloxidase | *P. stylirostris* | | | | a | b | b | b | | Le Moullac *et al.* (1997) |
| Phenoloxidase | *P. vannamei* | | b | ab | a | ab | ab | ab | ab | Liu *et al.* (2004) |
| Respiratory burst | *P. vannamei* | | b | a | a | a | a | a | a | Liu *et al.* (2004) |
| Phagocytic activity | *P. vannamei* | *V. alginolyticus* | b | b | a | b | b | b | b | Liu *et al.* (2004) |
| Clearance efficiency | *P. vannamei* | *V. alginolyticus* | b | a | a | ab | ab | ab | ab | Liu *et al.* (2004) |
| Haemocyanin | *P. japonicus* | | | b | a | a | ab | ab | ab | Chen & Cheng (1993) |
| Oxyhaemocyanin | *P. vannamei* | | c | b | a | a | a | a | a | Cheng *et al.* (2002) |
| Haemolymph protein | *P. japonicus* | | | c | ab | a | abc | abc | bc | Chen & Cheng (1993) |
| Haemolymph protein | *P. vannamei* | | c | b | a | a | a | a | a | Cheng *et al.* (2002) |
| Resistance | *P. stylirostris* | *Vibrio* AM23 | | | a | b | b | b | | Le Moullac *et al.* (1997) |
| Resistance | *P. vannamei* | *V. alginolyticus* | b | b | a | ab | ab | ab | ab | Liu *et al.* (2004) |

Data in the same row with different letter was significantly different ($P < 0.05$) among different moult stages.

The highest , 2nd; ,3rd; , 4th; , 5th; , 6th; the lowest.

Even though Le Moullac *et al*. (1997) and Liu *et al.* (2004) agreed in the correlation between the resistance to vibrio and phenoloxidase, but controversy arose when the THC was examined in relation to pathogen resistance.

It appeared that moult related changes in immunoreactive components of penaeid prawns varied within individuals and between species. Infected and uninfected prawns (health status) and environmental condition might also contribute to individual variability (Hose *et al.*, 1992). Furthermore, variations in methods could also cause discrepancy in the results. However in all the disparities, similarities also appeared. It was apparent that most of immunocompetence or haematological factors of the prawns were highest around intermoult stage (C) and early premoult stage (D0) (Table 5.6).

It is commonly believed that the lunar cycle affects the moult cycle of crustaceans. In turn, it might also affect the amount of the LOS in prawns. This study revealed evidence for the effect of the lunar cycle and its interaction with time after moulting on the spheroid to total tissue ratio. However, this evidence only appeared in the second experiment but was absent in the first. Therefore, there were two possibilities that might describe these differences between the experiments. Firstly, this might be due to the differences in age or size of the animals leading to the differences in moult periodicity. It was found that experimental animals in the second trial were larger and had a longer moult periodicity than those in the first experiment. Therefore, the moulting period of animals in the second study could approach the lunar months (29.5 days) that in turn affect the fluctuation of LOS cells (Table 5.1).

Moreover, it was clearly demonstrated in the second trial that elimination of the LOS cells occurred at the new moon and built up slightly across the lunar phases. It was found that during the new moon penaeid prawns became more active (Griffiths, 1999), triggered either by the increase in hydrostatic pressure due to spring tide or by the decrease in light intensity due to the dark phase of the moon. Thus, it enhanced the immune capability of the prawn to eliminate LOS cells as a reactive form of viral infections. How could the animal experiments in the laboratory, which looked independent from light intensity and hydrostatic pressure of the moon, be affected?

This rhythm might be endogenous and persistent even when the environmental cue was absent because it was controlled by the biological clock of the animals (Withers, 1992; Griffith & Wigglesworth, 1993).

Secondly, this discrepancy might appear as a result of a type I (alpha) error in statistics. Therefore, a significant difference was found in the second trial with the factor of lunar phase and its interaction with time after moulting, when in fact no significant differences actually existed. This hypothesis can only be discounted with further work (see Chapter 6).

The spheroids were produced in haemal sinus and were separated from the stromal matrix cells within the lymphoid organ. If these spheroids accumulated in the haemal sinus as the prawn grew, the area of the spheroid would enlarge with increasing animal size. However, the spheroid area ratio was not associated with the size of the animal (Anggraeni & Owens, 2000). Similar results were also found in the present study in which weight and total length of the prawn were not associated with the ratio of the spheroid cells, the prevalence of vacuolated spheroids and the number of vacuoles within the spheroid cells in the lymphoid organ. This study also demonstrated the absence of the effect of sex on the spheroid to total tissue ratio, the prevalence of vacuolated spheroids and the number of vacuoles within the spheroid cells.

It was found that the ratio of the LOS cells generally decreased in the second experiment. Therefore, there were two implications that could be made. Firstly, the LOS cells were formed during the early stage of these prawns. As the animals grew, the number of the normal tubules (stromal matrix) in the LO increased while the number of the LOS cells remained static. As a result the ratio of STT declined with the time of observation made in this study. If this was the case, then the spheroid cells would be in the LO during the life span of the animals and there was no recovery from the disease once the animals got infected. However, we found that some prawns had more LOS than the stromal matrix cells; some prawns had less LOS than the normal tubules; and some animals had no spheroids in the LO. This indicating that there was a fluctuation of LOS during the life span of the animals.

Secondly, there was an elimination mechanism of LOS within the lymphoid organ during the life span of the animals, as the animals for both experiments came from the same farm (same batch) and were captured and transported on the same day. However, the timing of the experiments was different. The first experiment was conducted 2 – 3 months earlier than the second one. The elimination process might have occurred when the prawns were kept in the 1000 L tanks and when the prawn might not be exposed to viruses that caused the changes to the LO. It seemed more likely that the LOS was eliminated gradually and as a result there was a fluctuation of LOS cells during the life span of the animals.

In conclusion, the present investigation showed that there might be an elimination mechanism of spheroid cells as prawns aged. Moult stages, sex, and animal size (weight and total length) did not affect the fluctuation of spheroid cells in laboratory held animals. This mechanism may have been associated with lunar rhythms. Since this pattern was only evident in the second experiment, then further investigations were needed to confirm this finding.

# CHAPTER 6
# THE RELATIONSHIP BETWEEN THE LOS CELLS AND MOULTING STAGES IN PRAWNS FROM COMMERCIAL FARMS

## 6.1. Introduction

Rhythmic activities exist at every level of organisation of animals, from the subcellular level, physiological and behavioural, to the coordination of population activities (Withers, 1992). Most of this rhythmicity is associated with the major geophysical cycles of the earth. These rhythms vary from one day, ebb and flow tides (12.4 hours), the lunar day or intertidal periodicity (24.8 hours), the solar day (24 hours), the fortnight tides (interval between spring and neap tides; 14.75 days), the lunar month (29.5 days), to the circannual rhythms (365 days) (Palmer, 1990; Withers, 1992). It is apparent that these rhythms are controlled either by exogenous or endogenous factors (Reaka, 1976). Many rhythms are endogenous and persist even in the absence the environmental cues, and are therefore controlled by a biological clock. This biological clock was suggested to be located in the nervous system and its effector control was mediated by the nervous system (Withers, 1992).

Activities related to lunar rhythmicity are evident in aquatic animals. Some studies revealed the relationship between moon phases and moulting as observed in stomatopod crustacea (Reaka, 1976), sand beach isopod (Klapow, 1972), intertidal amphipod (McCurdy *et al.*, 2000), lobster (Skewes *et al.*, 1994; Ferrero *et al.*, 2002), blue swimming crab (Hamsa, 1978) and penaeid prawn (White, 1973; Nascimento *et al.*, 1991). The growth increment of *P. vannamei* in ponds showed lunar periodicity (Griffith & Wigglesworth, 1993). Lunar variability on the reproductive activity of crustaceans was also evident (Paula, 1989; Nascimento *et al.*, 1991; Courtney *et al.*, 1996; Ferrero *et al.*, 2002; Flores *et al.*, 2002). Additionally, crustaceans also exhibited lunar dispersal patterns (Montfrans *et al.*, 1990; Enggleston *et al.*, 1998; Rios-Jara & Gonzales, 2000). Furthermore, a commercial penaeid prawn fishery showed variability in catchability indicating lunar effect (White, 1973; Courtney *et al.*, 1996; Griffiths, 1999; Salini *et al.*, 2001).

Few researchers have studied the endogenous nature of tidal and lunar rhythmicity on the immunodefence components of aquatic invertebrates (Williams, 1985; Truscott & White, 1990; Hawkins *et al.*, 1993; Hauton *et al.*, 1995). Williams (1985) detected the endogenous nature of the tidal rhythm on blood sugar concentration of the shore crab, *Carcinus maenas*. He found that the blood sugar level was around 40 – 50% higher at low water than at high water. The total haemocyte count of *C. maenas* significantly showed daily tidal and semi-lunar (spring neap tidal cycle) variation. The mean of THC was significantly higher at times of high tide than at low tides (Truscott & White, 1990). They found evidence that the THC increased 70% when the tidal range increased 4 m. In addition, a significant negative correlation between phenoloxidase activity and the tidal high was also detected in the shore crab (Hauton *et al.*, 1995).

Furthermore, the hard clam *Mercenaria mercenaria* also showed circatidal rhythm with metabolic and immunological activity. It was found that chronic tidal exposure increased hydrogen peroxidase concentration and small granulocyte number on re-immersion, and reduced lysozyme activities and rates of granulocyte locomotion (Hawkins *et al.*, 1993). Either daily cycle of high and low tides or biweekly cycle of spring and neap tides as a result of the lunar cycle, the disparity in hydrostatic pressure might function as a stimulus (Williams, 1985; Truscott & White, 1990; Hawkins *et al.*, 1993; Hauton *et al.*, 1995; Ferrero *et al.*, 2002).

From the above it is clear that lunar periodicity affects many aspects of invertebrate's lives. However, there are no studies determining the relationship between the lunar periodicity and lymphoid organ spheroid cells as a defence mechanism in penaeid prawns. The present study investigated the association between the moult stages and the lunar-related pattern on the abundance of lymphoid organ spheroid cells in cultured penaeid prawns from commercial farms.

## 6.2. Materials and Methods

### 6.2.1. Experimental animals

*P. monodon* were collected from commercial farms in northern Queensland. In the first trial, prawns were caught with a trap on four occasions from the primary sedimentation ponds while in the last sampling, prawns were caught with a cast net in the production pond. In the second trial, prawns were caught with a cast net from the production ponds. The experimental animals were transported to the Fish Laboratory, School of Veterinary and Biomedical Sciences, James Cook University, Townsville to determine the stage of moult and to histologically section.

### 6.2.2. Sampling procedure

Sampling was carried out following the lunar cycle i.e. new moon, first quarter moon, full moon and last quarter moon. Sampling was carried out 5 times in each trial. In the first experiment, sampling started and finished at the new moon. While in the second experiment, sampling began and ended at the full moon. Fifteen prawns were collected at each sampling. Therefore, 75 prawns with a mean (± SD) body weight of 11.19 ± 3.73 g and total length of 11.4 ± 1.1 cm were utilized for the first trial. In the second trial, 75 prawns with a mean body weight of 33.66 ± 6.91 g and a mean total length of 16.0 ± 1.0 cm were examined.

### 6.2.3. Moult staging

Moult was staged by examining the setogenesis and the withdrawal of epidermal tissue from the base of setae in the inner uropod close to the telson tip (Smith & Dall, 1985; Promwikorn *et al.*, 2004) (Chapter 5). Moult stages were divided into stages A, B, C, D0, D1, and D2 which was the last stage found during the field trials. Moulting activity was determined as the number of prawns that showed signs of imminent or were currently moulting (Skewes *et al.*, 1994). Prawns in late premoult D2 and in the postmoult A and B were classified as moulting. These moult stages lasted from one day before and after moulting (Chapter 5).

### 6.2.4. Histology

Prawns were anaesthetised with iced water prior to histological examination. The prawns were injected with 0.5 mL Davidson's fixative into the adjacent area of hepatopancreas and immersed in the fixative for 48 hours. The cephalothorax was cut in half longitudinally, put into a histocassette and stored in 70% ethanol for routine histological examination (Bell & Lightner, 1988) (see Chapter 3).

### 6.2.5. Data analysis

The abundance of the lymphoid organ spheroid cells was determined as spheroid to total tissue (STT) ratio (see Chapter 3). Data was analysed using univariate analysis of variance (ANOVA) on SPSS version 11 to examine the relationship between STT ratio and moult stages, lunar cycles, sex, weight and total length of the experimental animals (see Chapter 3). ANOVA was run twice with each LOS measurement (STT ratio, prevalence of vacuolated spheroids and number of vacuoles in spheroid) and the two different moult categories; one with moult stages (A, B, C, D0, D1, D2) and the second with three main moult stages (TMMS) (postmoult, intermoult and premoult). Post-hoc least significant difference (LSD) was performed to analyse differences between the means of individual variables at a critical value of $P < 0.05$.

## 6.3. Results

### 6.3.1. Moult stages and lunar phases

Stage A, B, C, D0, D1, and D2 could be determined during the experiment by using the setal development (setogenesis) and the withdrawal of epidermis from the setal bases (apolysis) in the median part of the inner uropod near the telson tip of the prawn. During experiments, several prawns showed unsynchronised development between setal development and epidermal withdrawal of the uropod. This might indicate the slow growth of the animals (see Chapter 5). As a result, it was difficult to distinguish between stage B, C and D. Therefore, the epidermal retraction was used to classify the stage of moult.

Moult stages of the prawn varied within the sampling time (the lunar periodicities) in both experiments. It seemed that stage D0 and D1 were more commonly found across the moon phases. Stage A was less commonly found in these experiments. In the first trial (Table 6.1), the stage of the moult was more uniform in the first sampling and the last sampling (new moon), while sampling in the other moon phases the stage of the moult was more dispersed. However, stage A was never found throughout the samplings. In the second trial, the stage of the moult was more divergent throughout the lunar rhythms (Table 6.2). Stage A and D2 still were less frequently found in this experiment.

**Table 6.1**. The number and the moult stages of *P. monodon* collected at different lunar phases in the first experiment.

| Lunar phases | Moult stages | | | | | | Total |
|---|---|---|---|---|---|---|---|
| | A | B | C | D0 | D1 | D2 | |
| NM | 0 | 0 | 0 | 6 | 9 | 0 | 15 |
| FQM | 0 | 2 | 0 | 6 | 6 | 1 | 15 |
| FM | 0 | 1 | 1 | 4 | 8 | 1 | 15 |
| LQM | 0 | 1 | 0 | 6 | 7 | 1 | 15 |
| NM | 0 | 0 | 4 | 11 | 0 | 0 | 15 |
| Total | 0 | 4 | 5 | 33 | 30 | 3 | 75 |

**Table 6.2.** The number and the moult stages of *P. monodon* collected at different lunar phases in the second experiment.

| Lunar phases | Moult stages | | | | | | Total |
|---|---|---|---|---|---|---|---|
| | A | B | C | D0 | D1 | D2 | |
| FM | 0 | 0 | 3 | 9 | 3 | 0 | 15 |
| LQM | 0 | 1 | 2 | 4 | 7 | 1 | 15 |
| NM | 1 | 2 | 2 | 4 | 6 | 0 | 15 |
| FQM | 1 | 4 | 2 | 1 | 5 | 2 | 15 |
| FM | 1 | 2 | 1 | 4 | 7 | 0 | 15 |
| Total | 3 | 9 | 10 | 22 | 28 | 3 | 75 |

It was apparent that in the first trial, moulting occurred in or near the first quarter, full and last quarter moon. While in the second trial, it seemed that moulting occurred around new, first quarter and full moon. However, this data was not robust enough to conclude the tendency of moulting rhythmicity of prawn based on moon phases due to the unbalanced numbers at sampling and the short period of sampling.

### 6.3.2. Moult stages and the LOS cells

There were 70.67% of the prawns tested during the first trial, displayed spheroid cells within their lymphoid organ. The STT ratio of 75 prawn samples varied from 0.00 – 55.56% with a mean (± SD) of 10.40 ± 11.16%. Forty eight percent of the prawns showed vacuolated spheroids. The number of vacuoles for all prawns tested ranged from 0.00 – 6.00 with a mean of 0.60 ±1.05. The prevalence of vacuolated spheroids varied between 0.00 and 100.00% with a mean of 26.93 ± 34.74%.

In the second experiment, 92.00% of the samples demonstrated spheroid cells within their lymphoid organ. The STT ratio of the 75 prawns ranged from 0.00 to 83.64% with a mean of 38.53 ± 25.14%. It was found that 74.67% of the experimental animals exhibited vacuolated spheroids. The number of vacuoles within the spheroid cells from all samples ranged from 0.00 to 4.35 with a mean of 0.75 ± 0.99. The prevalence of vacuoles in the spheroid cells also varied from 0.00% to 100.00% with a mean of 28.02 ± 27.99%.

The abundance of lymphoid organ spheroid cells varied within the stage of moult in each trial. In the first trial (Figure 6.1a), the STT ratio reached a peak at stage B and went down dramatically at stage C. Furthermore, the STT ratio climbed slightly at stage D0 and D1 before falling to the lowest ratio at stage D2. In the second trial (Figure 6.1b), the STT ratio had a tendency to increase slightly across the moult stages. Univariate analysis of variance showed that moult stages had no significant effect on the STT ratio either in the first experiment ($F = 2.179$; $df = 4, 74$; $P > 0.05$) or in the second experiment ($F = 0.464$; $df = 5, 74$; $P > 0.05$).

However, there was a relationship between the abundance of LOS cells and moult stages in the interaction between moult stages and lunar cycles. Statistical analysis demonstrated that this interaction was significant ($F = 3.865$; $df = 7, 74$; $P < 0.05$) in the first trial (Figure 6.2) but not in the second ($F = 0.720$; $df = 11, 74$; $P > 0.05$).

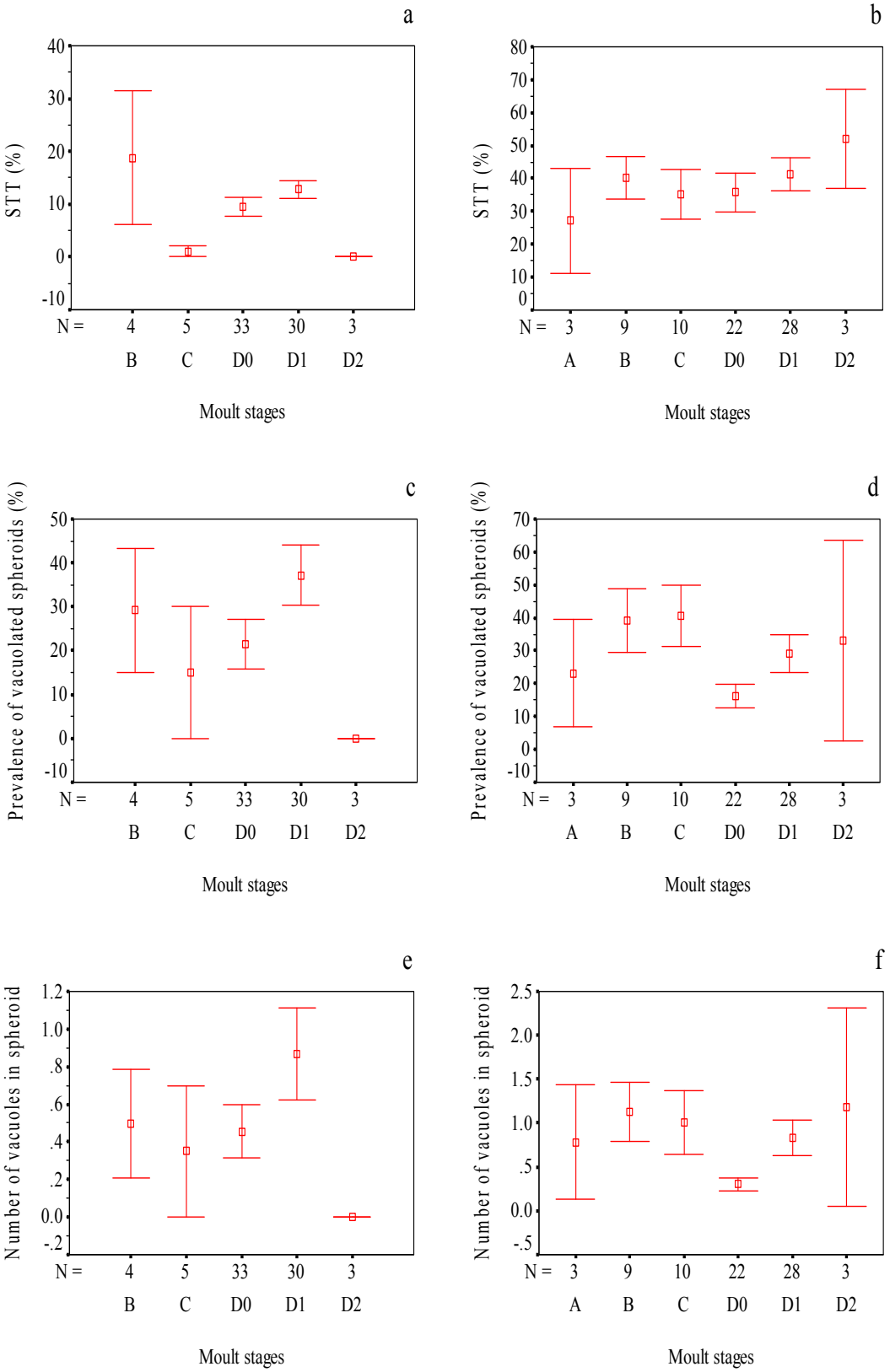



**Figure 6.1.** The mean error bar (± 1 SE) of the STT ratio (a and b), the prevalence of vacuolated spheroids (c and d) and the number of vacuoles in spheroids (e and f) of *P. monodon* with moult stages in the first (a, c and e) and second experiments (b, d and f).

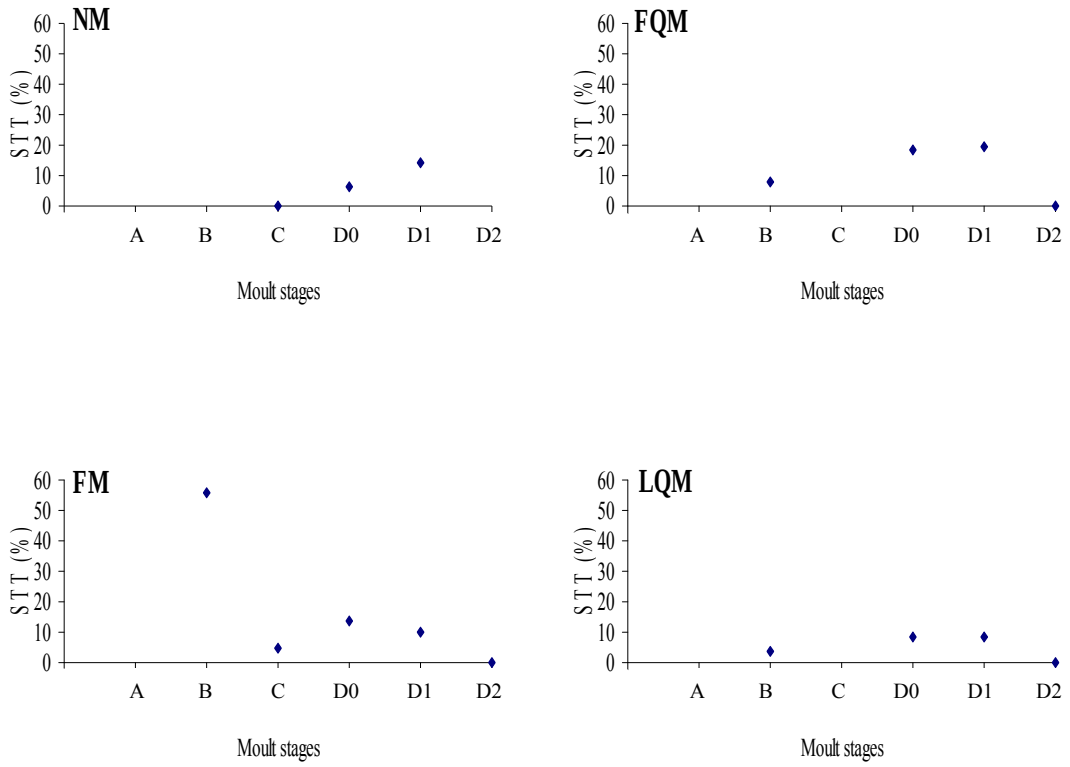



**Figure 6.2.** The spheroid to total tissue (STT) ratio of *P. monodon* with moult stages at different lunar phases (NM, new moon; FQM, first quarter moon; FM, full moon and LQM, last quarter moon) in the first experiment.

There was no significant difference in the interaction between moult stages and sex on the STT ratio both in the first ($F = 0.586$; $df = 3, 74$; $P > 0.05$) and in the second trial ($F = 0.784$; $df = 3, 74$; $P > 0.05$). Neither in the first study ($F = 0.105$; $df = 3, 74$; $P > 0.05$) nor in the second ($F = 0.815$; $df = 5, 74$; $P > 0.05$) were the interactions between moult, sex and lunar cycles significant.

The prevalence of vacuolated spheroids within the spheroid cells also showed inconsistency throughout the cycles of the moult. Stage D1 had the highest prevalence of vacuolated spheroids in the first tria1 (Figure 6.1c). Stage D2 had the lowest prevalence of vacuolated spheroids. In the second trial (Figure 6.1d), the highest prevalence of vacuolated spheroids was in stage C, whilst stage D0 was the lowest. However, univariate analysis of variance revealed that moult stages had no significant effect on the prevalence of vacuolated spheroids either in the first study ($F = 1.231$; $df = 4, 74$; $P > 0.05$) or in the second ($F = 1.022$; $df = 5, 74$; $P > 0.05$).

There was no significant effect in the interaction between moult stages and sex ($F = 0.863$; $df = 3, 74$; $P > 0.05$ and $F = 0.312$; $df = 3, 74$; $P > 0.05$ in the first and second trial, respectively), nor was the interaction between moult stages and lunar phase significant ($F = 1.011$; $df = 7, 74$; $P > 0.05$ and $F = 0.917$; $df = 11, 74$; $P > 0.05$). Moreover, interaction between moult stages, sex and lunar phase demonstrated the absence of significant effect on the prevalence of vacuolated spheroids in both trials ($F = 2.643$; $df = 3, 74$; $P > 0.05$ and $F = 0.659$; $df = 5, 74$; $P > 0.05$).

The pattern of the number of vacuoles in spheroids was similar to the pattern of prevalence of vacuolated spheroid cells (Figure 6.1e and f) across the moult stages. There was no significant difference of the number of vacuoles from different moult stages in both experiments ($F = 0.601$; $df = 4, 74$; $P > 0.05$ and $F = 0.810$; $df = 5, 74$; $P > 0.05$). Moult stages interaction with sex also had no significant effect in both studies ($F = 0.554$; $df = 3, 74$; $P > 0.05$ and $F = 1.735$; $df = 3, 74$; $P > 0.05$), nor were they significant in their interaction with lunar phases ($F = 0.649$; $df = 7, 74$; $P > 0.05$ and $F = 0.808$; $df = 11, 74$; $P > 0.05$). In addition, the interaction between moult stages, sex and lunar cycles again revealed no significant effect on the number of vacuoles both in the first study ($F = 2.527$; $df = 3, 74$; $P > 0.05$) or in the second ($F = 1.827$; $df = 5, 74$; $P > 0.05$).

When the moult stages were simplified into three main moult stages (TMMS), the variability of the STT ratio, the prevalence of vacuolated spheroids and the number of vacuoles also were analysed in both experiments (Figure 6.3). In the first trial, postmoult had the highest STT ratio, followed by premoult and intermoult accounted for the lowest ratio of STT. Whilst in the second trial, the highest STT ratio was in the premoult stage, followed by postmoult and the lowest was also in the intermoult stage. Univariate analysis of variance revealed no significant difference of the TMMS on the STT ratio either in the first trial ($F = 1.217$; $df = 2, 74$; $P > 0.05$) or in the second ($F = 0.012$; $df = 2, 74$; $P > 0.05$).

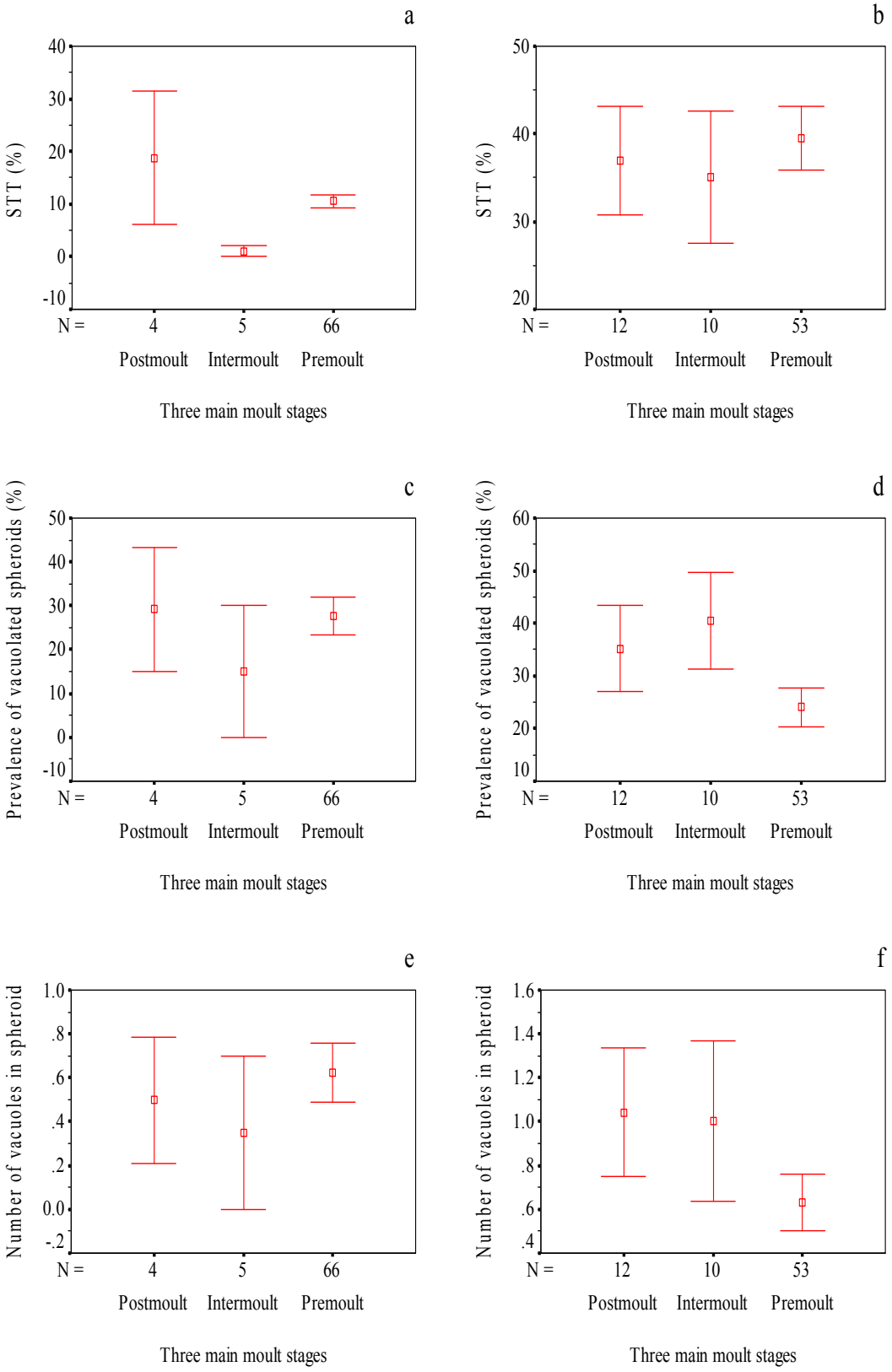



**Figure 6.3.** The mean error bar (± 1 SE) of the STT ratio (a and b), the prevalence of vacuolated spheroids (c and d) and the number of vacuoles in spheroids (e and f) of *P. monodon* with three main moult stages in the first (a, c and e) and second experiments (b, d and f).

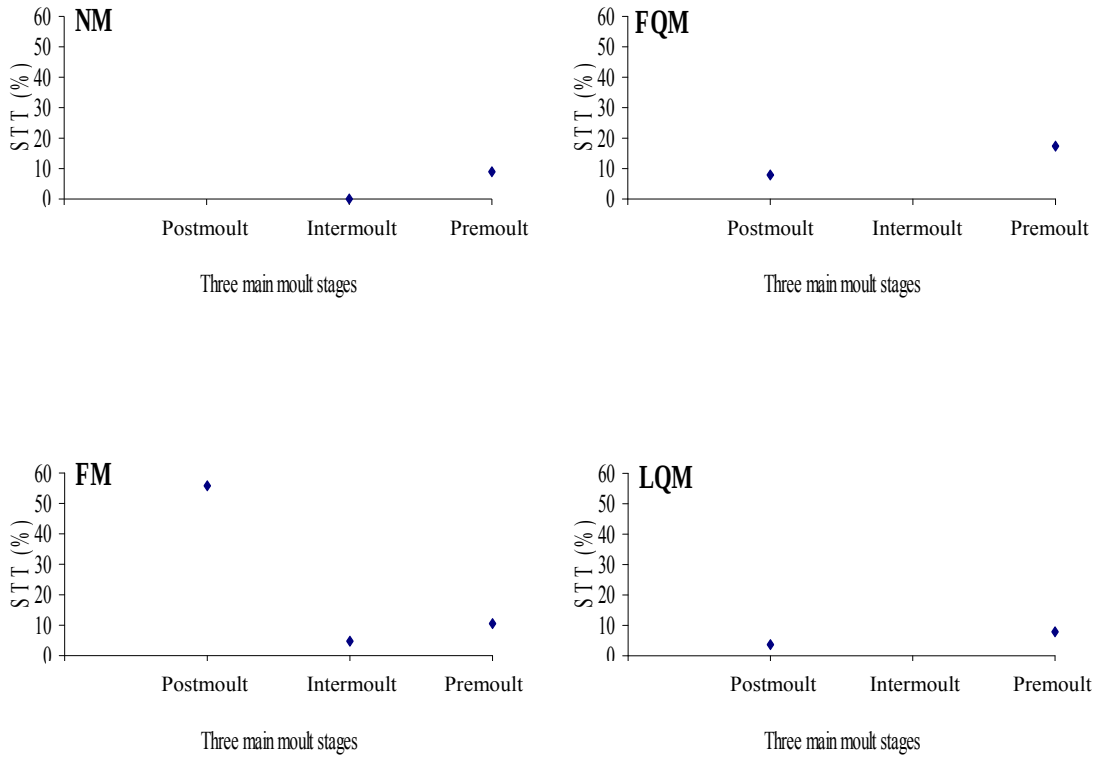



**Figure 6.4.** The spheroid to total tissue (STT) ratio of *P. monodon* with three main moult stages at different lunar phases (NM, new moon; FQM, first quarter moon; FM, full moon and LQM, last quarter moon) in the first experiment.

However, again the effect of the TMMS on the ratio of STT might be different within the moon phases (Figure 6.4). Statistically, this effect was significant in the first ($F = 7.245$; $df = 3, 74$; $P < 0.05$) but not in the second trial ($F = 0.439$; $df = 6, 74$; $P > 0.05$). Moreover, there was no significant effect in interaction between TMMS and sex ($F = 0.830$; $df = 2, 74$; $P > 0.05$ and $F = 0.806$; $df = 2, 74$; $P > 0.05$) nor was the interaction between TMMS, sex and lunar phases in the second experiment ($F = 0.286$; $df = 3, 74$; $P > 0.05$). The three way interactions between these three independent variables (TMMS * sex * lunar cycle) and the ratio of STT, prevalence of vacuolated spheroid and the number of vacuoles in spheroid in the first study could not be analysed due to lost degrees of freedom (df).

In terms of prevalence of vacuolated spheroid cells, the postmoult stage was higher than the other stages in the first experiment (Figure 6.2c). The prevalence of vacuolated spheroids was higher in the intermoult stage than postmoult and premoult

stages in the second experiment (Figure 6.2d). However, there was no significant difference of TMMS on the prevalence of vacuolated spheroids both in the first trial ($F = 0.155$; $df = 2, 74$; $P > 0.05$) or in the second ($F = 2.467$; $df = 2, 74$; $P > 0.05$). Interaction between TMMS and sex also revealed no significant effect ($F = 0.787$; $df = 2, 74$; $P > 0.05$ and $F = 0.363$; $df = 2, 74$; $P > 0.05$), nor was it significant in its interaction with lunar phase ($F = 1.677$; $df = 3, 74$; $P > 0.05$ and $F = 1.557$; $df = 6, 74$; $P > 0.05$). Furthermore, the interaction between TMMS, sex and lunar phase demonstrated no significant effect on the prevalence of vacuolated spheroids in the second study ($F = 1.047$; $df = 3, 74$; $P > 0.05$).

The discrepancy also appeared when the number of vacuoles within the lymphoid organ spheroid cells was presented throughout the three main moult stages. The pattern of the number of vacuoles within the LOS cells was also similar with the pattern of the prevalence of vacuolated spheroids except in the second experiment. In this experiment, the number of vacuoles was higher in the postmoult stage than intermoult and premoult stages (Figure 6.3.d). Univariate analysis of variance showed that there was no significant effect of the TMMS on the number of vacuoles in both experiments ($F = 0.099$; $df = 2, 74$; $P > 0.05$ and $F = 2.287$; $df = 2, 74$; $P > 0.05$). In addition, there was no significant difference of interaction between TMMS and sex ($F = 0.506$; $df = 2, 74$; $P > 0.05$ and $F = 2.762$; $df = 2, 74$; $P > 0.05$), nor was interaction between TMMS and lunar phases on the number of vacuoles in spheroid in both trials ($F = 0.891$; $df = 3, 74$; $P > 0.05$ and $F = 1.542$; $df = 6, 74$; $P > 0.05$). However, the effect of TMMS on the number of vacuoles was complex as shown by the interaction between TMMS, sex and lunar cycle. This interaction was significant ($F = 3.163$; $df = 3, 74$; $P > 0.05$) in the second experiment.

### 6.3.3. Lunar phases and the LOS cells

The variability of the spheroid to total tissue ratio appeared within the lunar rhythms in each trial (Figure 6.5a and b). Both trials had the same pattern in the STT ratio within the phases of the moon. It seemed that the build up of the LOS cells occurred in interval between new moon and first quarter moon, and then reached a maximum ratio at first quarter moon. While during full moon and last quarter moon, the LOS cells gradually decreased.

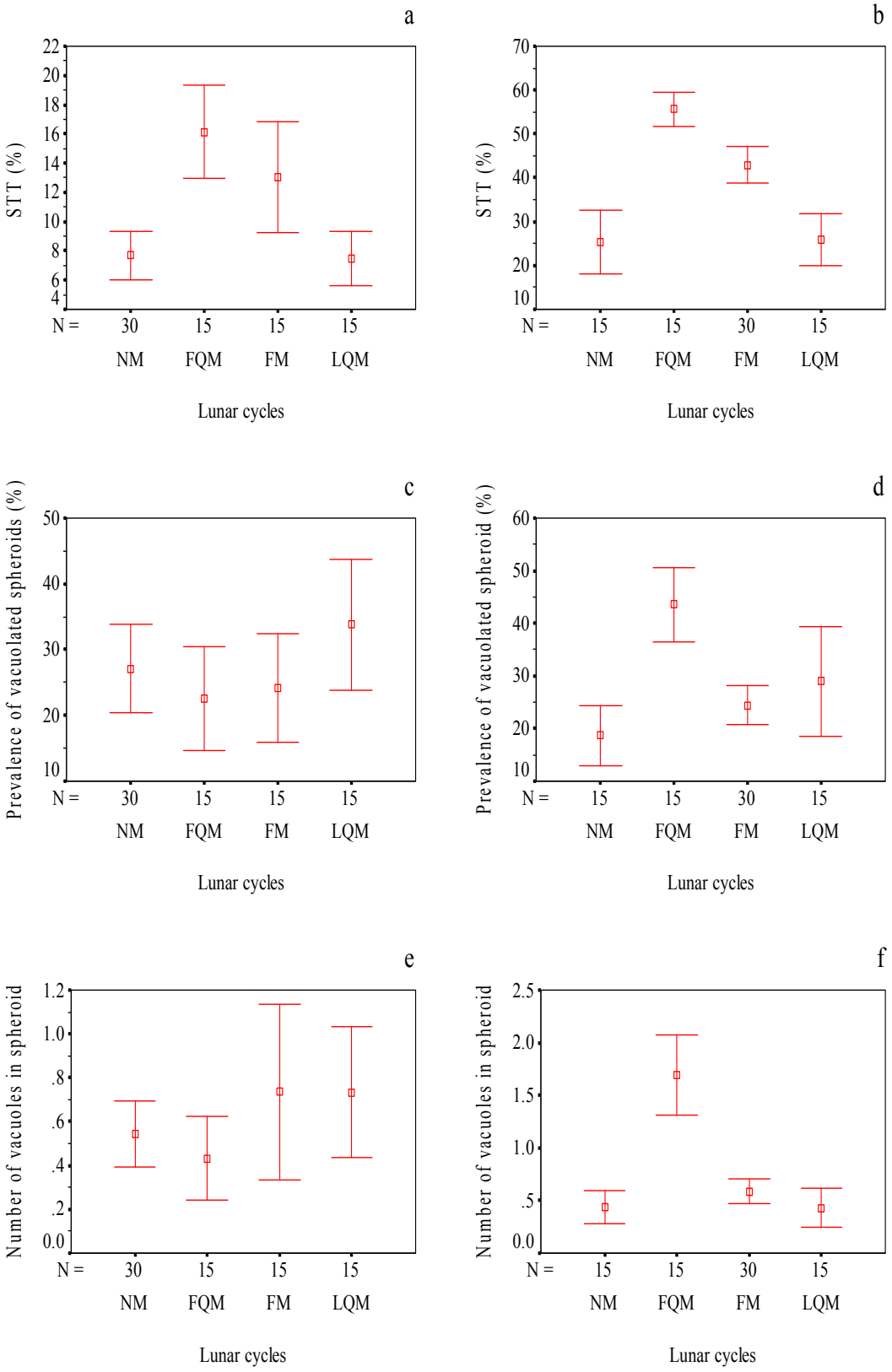



**Figure 6.5.** The mean error bar (± 1 SE) of the STT ratio (a and b), the prevalence of vacuolated spheroids (c and d) and the number of vacuoles in spheroids (e and f) of *P. monodon* in the first (a, c and e) and second experiments (b, d and f) with lunar phases. NM, new moon; FQM, first quarter moon; FM, full moon; LQM, last quarter moon.

A univariate analysis of variance revealed that neither in the first experiment ($F = 2.784$; $df = 3, 74$; $P > 0.05$) or in the second ($F = 1.995$; $df = 3, 74$; $P > 0.05$) was there a significant difference on the ratio of STT with the lunar phase. The interaction between lunar cycles and sex also showed no significant effect on the STT ratio in the first experiment ($F = 0.723$; $df = 3, 74$; $P > 0.05$), nor in the second ($F = 0.740$; $df = 3, 74$; $P > 0.05$).

The prevalence of vacuolated spheroids also showed inconsistency throughout the lunar phases in both experiments (Figure 6.5c and d). In the first experiment, the prevalence of vacuolated spheroids decreased from the new moon to the first quarter moon, increased at the next phase (full moon) and reached a peak at the last quarter moon. In the second experiment, the prevalence of vacuolated spheroids was highest during the first quarter moon, followed by last quarter moon and full moon. Whilst at the new moon period, the prevalence was the lowest. No significant difference in the prevalence of vacuolated spheroids by the lunar rhythms was found in both trials ($F = 0.734$; $df = 3, 74$; $P > 0.05$ and $F = 1.036$; $df = 3, 74$; $P > 0.05$). In addition, there was no significant effect of the interaction between moon phases and sex on the prevalence of vacuolated spheroids in the first study ($F = 1.379$; $df = 3, 74$; $P > 0.05$), nor in the second ($F = 0.\ 110$; $df = 3, 74$; $P > 0.05$).

The number of vacuoles within the spheroid cells also varied throughout the lunar cycles in both experiments (Figure 6.5e and f). The pattern of the number of vacuoles was almost similar to the pattern of prevalence of vacuolated spheroids. In the first experiment, the number of vacuoles was highest at full moon followed by last quarter moon then the new moon and the first quarter moon was the lowest. In contrast, in the second experiment, the number of vacuoles was highest at the first quarter moon followed by the full moon, then the new moon and the last quarter moon was the lowest. Unfortunately, no significant difference on the number of vacuoles in the spheroid with moon phases was found in the first trial ($F = 0.532$; $df = 3, 74$; $P > 0.05$), or in the last ($F = 2.659$; $df = 3, 74$; $P > 0.05$). Neither in the first study ($F = 0.987$; $df = 3, 74$; $P > 0.05$) or in the second ($F = 0.265$; $df = 3, 74$; $P > 0.05$) did the interaction between moon phases and sex have a significant effect on the number of vacuoles in the spheroid.

However, when the TMMS was used, significant differences on the STT ratio within the lunar phase was evident in the first experiment ($F = 5.230$; $df = 3, 74$; $P < 0.05$) but not in the second ($F = 2.552$; $df = 3, 74$; $P > 0.05$). Post-hoc LSD demonstrated that the STT ratio at first quarter moon was significantly higher than new moon and last quarter moon in the first study (Table 6.3). In contrast, in terms of the number of vacuoles in spheroids, a significant difference on the number of vacuoles with moon phase was obvious in the second trial ($F = 3.499$; $df = 3, 74$; $P < 0.05$) but not in the first ($F = 0.758$; $df = 3, 74$; $P > 0.05$). LSD multiple comparison revealed that the number of vacuoles in spheroids at first quarter moon was significantly higher than at other moon phases (Table 6.4).

**Table 6.3.** Significant differences (P values) of the STT ratio of *P. monodon* at different lunar phases in the first experiment.

| | NM | FQM | FM | Mean ± SE (%) |
|---|---|---|---|---|
| NM | | | | 7.694 ± 1.670 |
| FQM | 0.016 | | | 16.145 ± 3.193 |
| FM | 0.121 | 0.434 | | 13.046 ± 3.764 |
| LQM | 0.942 | 0.030 | 0.160 | 7.447 ± 1.848 |

**Table 6.4.** Significant differences (P values) of the number of vacuoles in spheroids of *P. monodon* at different lunar phases in the second experiment.

| | NM | FQM | FM | Mean ± SE (%) |
|---|---|---|---|---|
| NM | | | | 0.439 ± 0.158 |
| FQM | 0.000 | | | 1.689 ± 0.382 |
| FM | 0.596 | 0.000 | | 0.587 ± 0.116 |
| LQM | 0.975 | 0.000 | 0.572 | 0.429 ± 0.187 |

Univariate analysis of variance demonstrated that there was no significant effect of weight ($F = 2.214$; $df = 1, 74$; $P > 0.05$ and $F = 0.194$; $df = 1, 74$; $P > 0.05$), total length ($F = 3.899$; $df = 1, 74$; $P > 0.05$ and $F = 0.012$; $df = 1, 74$; $P > 0.05$), or sex ($F = 1.924$; $df = 1, 74$; $P > 0.05$ and $F = 0.002$; $df = 1, 74$; $P > 0.05$) on the STT ratio in both experiments. Weight ($F = 0.336$; $df = 1, 74$; $P > 0.05$ and $F = 0.048$; $df = 1, 74$; $P > 0.05$), total length ($F = 0.478$; $df = 1, 74$; $P > 0.05$ and $F = 0.356$; $df = 1, 74$; $P > 0.05$) and sex ($F = 0.108$; $df = 1, 74$; $P > 0.05$ and $F = 0.415$; $df = 1, 74$; $P > 0.05$) had no significant effect on the prevalence of vacuolated spheroids.

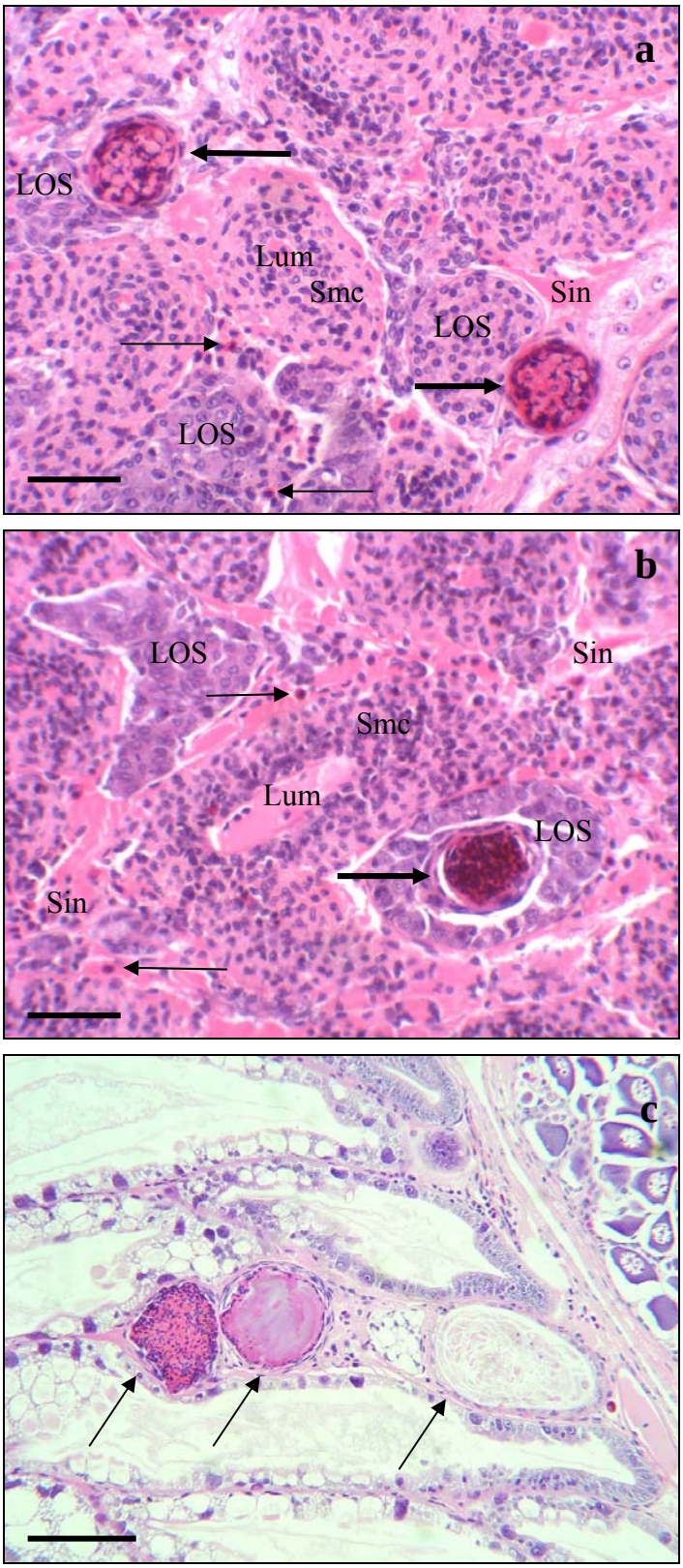



**Figure 6.6.** Longitudinal section of various tissues of *P. monodon.* (a) Eosinophilic foci (bold arrow) within the lymphoid organ, scale bar = 50 µm; (b) bacterial granuloma inside the lymphoid organ spheroid cells (bold arrow), note flattened epithelial cells encapsulating the melanized zone, scale bar = 50 µm; (c) bacterial granuloma within the hepatopancreas (arrow), scale bar = 100 µm. H & E stain. Lum, lumen; Smc, stromal matrix cells; Sin, haemal sinus, and haemocyte (arrow).

Similar results were also found in terms of the number of vacuoles in spheroids. Weight ($F = 0.088$; $df = 1, 74$; $P > 0.05$ and $F = 0.199$; $df = 1, 74$; $P > 0.05$), total length ($F = 0.116$; $df = 1, 74$; $P > 0.05$ and $F = 0.866$; $df = 1, 74$; $P > 0.05$) and sex ($F = 0.030$; $df = 1, 74$; $P > 0.05$ and $F = 0.094$; $df = 1, 74$; $P > 0.05$) had no significant effect on the number of vacuoles.

In the first trial, there was no significant correlation between STT ratio and prevalence of vacuolated spheroids ($r = 0.206$; $P = 0.076$), nor was there an effect between STT ratio and the number of vacuoles in the spheroids ($r = 0.106$; $P = 0.364$) (Pearson correlation). However, there was a strong significant correlation between the number of vacuoles and prevalence of vacuolated spheroids ($r = 0.841$; $P = 0.000$). In the second trial, STT ratio and prevalence of vacuolated spheroids had no significant correlation ($r = 0.195$; $P = 0.093$). However, a weak correlation appeared between STT ratio and prevalence of vacuolated spheroids ($r = 0.338$; $P = 0.003$) and a strong correlation occurred between the number of vacuoles and the prevalence of vacuolated spheroids ($r = 0.834$; $P = 0.000$).

The presence of eosinophilic/magenta and basophilic inclusions within the lymphoid organ spheroid cells was evidenced during the experiment. These inclusion bodies were only observed within the LOS cells and were never found in the stromal matrix cells (normal lymphoid tubules). Ectopic spheroid cells were found in antennal gland and heart. Furthermore, eosinophilic foci were sometimes also observed within the lymphoid organ during the experiments (Figure 6.6a). Bacterial granulomas were found within the lymphoid organ, inside the LOS cell (Figure 6.6b) and in the hepatopancreas (Figure 6.6c) suggesting bacterial septicaemia occurred in the farmed prawns.

### 6.4. Discussion

The method of sampling (catchability) may have influenced the moult stages during the experiments, particularly in the first trial. In this experiment, sampling was by trapping four times. Trapping may have affected the randomization of the sample. This method sampled prawns which were attracted to the food within the trap. Prawns soon after and before moulting do not feed as seen in *P. esculentus*

(Wassenberg & Hill, 1984) and *P. indicus* (Vijayan *et al.*, 1997). As a result, it was unlikely to find early postmoult stage (stage A) and late premoult stage (stage D2, D3 or D4) (Table 6.1). However, this was the management strategy of the farm, with which we had to comply.

In the second experiment in which sampling was done by a cast net, most moult stages could be found (Table 6.2). This indicated that a cast net was more applicable than a trap in that it randomised the sampling of penaeids in the ponds. However, early postmoult and late premoult stages were still found in small numbers compared to the other stages. Burying of *P. esculentus* during the day (Wassenberg & Hill, 1984) might also occur in *P. monodon* during these experiments that in turn, affected sampling. In addition, they found that the emergence time of *P. esculentus* declined around ecdysis. A cast net was more likely to catch prawns above the substrate of the ponds or when prawns were more active. It is clear that sampling methodology could influence the mix of moulted prawns in any collection.

It was unlikely that the moult stages were directly responsible for the variability of the spheroid cells within the lymphoid organ. However, the first trial showed that the effect of moult stages on the ratio of STT was within the interaction between moult stages and lunar rhythms. This result might be affected by the high variability of STT ratio in individual prawns and the relatively small sample size in each category of moult stages especially in stage B (postmoult) and C (intermoult). Particularly at the full moon, only one prawn at stage C (intermoult) was collected which had the highest STT ratio (55.56%) during the experiment. As a result the occurrence of type I (alpha) error in this case was possible, i.e. it might not really be statistically different, just a result within the accepted error rate of the test. Therefore, it is suggested that other mechanisms worked stimulating the fluctuation of the spheroid cells throughout the life of the animals.

The tendency of moulting related to phase of the moon was varied between the two experiments. In the first trial, ecdysis occurred in or near first quarter moon, full moon and last quarter moon. In the second trial, it was apparent that prawns moulted around new moon, first quarter moon and full moon. The discrepancy might have occurred due to the differences in age and size of the prawns (Robertson *et al.*,

1987), the duration of moult, the small number of samples and the short period of the sampling. The method of sampling, as mentioned above, might also have contributed to the inconsistency. As a result, it was hard to find correlation between the moon phases and moult stages in the current study.

Some studies have presented evidence for the correlation between moulting and lunar periodicities. *Gonodactylus zacae* showed strong bimodal patterns in numbers of moults throughout the moon phases. Most moults occurred around full moon and new moon (Reaka, 1976). Synchronized fortnightly moulting in the sand beach isopod, *Excirolana chiltoni*, showed that the actual moulting activity reached a peak one week prior to both the new and full moon (Klapow, 1972). Reaka (1976) revealed that *Pseudosquilla ciliata* mostly moulted around last quarter moon. Nascimento *et al.* (1991) found that in *P. schimitti*, moulting predominantly occurred in intervals between full and new moons. In *P. esculentus*, the peak of moulting frequency occurred at full moon (White, 1973). In *Panulirus ornatus* moulting activity peaked around one week after the full moon (Skewes *et al.*, 1994).

In contrast, the moulting frequency of *Homarus gammarus* peaked around the new moon and declined around the full moon period (Ferrero *et al.*, 2002). In the blue swimming crab, *Portunus pelagicus*, moulting activity was also relatively higher during the new moon than at the full moon (Hamsa, 1978). Similarly, McCurdy *et al*., (2000) found evidence that in the intertidal amphipod *Corophium volutator*, moulted females were more numerous at the new moon and assumed that this could be associated with the dispersal opportunities for this amphipod. To summarise, it has been suggested that the variations in moulting activity with lunar rhythms in these studies might be affected by species differences, animal size, competition for space, and behavioural mechanism to avoid predation or cannibalism, feeding and foraging behaviour, and mating or spawning synchronicities.

The effect of the endogenous factor of lunar periodicities has also been studied on the growth increments, behaviour, and the reproductive activities of crustaceans. Cultured *P. vannamei* from wild larvae caught in Ecuadorean ponds showed significantly higher ($P < 0.05$) wet weight increments during new and full moon than during first and last quarter moon (Griffith & Wigglesworth, 1993). They claimed

that this prawn might have previous exposure to entraining cue or zeitgeber, such as temperature or salinity or other unidentified factors, which might contribute to the relationship between growth increments and lunar rhythms. Furthermore they suggested that this growth rhythm might be endogenous or inherited without an environmental cue being necessary.

Mating in *P. schmitti* reached a peak around full and new moon phases and no mating was observed during the moulting peaks (in the intervals between) (Nascimento *et al.*, 1991). In *P. plebejus*, there was a tendency to increase the mean percentage of mature females (vitellogenic oocytes) around new moon and full moon. The mean percentage of ripe females (oocytes with peripheral body) reached maximum between the new and full moon phases (Courtney *et al.*, 1996). A study on sesarmid crabs, *Pesiserma guttatum*, also showed lunar variability on its reproductive activity. Larvae were released around the new and full moon while the ovigerous ratio peaked preceding the full moon (Flores *et al.*, 2002). In *H. gammarus* the release of hatchlings was common around new moon and full moon (Ferrero *et al.*, 2002). They suggested that at the new moon, larvae were not easily recognised by predators. On the other hand at the full moon, the increasing turbidity due to the stronger spring tidal currents could enhance the mimetic effect of larval colour to avoid predation. In contrast, work of Paula (1989) showed that larval release of decapod crustaceans mostly occurred during the first quarter moon suggesting this might be a combination of oviposition time, incubation time, and tidal rhythms.

The settlement of the Caribbean spiny lobster (*Panulirus argus*) postlarvae was highest between the new moon and the first quarter moon (Enggleston *et al.*, 1998). In contrast, the settlement of the blue crab *Callinectes sapidus* megalopae was highest during the full moon (Montfrans *et al.*, 1990). Furthermore, both adult and copepodite instar of zooplankton *Pseudodiaptomus cokeri* had a full moon peak in abundance at the surface (Rios-Jara & Gonzales, 2000). Tidal transport as a result of new moon spring tides allowed the lobster postlarvae to move to inshore nursery habitats and predation could be minimized due to the darkest phases of the moon (Enggleston *et al.*, 1998).

Some reports showed the effect of moon phases on the penaeid catch rate of the commercial fishery. The maximum catch per effort values for *P. esculentus* and *P. plebejus* occurred a couple days before the full moon, while the minimum occurred several days prior or at the new moon phases (White, 1973; Courtney *et al.*, 1996). However, White (1973) failed to determine whether the fluctuation of the catches was a result of moon cycles or another response such as the fluctuation of some prey species. Furthermore, water temperature, population density, population age structure, depth or tidal cycle was unlikely to affect the rhythm. Courtney *et al*. (1996) suggested that the relationship between catch rate and lunar periodicity varied with prawns size and/or depth. Another study on the relationship between lunar cycles and catch rate of penaeids showed that endeavour prawns (*M. endeavouri* and *M. ensis*) had largest catch rate at full moon while tiger prawns (*P. esculentus* and *P. semisulcatus*) peaked at last quarter moon (Salini *et al.*, 2001).

Different results in catch rate of penaeid prawn associated with the lunar cycle were presented by Griffiths (1999). He found that catch per unit of effort (CPUE) of *P. plebejus* in an Australian coastal lagoon was significantly higher during the new moon than the full moon. He suggested that this result might be driven by the behaviour of the prawn that was more active during dark moon and remained buried in the substratum during moonlight to avoid predation. Wassenberg and Hill (1994) revealed that penaeid species have different responses to light intensity. *P. plebejus* was very sensitive to the light intensity compared to the other species. Except for *P. merguiensis*, all other species (*P. plebejus, P. latisulcatus, P. semisulcatus, Metapenaeus ensis, P. esculentus, M. endeavouri,* and *M. bennetae*) emerged from the substratum when the light intensity decreased and buried themselves when the light intensity increased. Furthermore, burial activity before dawn might be controlled by an internal clock. This behavioural mechanism might explain the differences in catchability in prawn fisheries. Similarly, the catch rate of the blue swimming crab, *Portunus pelagicus*, was also higher during the full moon (Hamsa, 1978). Clearly, catchability is affected by the lunar phases.

In summary, lunar phases influence many aspect of crustacean life. However, the actual timing varies across the different species involved. The first experiment showed that the variability of STT ratio in *P. monodon* appeared to be a result of

lunar rhythms. It was found that the STT ratio at first quarter moon was significantly higher (LSD multiple comparison, $P < 0.05$) than at new moon and last quarter moon. This implied that at intervals between new moon and first quarter moon, the spheroid cells built up and reached a peak at first quarter moon, then gradually declined at full moon and last quarter moons. Thus, how do the moon phases affect the fluctuation of the spheroid cells within the lymphoid organ of penaeid prawns?

Lunar cycles influence the animal activities in two ways: firstly, with the light intensity in the night related to nocturnal animals and secondly with the ocean tides (either low and high tides or amplitude with spring neap tides) (Withers, 1992). It seems more likely that the lunar patterns of the abundance of lymphoid organ spheroid cells can best be explained by the behavioural mechanism of penaeid prawns to light intensity. Wassenberg and Hill (1994) found evidence that most of penaeid prawns were sensitive to the light intensity (varied with species), emerged at dusk and buried themselves before dawn. It is not surprising that prawns were active during the dark moon and inactive (buried in the substratum) during the full moon (Griffiths, 1999).

Whether spheroid cells are disposed of or destroyed within the lymphoid organ is still debatable. Hasson *et al.* (1999b) hypothesised that two processes might occur at the same time in chronic TSV infected *P. vannamei*, depending on the nutritional and health status of the prawn. Firstly, viral replication might continue within type B LOS which escape the organ, enter the circulatory system, and return to the LO where phagocytosis occurred. As a result, the cyclic phase of LOS persisted in the chronic infection. Secondly, LOS might develop into type C eliminating the virus by self-destruction (apoptosis) and return the LO to the normal form. If the first was occurring, then the LOS cells would persist in the LO during the life of the prawn and the number of LOS would increase with animal size or age due to the continued viral replication. However, the present study found that the STT ratio varied throughout the life of the animals and there was no association with the animal size (total length and weight) or age (Chapter 5) as previously observed by Anggraeni and Owens (2000).

Anggraeni and Owens (2000) suggested that LOS might be disposed of possibly during the ecdysis. Unfortunately, there was no evidence from the current study either from the field trials or laboratory maintained animals (Chapter 5) of the effect of the moulting cycle on the changes of the STT ratio during the life of the prawn. However, the present study strongly showed lunar related patterns on the abundance of spheroid cells in the lymphoid organ. Thus, it was suggested that the spheroid cells were gradually disposed of, stimulated by the lunar rhythmicity. Once again, what factor or process is driving the variability of the spheroid cells within the moon rhythms is unknown and needs further investigation.

# CHAPTER 7
# EFFECT OF VIRAL INFECTION ON THE RELATIONSHIP BETWEEN THE LOS CELLS AND MOULTING STAGES

## 7.1. Introduction

One of the most catastrophic impacts on the world aquaculture industry was viral diseases in cultured penaeid prawns. At least 20 viruses of penaeid prawns have been identified since 1995 (Lightner, 1996; Lightner & Redman, 1998) and the number has increased in recent years as new viruses were found. In Australia alone, viral infections such as hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), lymphoidal parvovirus (LPV), hepatopancreatic parvovirus (HPV), infectious hypodermal and haematopoietic necrosis virus (IHHNV), spawner-isolated mortality virus (SMV), lymphoid organ virus (LOV) later named gill associated virus (GAV), bennetae baculovirus (BBV), parvo-like virus of *P. japonicus* (P-PJ), penaeid haemocytic rod-shaped virus (PHRV) and gut nerve syndrome have emerged in wild and farmed penaeid prawns (Owens 1997; Spann & Lester, 1997; Munday & Owens, 1998).

Since 1994, viruses became a major concern when mass mortality of 12 – 15 g prawns occurred and was named mid crop mortality syndrome, MCMS (Owens 1997). Predominantly 2 viruses were identified as involved in MCMS, a parvo-like virus, SMV and a yellowhead-like virus, GAV (Cullen & Owens, 2003). Currently, the infestations of these two viruses are still significant to the Australian prawn industry. Owens *et al.* (2003) reported that approximately 24% of spawners and 44.7% of *P. monodon* ponds were infected with SMV. Meanwhile, the prevalence of GAV in the black tiger prawn was much higher; around 95% (Munro & Owens, 2005). Therefore, it is not surprising that GAV received plenty of attention from those who are involved in the aquaculture industry (farmers, scientists and policy makers).

GAV has been studied by applying routine histology; transmission electron microscopy (TEM); reverse transcriptase nested polymerase chain reaction (RT-

nPCR); *in situ* hybridization (ISH); immunohistochemistry; real-time quantitative RT-PCT (qRT-PCR) and currently with haemoagglutinin assay using chicken erythrocytes (Spann & Lester, 1997; Cowley *et al.*, 2000a; Cowley *et al.*, 2002b; Callinan & Jiang, 2003; Spann *et al.*, 2003; de la Vega *et al.*, 2004; Munro & Owens, 2005). The GAV genome has also been sequenced (Cowley *et al.*, 2000b; Cowley *et al.*, 2002a). Data from experimentally infected prawns showed that GAV could be transmitted vertically from broodstock to their progeny and that *P. monodon* was the natural host of the virus (Spann *et al.*, 2000; Cowley *et al.*, 2002b). Histopathologically, GAV infected prawns had spheroid cells in the lymphoid organ (Spann *et al.*, 1997; Spann *et al.*, 2003). This chapter examines the abundance of LOS cells within the lymphoid organ in association with the moulting stages in *P. monodon* that were experimentally infected with GAV.

## 7.2. Materials and Methods

### 7.2.1. Experimental animals

Black tiger prawns, *P. monodon*, were transported from a commercial farm in northern Queensland to aquaria at the School of Veterinary and Biomedical Sciences, James Cook University, Townsville on November 16$^{th}$, 2005. The prawns were reared in two 1,000 L plastic bins with a recirculating system and two aerators in each bin. Salinity was held at 35 ppt and temperature varied between 26 – 28 ºC.

For experimental study, the prawns were transferred and individually kept in recirculating aquaria. Prior to use, the filter system and experimental aquaria were chlorinated with liquid chlorine (100 g/l) at 30 ppm overnight. The next day, the equipment was rinsed with fresh water overnight and once again the following day. During the experiment, salinity varied from 28 – 33 ppt and temperature ranged from 26 – 30 ºC. The experimental animals were fed with commercial prawn pellets at 10% total body weight divided into 2 daily feedings. Siphoning was conducted once every two days to remove the waste from the aquaria. Prawns with a mean (± SD) body weight of 13.25 ± 3.82 g (6.91 – 26.31 g) and total length of 11.8 ± 1.0 cm (9.8 – 14.2 cm) were used during the experiment.

### 7.2.2. Viral extraction

Frozen prawns from northern Queensland infected with gill associated virus (GAV) and other viruses were thawed in a 37 ºC water bath. Viral extract was prepared from 5 g of head soft tissues from 3 prawns after the hepatopancreas was removed. The tissues were put in stomacher bags and homogenised in 20 ml double strength phosphate buffer saline (PBS). The homogenized tissues were clarified by centrifugation at 1500 rpm for 10 minutes and the supernatant was further centrifuged at 5000 rpm in an Eppendorf Centrifuge 5804 for 15 minutes. The extract was added with 1% Bovine Donor Serum (BDS) and filtered through 0.45 µm filter using a 5 mL syringe to form a cell-free extract. Furthermore, the extract was divided into 70 µL aliquots in 1.5 mL Eppendorf tubes and stored at – 80 ºC prior to use.

### 7.2.3. Viral injection

The experimental prawns were divided into 2 groups: a control group and a GAV infected group. Prior to use, aliquots of virus were rapidly thawed in a 37 ºC water bath. For each group, prawns on one day after moulting (intermoult stage) were individually injected into the first abdominal segment, either with 50 µL double strength phosphate buffer saline for control, or 50 µL cell-free viral extract for viral infected group using a 0.5 mL Terumo insulin syringe with a needle (29 gauge x ½").

### 7.2.4. Sampling procedure and moult staging

Prawns were sampled on 1, 2, 3, etc. days post-injection (dpi). Three prawns would be sampled every sampling time from each group. The number of samples for each treatment depended on the duration of the moult cycle. The day post-injection was classified in three categories: (1) 1 – 5 dpi; (2) 6 – 10 dpi and (3) 11 – onward dpi. The stage of moult was determined by using the setal development of new setae and the withdrawal of epidermis from the base of setae (Chapter 5). Four moult stages could be determined during the experiment, stage A/B, C, D0, and D1/D2. Stage A and B were categorised as one stage because stage A was only found in one prawn and morphologically and physiologically stage A and B were almost similar and

presented after moulting. Similarly, stage D2 was also only found in one prawn and expressed as a single stage with D1. Histological sections were analysed as described in Chapter 3.

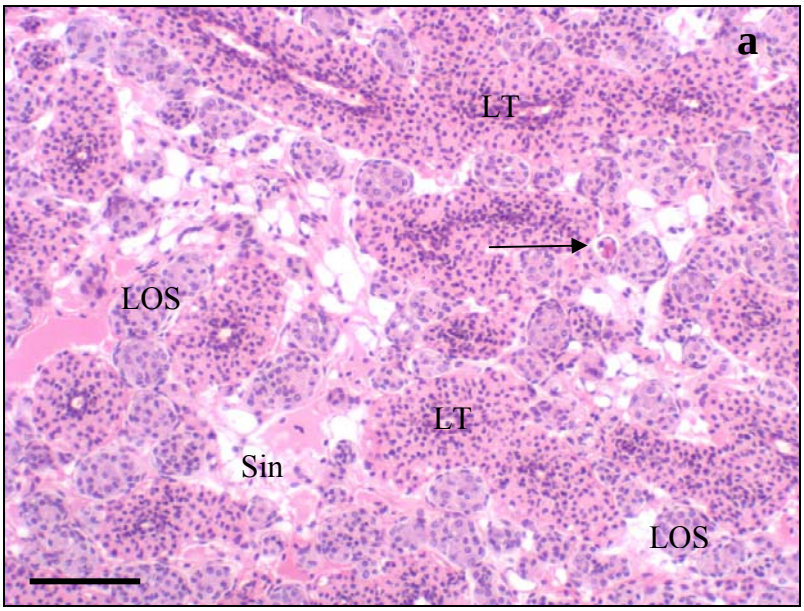
### 7.2.5. Data analysis

To examine the effect of each treatment, moult stages, lunar cycles, day post-injection, sex, and the size of the animals (weight and total length) on the spheroid to total tissue ratio, the prevalence of vacuolated spheroids and the number of vacuoles in spheroid, the data was analysed using univariate analysis of variance with SPSS program version 11. Post-hoc analysis was performed using least significant differences (LSD) to examine the differences between individual mean of the variables. Differences between means were considered significant at $P < 0.05$ level.

## 7.3. Results

### 7.3.1. Clinical signs and histopathology

Two weeks after the experiment started, mass mortality occurred in the stock tanks. After that, it was difficult to find healthy prawns for transfer to the experimental aquaria. As a result, mortality also occurred in the experimental aquaria before treatment and the experiment ended before sampling was completed (one moult cycle). Seven and five prawns were moribund or dead in control and infected group respectively before treatment (injection). One prawn was moribund and five prawns died just after injection in inoculated group, whilst there were no prawns found moribund or dead just after injection in control group. Furthermore, 1 prawn died in control group 3 dpi and 1 prawn was dead 4 dpi in the infected group. Therefore, only 46 prawns (23 prawns from each treatment) could be assessed during the experiment.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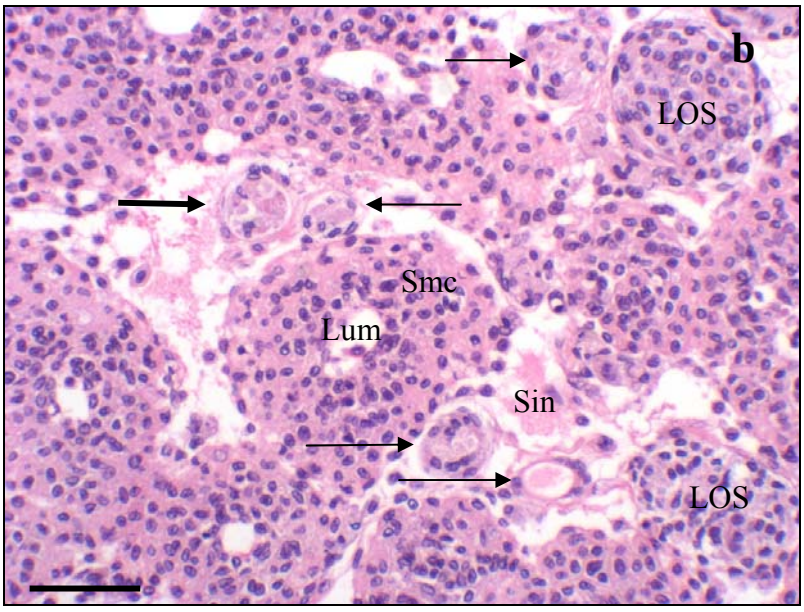
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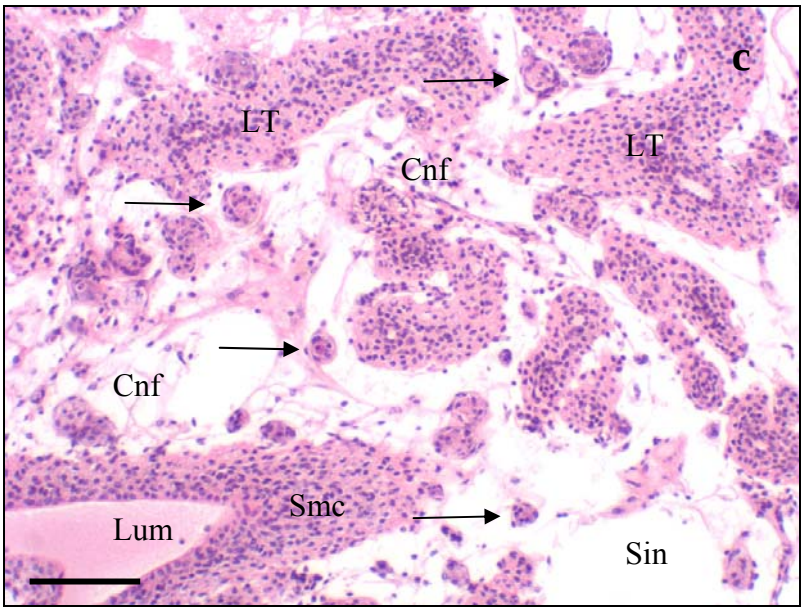
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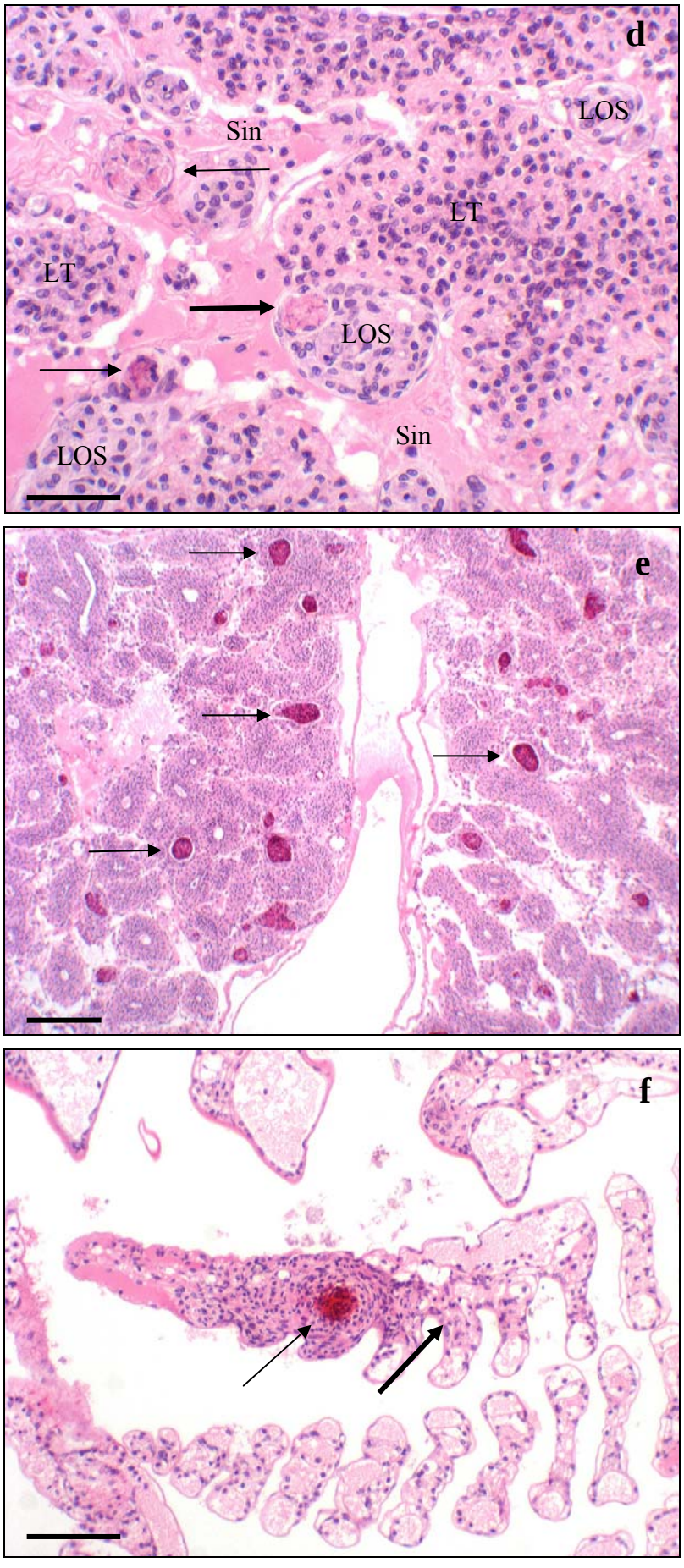
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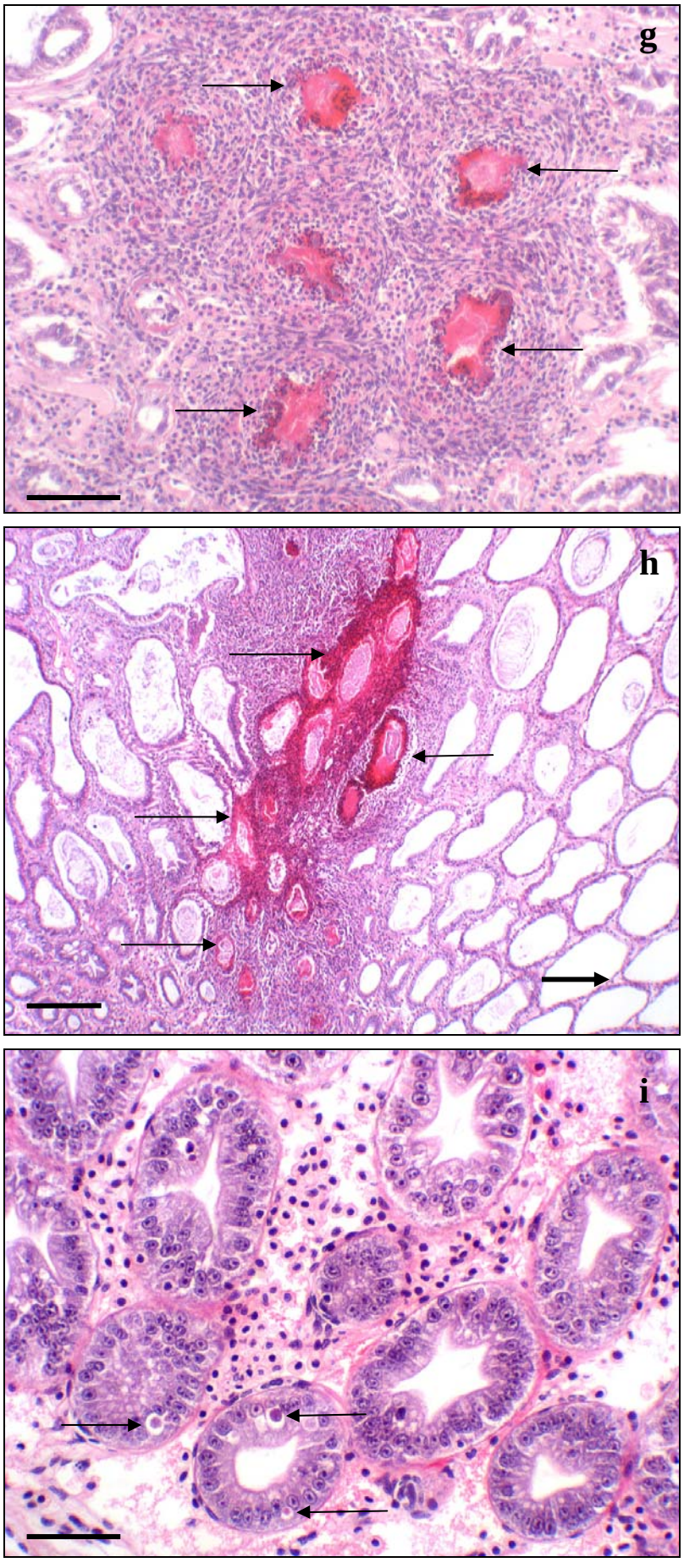
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**Figure 7.1.** Various tissue section of *P. monodon* with H & E stain. (a) Numerous highly encapsulated LOS cells with fibrocytes in haemal sinuses (Sin) between lymphoid organ tubules and one eosinophilic focus (arrow), scale bar = 100 µm; (b) highly degenerated LOS cells (arrow) and necrotic eosinophilic foci (bold arrow), scale bar = 50 µm; (c) abnormal interstitial space (haemal sinuses)/gapping between tubules, note the LOS cells (arrow), scale bar = 100 µm; (d) eosinophilic foci (arrow) and one focus inside the LOS cell (bold arrow) suggesting that originally these foci developed from the LOS cells, scale bar = 50 µm; (e) multiple formation of bacterial granulomas (arrow) in the two lobes of lymphoid organ, cross section, scale bar = 200 µm; (f) melanized nodule in the gill of the prawn with multiple layer of haemocytes encapsulated the nodule (arrow), note the haemocytic infiltration (bold arrow), scale bar = 100 µm; (g and h) massive melanized nodules (arrow) in the hepatopancreas surrounded by multiple layer of haemocytes resulted in the inflammation of the tissue and atrophy of hepatopancreatic tubules (bold arrow), scale bar = 100 µm (g) and 200 µm (h); (i) massive haemocytic aggregations in the haemal sinuses between tubules (bold arrow) and apoptotic cells (arrow) in the hepatopancreas tubules, scale bar = 50 µm.

Some prawns showed amputated and reddish appendages, red body colouration, pinkish gill, lethargy, anorexis, out of balance (swimming in their side), soft cuticle, poorly developed setae in the inner uropod and have biofouling with ciliates. Histopathologically, every single prawn demonstrated spheroid cells within the lymphoid organ. Some spheroids were highly encapsulated by fibrocytes or flattened epithelial cells (Figure 7.1a) while some appeared highly degenerated or less dense (lack of granule material inside) (Figure 7.1b). Abnormal interstitial space (gapping) between tubules was more frequently observed (Figure 7.1c). Necrotic eosinophilic foci were also often found in the lymphoid organ during the experiment (Figure 7.1d). Ectopic spheroids were observed in antennal gland, tegmental gland, hepatopancreas, connective tissue, heart and gills.

Another pathological feature was multiple formations of bacterial granulomas in the lymphoid organ (Figure 7.1e). Generally, these nodules consisted of bacterial colonies in the centre and surrounded by melanized zones. The melanized zones were encapsulated by multiple layers of haemocytes. This inflammatory type of reaction was also observed in the gills (Figure 7.1f) and the hepatopancreas (Figure 7.1g and h). In the hepatopancreas, infiltration of haemocytes around bacterial colonies in tubules (infected sites) resulted in inflammation. Furthermore, haemocytes walled off the invaded tubules and formed granulomatous nodules. Melanization was demonstrated in old granulomatous lesions. Interstitial space between tubules was extensively expanded with enlarged haemal sinuses and

haemocytes (Figure 7.1i). All these histopathological changes were found both in control and in the infected group.

### 7.3.2. Moult stages and lunar phases

Sometimes, staging the moult was difficult due to poorly developed setae and cuticle in the inner uropod in some prawns. Unsynchronised development between setae including the retraction of the cellular matrix, the formation of setal cones, and the retraction of epidermis from the setal bases was seen during the experiment. It seemed that the moult stages developed slowly compared to normal prawns. For instance, in the previous experiment (Chapter 5) in larger prawns (15.2 ± 3.3 g), stage D0 was observed between 1 and 4 days after moulting whilst in this experiment smaller prawns (13.3 ± 3.8 g) at stage D0 was observed for longer between 1 and 7 days after moulting. The moulting period of the prawns in this experiment was also longer than the previous experiment (Chapter 5) and took around 12 – 20 days (15.3 ± 2.4 days) and compared with 8 – 22 days (14.3 ± 3.7 days) previously.

**Table 7.1.** The percentage of moulting (ecdysis) of *P. monodon* at four lunar phases. NM, new moon; FQM, first quarter moon; FM, full moon; LQM, last quarter moon.

| Lunar phases | Number of animals | Ecdysis (%) |
|---|---|---|
| NM | 17 | 36.96 |
| FQM | 9 | 19.57 |
| FM | 9 | 19.57 |
| LQM | 11 | 23.91 |
| Total | 46 | 100.00 |

**Table 7.2.** The percentage of *P. monodon* sampled at different moult stages at four lunar phases. NM, new moon; FQM, First quarter moon; FM, full moon; LQM, last quarter moon.

| Lunar phases | Moult stages (%) | | | | |
|---|---|---|---|---|---|
| | A/B | C | D0 | D1/D2 | Total |
| NM | 2.17 | 2.17 | 2.17 | 8.70 | 15.22 |
| FQM | 4.35 | 2.17 | 8.70 | 4.35 | 19.57 |
| FM | 10.87 | 6.52 | 8.70 | 4.35 | 30.43 |
| LQM | 2.17 | 6.52 | 6.52 | 19.57 | 34.78 |
| Total | 19.57 | 17.39 | 26.09 | 36.96 | 100.00 |

The actual moulting activity (ecdysis) of the prawns varied within the lunar phases (Table 7.1). Most prawns moulted around new moon (36.96 %), followed by last quarter moon (23.91 %), and first quarter and full moon (19.57 % each). The moult stages also varied within the phases of the moon (Table 7.2) and all the stages could be observed within the moon phases. Stage A/B was highest at full moon, stage C was highest at full and last quarter moons and stage D0 was highest at first quarter and full moons, while stage D1/D2 was highest at last quarter moon. Most of the prawns were sampled around last quarter moon, followed by full and first quarter moons, and new moon was the lowest (Table 7.2).

### 7.3.3. The abundance of LOS cells

All prawns tested (100%) demonstrated spheroid cells within the lymphoid organ. In the experimental aquaria, the STT ratio ranged from 9.1 to 91.9% (46.6 ± 20.0%). There were 76.1% of the prawns showed vacuoles within the spheroid cells. The prevalence of vacuolated spheroids varied between 0.0 – 84.6% (18.8 ± 22.1%). The number of vacuoles per spheroid ranged between 0.0 and 2.3 (0.4 ± 0.6). Moribund or dead prawns before injection in control group had STT ratio of about 5.1 – 54.6% (26.5 ± 17.6%) while the GAV infected group before injection had STT ratio around 9.5 – 37.1% (24.5 ± 13.0%). Six prawns, which were moribund or dead just after inoculation with GAV, had an STT ratio ranging from 9.1– 67.9% (37.9 ± 15.1%). Six prawns from the stock tanks showed the ratio of STT varied from 36.7 – 70.4% (57.2 ± 14.2%).

#### 7.3.3.1. Days post-injection (dpi) and the LOS cells

The inconsistency of the STT ratio during the days post-injection (dpi) appeared during the experiment (Figure 7.2). The STT ratio was lowest at 1 – 5 dpi, increased and reached a peak at 6 – 10 dpi and slightly decreased at 11 – onward dpi. While the prevalence of vacuolated spheroid and the number of vacuoles within the spheroids increased with the number of days post-injection.

It was apparent that the more days post-injection of the prawns, the higher the prevalence of vacuolated spheroids and the number of vacuoles in the spheroids.

However, a univariate analysis of variance demonstrated that there was no significant effect of days post-injection on the STT ratio ($F = 0.228$; $df = 1, 45$; $P > 0.05$), the prevalence of vacuolated spheroids ($F = 0.332$; $df = 1, 45$; $P > 0.05$) or the number of vacuoles in the spheroid cells ($F = 0.212$; $df = 1, 45$; $P > 0.05$). The interaction between the days post-injection and the other independent variables could not be analysed due to losses of degrees of freedom (df).

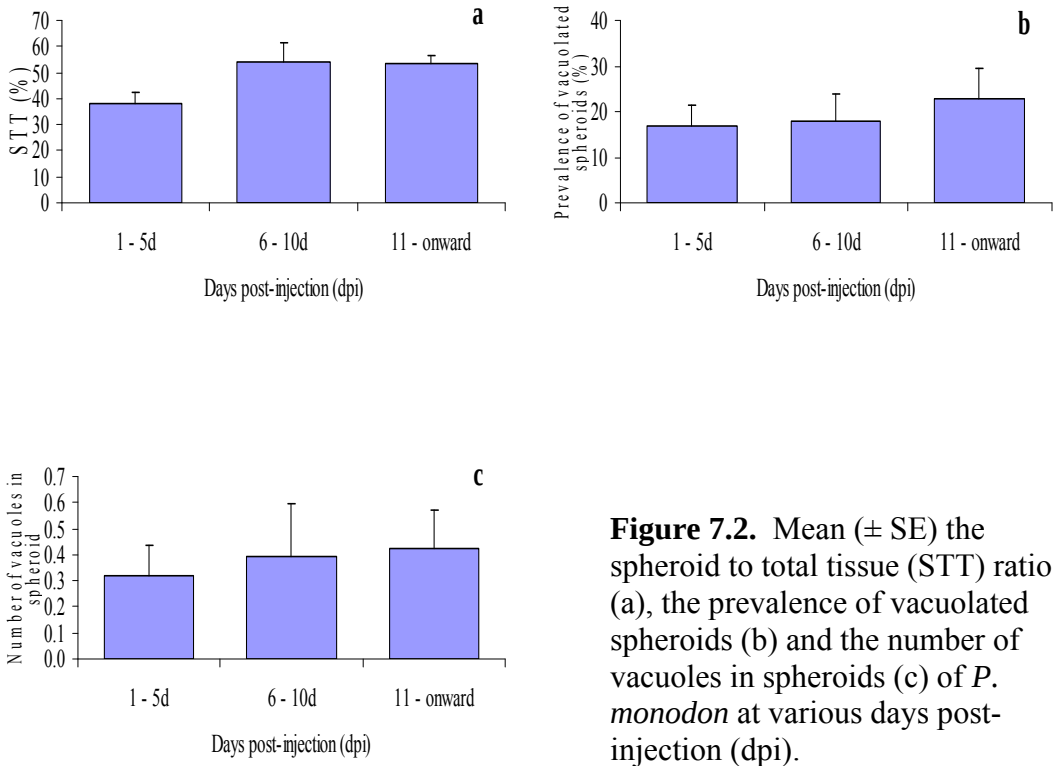


**Figure 7.2.** Mean (± SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in spheroids (c) of *P. monodon* at various days post-injection (dpi).

7.3.3.2. Moult stages and the LOS cells

The ratio of STT, the prevalence of vacuolated spheroid and the number of vacuoles in spheroid cells varied within the stage of moult. The ratio of STT, the prevalence of vacuolated spheroids and the number of vacuoles were highest in stage C (Figure 7.3). The lowest of the STT ratio and the prevalence of vacuolated spheroids were in stage D0, while the lowest number of vacuoles was in stage A/B.

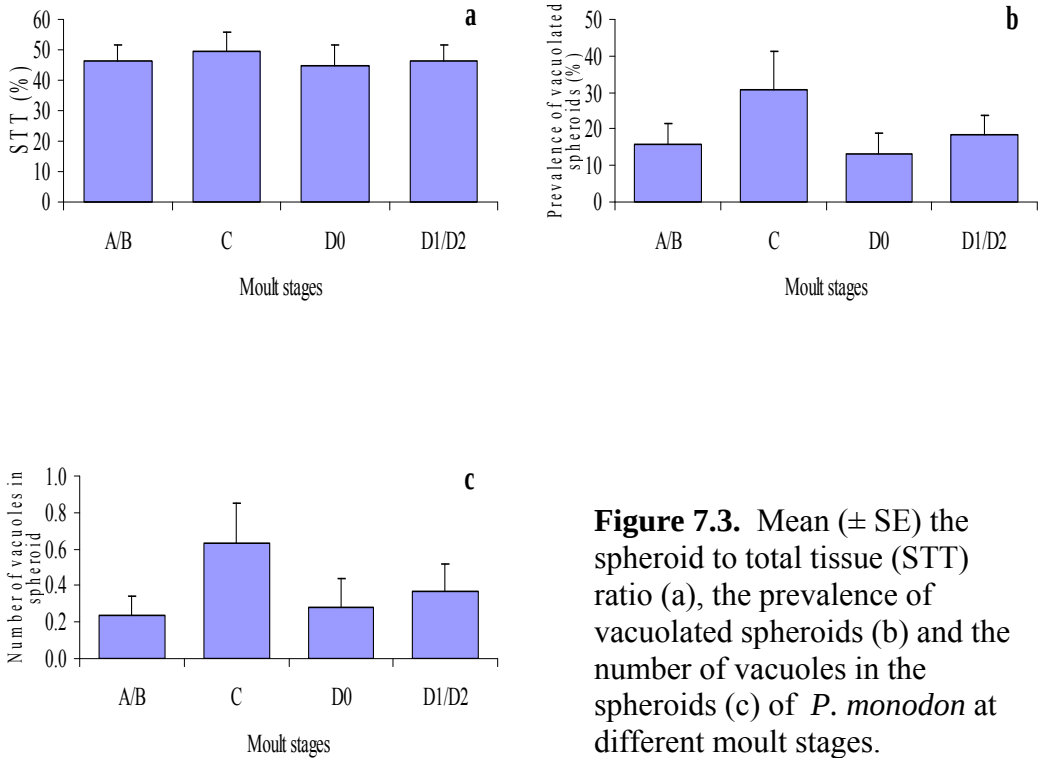


**Figure 7.3.** Mean (± SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in the spheroids (c) of *P. monodon* at different moult stages.

However, statistical analysis showed that moult stages had no significant effect on the ratio of STT ($F = 1.438$; $df = 3, 45$; $P > 0.05$), the prevalence of vacuolated spheroid ($F = 0.888$; $df = 3, 45$; $P > 0.05$), or the number of vacuoles in the spheroids ($F = 0.974$; $df = 3, 45$; $df > 0.05$). The interaction between moult stages and treatments also revealed no significant effect on the ratio of STT ($F = 0.045$; $df = 1, 45$; $P > 0.05$), the prevalence of vacuolated spheroids ($F = 1.297$; $df = 1, 45$; $P > 0.05$) or the number of vacuoles in spheroids ($F = 2.244$; $df = 1, 45$; $P > 0.05$). However, moult stages interactions with the other independent variables could not be analysed due to loss degrees of freedom.

### 7.3.3.3. Lunar phases and the LOS cells

The variability of the STT ratio, the prevalence of vacuolated spheroids and the number of vacuoles in the spheroid within the lunar phases was also evident during the experiments (Figure 7.4). It was found that the ratio of STT was highest at last quarter moon, while the prevalence of vacuolated spheroids and the number of vacuoles inside the LOS were highest at new moon.

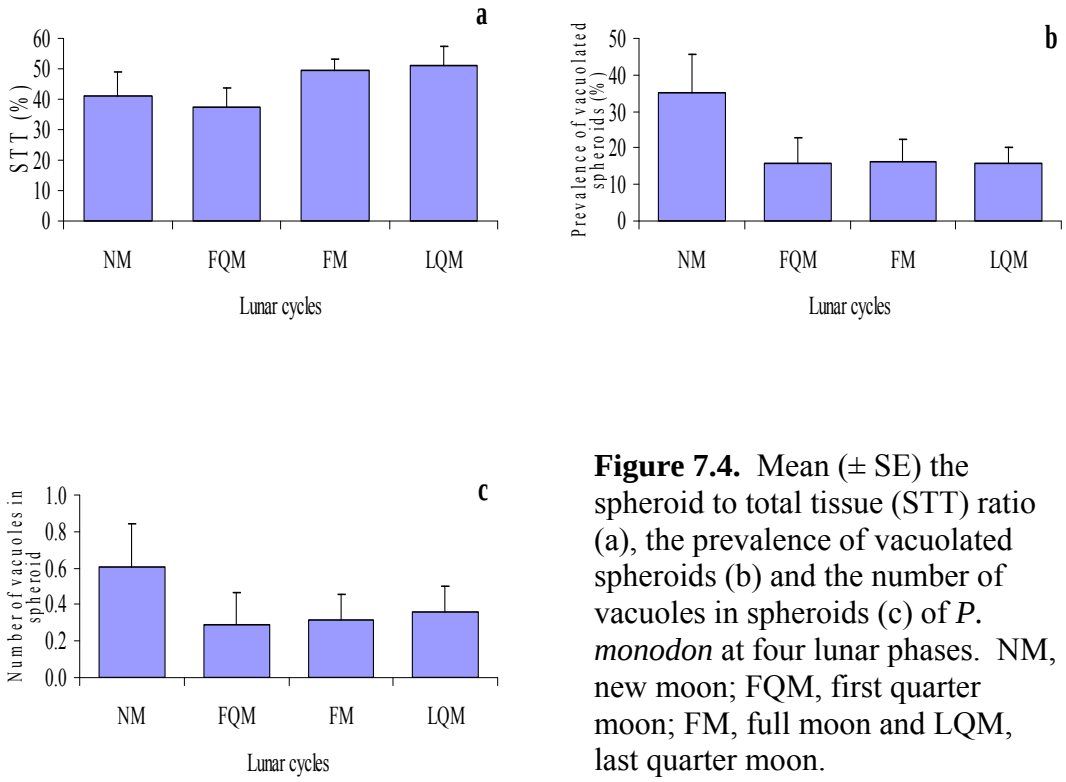


**Figure 7.4.** Mean (± SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in spheroids (c) of *P. monodon* at four lunar phases. NM, new moon; FQM, first quarter moon; FM, full moon and LQM, last quarter moon.

As a single factor, lunar phase had no significant effect on the ratio of STT ($F = 2.434$; $df = 3, 45$; $P > 0.05$), the prevalence of vacuolated spheroids ($F = 1.302$; $df = 3, 45$; $P > 0.05$) or the number of vacuoles in the spheroid ($F = 1.385$; $df = 3, 45$; $P > 0.05$). However, as illustrated in Figure 7.5, the relationship between the LOS cells and lunar phase was being masked by the interaction between lunar cycle and the treatments. In the control group, the ratio of STT was lowest at the new moon and highest at the full moon. On the other hand, in the infected (GAV) group the ratio of STT was lowest at first quarter moon and highest at last quarter moon. This interaction between lunar phases and treatment on the ratio of STT was significant ($F = 6.938$; $df = 1, 45$; $P < 0.05$), but not on the prevalence of vacuolated spheroids ($F = 0.599$; $df = 1, 45$; $P > 0.05$) or the number of vacuoles in spheroids ($F = 0.487$; $df = 1, 45$; $P > 0.05$).

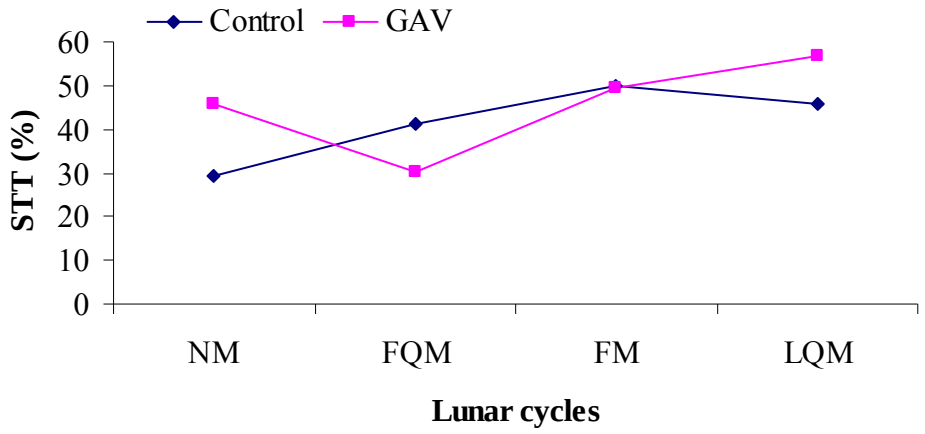


**Figure 7.5.** The spheroid to total tissue (STT) ratio of *P. monodon* at four lunar phases with two different treatments.

The STT ratio ($F = 3.010$; $df = 1, 45$; $P > 0.05$), the prevalence of vacuolated spheroids ($F = 0.077$; $df = 1, 45$; $P > 0.05$) and the number of vacuoles in spheroids ($F = 0.204$; $df = 1, 45$; $P > 0.05$), the lunar phase interaction with sex were not statistically significant. Again, due to loss degrees of freedom, the lunar phase interactions with the other independent variables could not be analysed.

7.3.3.4. Treatments and the LOS cells

The ratio of spheroid cells, the prevalence of vacuolated spheroids and the number of vacuoles in the spheroid cells was different in each treatment (Figure 7.6). ANOVA demonstrated that there was a significant difference between treatments on the ratio of STT ($F = 6.032$; $df = 1, 45$; $P < 0.05$). The STT ratio was significantly higher in infected (presumptive GAV) group than in the control group. The prevalence of vacuolated spheroids and the number of vacuoles in spheroid were also higher in GAV infected group than control group, but not significantly for the prevalence of vacuolated spheroids ($F = 0.041$; $df = 1, 45$; $P > 0.05$) or the number of vacuoles in spheroids ($F = 0.005$; $df = 1, 45$; $P > 0.05$). Treatment interactions with the other independent variables could not be analysed due to loss degrees of freedom.

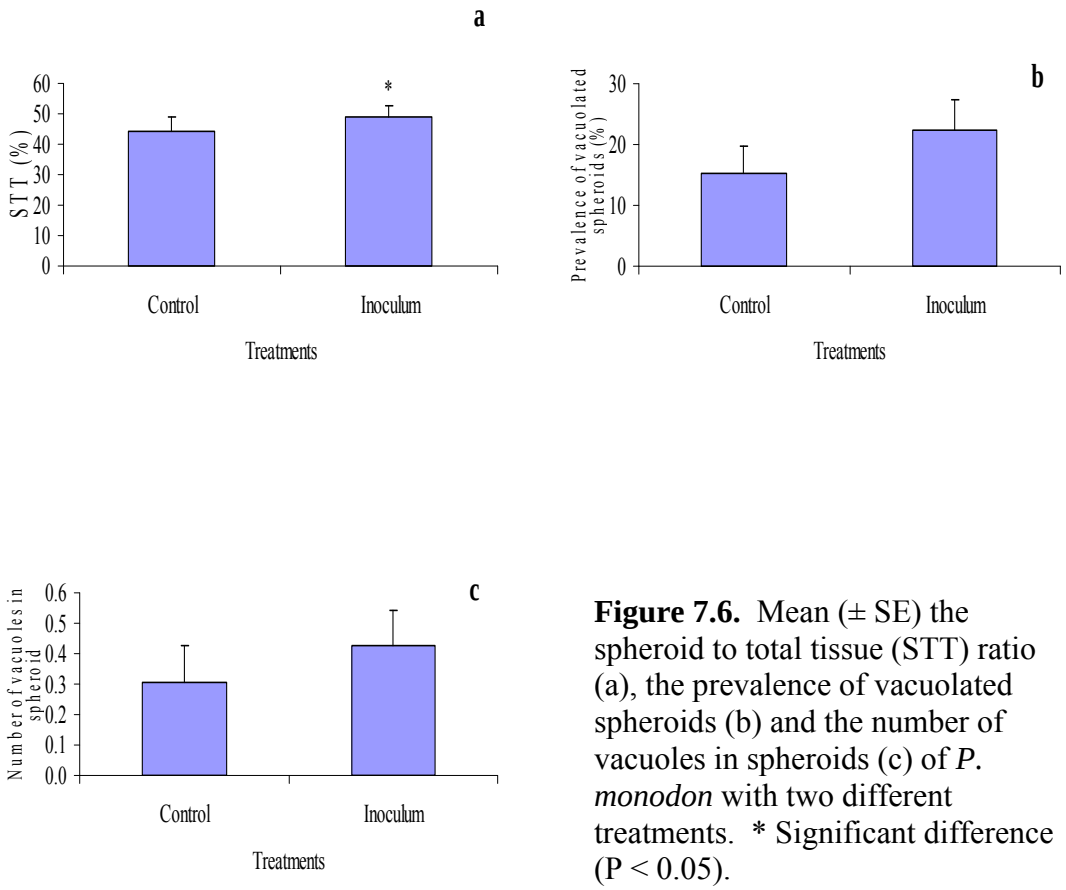


**Figure 7.6.** Mean (± SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in spheroids (c) of *P. monodon* with two different treatments. * Significant difference ($P < 0.05$).

Sex (Figure 7.7) had a significant effect on the spheroid to total tissue ratio of *P. monodon* ($F = 12.798$; $df = 1, 45$; $P < 0.05$) in this study. The STT ratio in females was significantly higher than in males. In contrast, the prevalence of vacuolated spheroids and the number of vacuoles in the spheroid were not significantly different ($F = 0.439$; $df = 1, 45$; $P > 0.05$ and $F = 0.910$; $df = 1, 45$; $P > 0.05$, respectively). Interactions between sex and other independent variables could not be analysed due to loss degrees of freedom.

The size of the animals (weight and total length) had no significant effect on the ratio of STT ($F = 0.000$; $df = 1, 45$; $P > 0.05$ and $F = 0.057$; $df = 1, 45$; $P > 0.05$), the prevalence of vacuolated spheroids ($F = 2.682$; $df = 1, 45$; $P > 0.05$ and $F = 2.231$; $df = 1, 45$; $P > 0.05$) and the number of vacuoles in the spheroids ($F = 1.931$; $df = 1, 45$; $P > 0.05$ and $F = 1.750$; $df = 1, 45$; $P > 0.05$). There was a weak Pearson's correlation between the STT ratio and the prevalence of vacuolated spheroids ($r = 0.318$, $P = 0.031$). A weak correlation also appeared between the ratio of STT and the number of vacuoles in LOS cell ($r = 0.318$, $P = 0.008$). However, there was a

strong correlation (r = 0.933, P = 0.000) between the prevalence of vacuolated spheroids and the number of vacuoles in the spheroids.

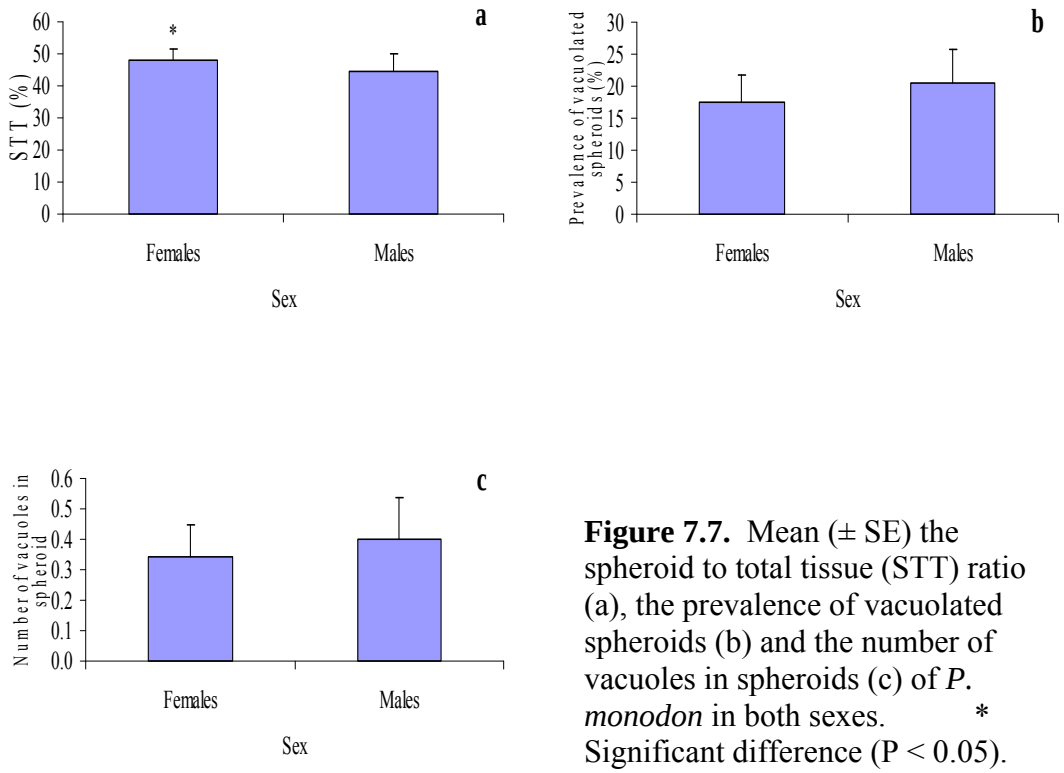


**Figure 7.7.** Mean (± SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in spheroids (c) of *P. monodon* in both sexes. * Significant difference ($P < 0.05$).

## 7.4. Discussion

Initiation of non-pathognomonic signs for GAV infection as described by previous researchers (Spann *et al.*, 1995; Spann *et al.*, 1997; Spann *et al.*, 2000; Callinan & Jiang, 2003; Callinan *et al.*, 2003) could also be observed during the experiment both in stock tanks and experimental aquaria. Moreover, undeveloped setae and unsynchronised development between setae and the retraction of epidermis from the setal bases suggested that these prawns might experience long moulting cycles and slow growth. Spann *et al*. (1997) observed that prawns infected with GAV ceased moulting. However, the present study suggested that prawns had prolonged moulting activity. Therefore, the gross sign of diseases in prawns are not a good indicator of viral infection due to inconsistency of the symptoms, which could lead to subjectivity and other viral diseases could produce the same symptoms (Cowley *et al.*, 2004; de

la Vega *et al.*, 2004). Furthermore, prawns infected by viral diseases sometimes do not show any gross sign of diseases (Bonami *et al.*, 1992; Nadala *et al.*, 1992; Spann *et al.*, 1995; Flegel *et al.*, 2004; Sritunyalucksana *et al.*, 2006) as seen in Chapter 5 and 6.

Mass mortality in the stock tanks, mortality in experimental aquaria before prawns were injected either with PBS or viral free extract and the presence of LOS cells in every single prawn indicated that these prawns have been exposed to viral infection related changes to the lymphoid organ at the farm before the experimental treatments. LPV (Owens *et al.*, 1991), LOVV(Bonami *et al.*, 1992), TSV (Hasson *et al.*, 1995), YHV (Lu *et al.*, 1995), and LOV later called GAV (Spann *et al.*, 1995; Spann *et al.*, 1997) were amongst viral diseases of penaeid prawns associated with spheroid cells within the lymphoid organ. Using RT-nPCR to screen the prevalence of GAV in *P. monodon* broodstock from different sources, Cowley *et al*. (2000a) found that around 98% (57/58) of the broodstock which supplied Australian hatcheries was infected with GAV. This promoted the vertical transmission of GAV at spawning to the larvae used in Australian farms (Cowley *et al.*, 2002b).

In addition, pathological and epidemiological studies of peripheral neuropathy and retinopathy (PNR) in farmed *P. monodon* strongly pointed out GAV as the causative agent of the disease (Callinan & Jiang, 2003; Callinan *et al.*, 2003). Spann *et al.* (2003) claimed that generally healthy cultured *P. monodon* in Australia had chronic GAV infection. A study by Munro & Owens (2005) found that the prevalence of GAV in *P. monodon* was 95%, while in *P. esculentus*, *P. merguiensis*, *P. longistylis*, and *Cherax quadricarinatus* it was 0%. Furthermore, Munro (pers. commun.) claimed that currently, most of cultured black tiger prawns in Australia had GAV infection without any gross signs of diseases. Furthermore, apparently *P. monodon* was the natural host and GAV was endemic in Australia (Cowley *et al.*, 2000a; Spann *et al.*, 2000). Therefore, it was most likely that these experimental prawns were also infected with GAV.

The possibility of bacterial infection as secondary pathogen in this experiment was indicated by the presence of granulomatous reaction in the lymphoid organ, hepatopancreas and gills. This inflammatory type reaction resembled melanized

nodule formation as described by Egusa *et al.* (1988) in *P. japonicus* infected *Vibrio* sp. These melanised nodules were formed as a protective response to bacterial infection, to confine the infected tubules and to prevent bacterial spreading (Egusa *et al.*, 1988; Jiravanichpaisal & Miyazaki, 1994). It was apparent that the lymphoid organ as well as hepatopancreas and gills were highly vulnerable to bacterial infection. Furthermore, this inflammatory reaction, once again confirmed that the spheroid cells were not produced due to bacterial infection, contrary to the published work of Van de Braak *et al.* (2002c) and Alday-Sanz *et al.* (2002) but as a major mechanical defence against viral infection (Anggraeni & Owens, 2000). It was not surprising that a perfect combination between GAV and bacterial infection might overwhelm the protective responses that led to the death of the infected animals.

Stress during transportation, a decrease of salinity from 40 ppt at the farm to 35 ppt and crowded stock tanks in the laboratory created stress and might have suppressed the immune capability of the prawns. As a result, virus might easily proliferate and bacteria as an opportunistic pathogen could also make a contribution to mortalities. Owens *et al*. (1998) found that rapid change of salinity and low level of oxygen were associated with mortalities in prawns infected with SMV. Furthermore, de la Vega *et al.* (2004) revealed that stress related to captivity, handling, invasive clinical procedures, and transportation could enhance viral propagation and mortalities. In the tanks, moulting and moribund infected prawns might be cannibalised by other healthy prawns that in turn infected themselves (Callinan & Jiang, 2003).

As previously described (Chapter 5 and 6), this present study also confirmed that the spheroid cells had no association with body weight and total length of animals. Consistent with the study of Anggraeni and Owens (2000) where the size of the prawns infected with MCMS did not correlate with the spheroid area. Furthermore, the present investigation clearly demonstrated that the spheroids to total tissue ratio, the prevalence of vacuolated spheroids and the number of vacuoles in the spheroid had no association with moult stages. This emphasised that the fluctuation of the abundance of the spheroid cells in the lymphoid organ was not driven by moulting as proposed by Anggraeni and Owens (2000) which was the hypothesis of the current study. This indicated that another factor or mechanism worked throughout the life of

the prawns causing fluctuation of the spheroid cells and it appeared it was linked to the lunar cycle (Chapter 5 and 6).

The ratio of STT influenced by the moon phase once again appeared in the interaction between moon phase and treatments. It was found that in the control group the STT ratio which was lowest at new moon as observed in previous studies (Chapter 5 and 6), rose for the next phase, reached a peak at full moon and then decreased at last quarter moon. However in the GAV infected group, the ratio of STT was lowest at first quarter moon, increased at the next phase and had a maximum at last quarter moon and reduced at full moon. It seemed that the STT ratio in the infected group developed and eliminated a week later than in the control group. This might indicate that in prawns highly infected with GAV or other viral diseases changes in the lymphoid organ react slowly in eliminating the LOS cells compared to less infected animals.

As expected, the STT ratio of the GAV injected group was significantly higher than the control group. This indicated that even though the experimental prawns already had infections (bacteria and virus); the additional injection of cell-free extract of GAV amplified the production of the spheroid cells within the lymphoid organ. This finding was supported by the work of Anggraeni (1998) who found that the increase of spheroid cells was only associated with viral infection and not correlated to bacterial infections as stated by Alday-Sanz *et al.* (2002) and Van de Braak *et al*., (2002c).

Sex related differences on the abundance of the spheroid cells in penaeid prawns were not consistent during the experiments. The first two trials (Chapter 5 and 6) showed that there was no significant difference on STT ratio between males and females. However, this last trial (Chapter 7) clearly demonstrated a female bias on the ratio of STT. Some studies revealed the inconsistency of relationship between sex and prawns' immunodefence. On one hand, Sequeira *et al.* (1995) reported the presence of sex related DHC in association within the cycle of the moult. In concert, Owens and O'Neill (1997) found female bias on haematological components of penaeid.

On the other hand, it was found that haematological variation was not significantly different between female and male prawns (Chen & Cheng, 1993; Cheng & Chen, 2001; Cheng *et al.*, 2002). Sheridan *et al*. (2000) who investigated sex related variation in arthropods infected with parasites showed that, in general, there was no sex bias among invertebrate hosts but it could exist in any specific host-parasite system. Furthermore, these authors suggested that the interaction between the immune and endocrine system in vertebrates might not exist in invertebrates. The negative feedback system between immunodefence and the expression of sexual features and behaviour provided by testosterone as observed in vertebrates was absent in invertebrates. Therefore, it was not surprising if higher parasitic infection in males was common in vertebrates but not in invertebrates. In short, it is suggested that sex-related changes in immunological components of penaeid prawns is still unclear. These discrepancies might be due to the differences in health status, pathogens, the maturation of animals, or species differences.

In summary, presumptive GAV infection enhanced the formation of spheroid cells in the lymphoid organ and this immune reactivity was only associated with viral infection but not with bacterial infection.

# CHAPTER 8
# GENERAL DISCUSSION

Setal development (setogenesis) and epidermal retraction of cuticle from the setal bases (apolysis) in the median part of inner uropod near the telson tip (Smith & Dall, 1985; Promwikorn *et al.*, 2004) allowed a finely graded classification of moult stages in *P. monodon* in both laboratory held animals (Chapter 5 and 7) and farmed prawns (Chapter 6). A rapid and repeatable moult staging of individual prawns could be undertaken without sacrificing animals. However, during the experiments, a repetitive moult staging could not be performed because the animals had to be sacrificed to examine the LOS by histology.

Since setogenesis was used to determine the postmoult stage (stage A and B) and intermoult stage (stage C) and apolysis was utilised for premoult stages (stage D0, D1, D2 and D3/D4), unsynchronised development of these two criteria in several prawns created confusion in determining their moult stages, particularly between stage B, C and D. Therefore, a combination of these two criteria and a histological section of exoskeleton (Smith & Dall, 1985) might be more appropriate in defining moult stages of abnormal prawns.

The effect of lunar-related patterns on moulting activity (ecdysis) of *P. monodon* was unclear since both laboratory held animals and field trials had different results in each experiment. Similar results also appeared when the stage of moult was compared within the lunar phases. It seemed apparent that moulting periodicity led to the discrepancies. In addition, the moult period of prawns depended on age and size. The bigger the prawns the longer the moulting periodicity (Vijayan *et al.*, 1997). The small number of samples and short period of sampling might also have affected the results.

Gross signs of diseases (Chapter 7) were not a good indicator to assess viral diseases in penaeid prawns due to inconsistency, subjectivity and similar symptoms could also be observed in other viral diseases (Tang *et al.*, 2002; Cowley *et al.*, 2004; de la Vega *et al.*, 2004). Furthermore, sometimes disease symptoms were absent in prawns infected by viruses (Bonami *et al.*, 1992; Spann *et al.*, 1995; Flegel *et al.*,

2004; Sritunyalucksana *et al.*, 2006). Moreover, spheroid cells as a non-specific reaction to viral disease in penaeid prawns were not only found in prawns that displayed clinical symptoms of diseases (Chapter 7) but also presented in the prawns with no signs of disease syndromes (Chapter 5 and 6). However, the presence of spheroid cells in the lymphoid organ of the prawns during the experiments (Chapter 5, 6 and Chapter 7 before treatments) suggested that it might be associated with GAV infection (Cowley *et al.*, 2000a; Cowley *et al.*, 2002b; Callinan & Jiang, 2003; Spann *et al.*, 2003; Munro & Owens, 2005). Prawn's response to GAV infection might be affected by dose of infection, transmission route, and the age of the prawns (Spann *et al.*, 2003). Moreover, persistent viral infection might have a protective function. It would be advantageous to understand why viral infection sometimes cause diseases and sometimes not (Flegel *et al.*, 2004).

As a result of immune reactivity to viral infection, the spheroid cells have been found in some penaeid prawn species such as *P. monodon, P. penicillatus* (Lightner et al., 1987)*, P. esculentus, P. merguensis* (Owens *et al.*, 1991), *P. vannamei* (Bonami *et al.*, 1992)*, P. stylirostris* (Nadala *et al.*, 1992)*, P. chinensis* (Shao *et al.*, 2004)*,* and *P. japonicus* (Kondo *et al.*, 1994). Moreover, these spheroid cells were claimed to be associated with hypertrophy and metastasis of "Oka organ" (Lightner *et al.*, 1987), LPV (Owens *et al.*, 1991), LOVV (Bonami *et al.*, 1992), and YHV (Boonyaratpalin *et al.*, 1993). The presence of LOS cells was also found associated with LOV/GAV (Spann *et al.*, 1995; Spann *et al.*, 1997), TSV (Hasson *et al.*, 1995), SMV (Fraser & Owens, 1996), and infectious viral diseases in *P. orientalis* (Park *et al.*, 1998), and recently found in monodon slow growth syndrome, MSGS (Anantasomboon *et al.*, 2006) and Laem-Singh virus, LSNV (Sritunyalucksana *et al.*, 2006).

A modified quantitative method of Littik (2003) offered an alternative robust method to asses the spheroidal state of the prawns (Chapter 4). This technique provided a simple, more rapid and accurate method to examine the histopathological changes of the lymphoid organ, the spheroid cells. Furthermore, serial longitudinal sections of the lymphoid organ proved that the half midsagittal section of the cephalothorax represented the abundance of the spheroid cells in the total lymphoid organ of penaeid prawns. The low cost and skill requirements of these techniques in assessing the immune reactivity of the prawns to viral infection, gives a good opportunity for

people in developing countries where advanced diagnostic equipment and facilities are very expensive and therefore limited.

Three experiments in the previous chapter (Chapter 5, 6 and 7) showed that the abundance of spheroid cells within the lymphoid organ varied during the life span of the prawns. However, the present study clearly showed the absence of an effect of size of the prawn (weight and total length) on the abundance of the LOS cells as has been observed by Anggraeni and Owens (2000) in MCMS of the giant black tiger prawn, *P. monodon*. Furthermore, since the physiological functions and immunocompetence of prawns were affected by moulting, it was hypothesised in this study as proposed by Anggraeni and Owens (2000) that the abundance of lymphoid organ spheroid cells was related to the moult stages of the prawns. If so, the correlation between the lymphoid organ spheroid cells and moulting stages could be used to enhance the health status of cultured penaeid prawns by stimulating moulting (ecdysis). In addition, this knowledge could also be used in manipulating water hardness to cut down feeding on exuviae soon after prawn moulting in order to prevent the transmission of diseases (Owens 1997). Unfortunately, it was found that there was not support for that supposition. Most of the experiments demonstrated the absence of a relationship between moult stages and the ratio of STT. This indicated that the fluctuation of the spheroid cells during the life of the prawns was not affected by the stage of moult. In other word, the spheroids cells were most likely not disposed of during ecdysis (moulting).

Lunar periodicities may be an important key to the variability of the spheroid cells during the life of the animals. It was clearly shown (Table 8.1) that changes in the abundance of the spheroid cells occurred within the moon phases both in laboratory held animals (Chapter 5 and 7) and in farmed animals (Chapter 6). The presence of lunar related patterns on the abundance of LOS cells in laboratory held animals strongly suggested that this effect was endogenous and hereditary, without environmental cues (Withers, 1992; Griffith & Wigglesworth, 1993). In farmed animals, the presence of the effect of light intensity of dark-light moon and the hydrostatical effect of spring-neap tide might provide environmental cues absent in the laboratory animals.

**Table 8.1.** Summary of significant effect (F values) of lunar phases and its interaction with the other variables on the STT ratio of *P. monodon* throughout the experiments.

| Source | First study | Second study |
|---|---|---|
| Laboratory trial (Chapter 5) | | |
| Lunar phases | 0.595 | 0.048 |
| Time after moulting * lunar phases | 0.853 | 0.005 |
| Field trial (Chapter 6) | | |
| Lunar phases | 0.003 | 0.066 |
| TMMS * lunar phases | 0.000 | 0.849 |
| Infection experiment (Chapter 7) | | |
| Lunar phases | 0.132 | NA |
| Treatment * lunar phases | 0.027 | NA |

In the laboratory held animals, the effect of the lunar cycle on the STT ratio was only present in the second trial but not in the first (Chapter 5), whilst in farmed animals it was present in the first and not the second study (Chapter 6). This might indicate that the relationship between lunar cycle and the abundance of spheroids might vary with moulting period due to size and age differences. The size of the prawns from the second trial of laboratory maintained animals were similar to the prawns from the first trial of farmed prawns which might have a fortnightly to three week moulting period. Thus, moulting in these prawns could approach the moon phasing, in turn affecting the changes in LOS cells. However, prawns were smaller in the first laboratory experiment and had a week moulting period, while larger prawns in the second farm study might have longer than a three week moulting period. Thus, they did not approach the cycle of the moon in terms of moulting and subsequently LOS elimination.

LOS cells cytochemically were similar to SGH and LGH because they contained PO and peroxidase activity but without granularity (Anggraeni & Owens, 2000; Shao *et al.*, 2004). Therefore, Anggraeni and Owens (2000) suggested that the LOS cells might be formed from spent (exocytosed) granular haemocytes. As phagocytic cells, the spheroid cells as spent haemocytes migrated from the lymphoid tubule lumen through the stromal matrix cells and into the haemal sinuses. Van de Braak *et al.* (2002c) suggested that the haemocytes might be degranulated after phagocytosis, leaving their granules with the degrading foreign substances in the tubule wall. Furthermore, in prawns experimentally infected with WSSV, Van de Braak *et al.*

(2002a) revealed that granular haemocytes migrated from the haematopoietic tissue to infected tissues. Thus, if the spheroid cells developed from granular haemocytes, then as the number of granular haemocytes in the lymphoid organ increased, the more or larger spheroids could be produced in the haemal sinuses during viral infections.

Hasson *et al.* (1999b) hypothesised paradoxical possibilities of the association between the LOS production and the normal LO tubules. Firstly, in concert with Nadala *et al.* (1992), they suggested that LOS cells were the degeneration phase of the LO tubules because the number of LO tubules declined when the number of Type A LOS cells increased. If it was the case, then the spheroids might be the only cells found in the LO when all the LO tubules have been transformed into LOS cells during viral infection. However, the present study never found that the LO was only occupied by the LOS cells. The presence of LOS was always accompanied by the presence of the LO tubules. Conversely, sometimes the LO tubules were the only structures observed in the LO without any LOS suggesting no viral infection to cause LOS formation in these animals.

Contrary to the above hypothesis, these authors also hypothesised that it might be just a perception of a decrease in the normal LO tubules during viral infections. The presence of LOS cells around the subgastric artery, the fact that the LOS cells were positive for peroxidase and PO activity while the normal LO tubules were not (suggesting the LOS cells had a haemocytic origin while LO tubules did not) and LOS cells were formed from accumulation of spent haemocytes in the haemal sinuses that migrate through the stromal matrix cells (Anggraeni, 1998; Hasson *et al.*, 1999b; Anggraeni & Owens, 2000; Shao *et al.*, 2004) support this later supposition. All these finding suggested that the increase in the number of spheroid cells did not cause a decreased number of the normal lymphoid tubules (stromal matrix cells), but resulted in hypertrophy of the lymphoid organ as has been observed by previous researchers (Lightner *et al.*, 1987; Owens & Hall-Mendellin, 1989; Owens *et al.*, 1991; Nadala *et al.*, 1992; Turnbull *et al.*, 1994; Hasson *et al.*, 1999b). However, Shao *et al.* (2004) did not realise this phenomena when they reportedly found LOS cells only appeared in prawns with larger lymphoid organs (more than 2 mm).

In addition, these experiments (Chapter 5, 6 and 7) obviously implied the presence of an elimination mechanism for spheroid cells in the lymphoid organ of penaeid prawns. Based on these findings, there were two possibilities relating to the fluctuation in the ratio of LOS that might occur. Firstly, the LOS cells were destroyed gradually or totally via self destruction (apoptosis) in the lymphoid organ as proposed by Hasson *et al.*, (1999b). Secondly, the spheroid cells might be disposed of gradually or totally during the life span of the animals as hypothesised by Anggraeni and Owens (2000). If either of these two mechanisms occurs; this present study suggests that it is stimulated by the lunar rhythmicity.

During the production of spheroid cells, the reticular connective tissue or haemal sinuses were extensively expanded because LOS were formed in the haemal sinuses (Anggraeni & Owens, 2000). In addition, if either of these elimination processes occurred, as a result, there could be a gapping between tubules before they became normal again. Gapping or abnormal interstitial spaces between tubules has been reported in GAV infected prawns (Spann *et al.*, 2003) and were often observed during the present study. According to Spann *et al*. (2003), due to heavy accumulation of infected cells, haemal sinuses and connective tissue became necrotic resulting in gapping. However, the current study proposed that the gapping occurred due to the elimination of LOS cells in the lymphoid organ. This abnormal interstitial space of haemal sinuses was more obvious when the number of spheroid cells was decreased.

Furthermore, if necrosis occurred, then histopathologically, the transition stage of the LOS before disappearing should be observed within the LO. Since Hasson *et al*. (1999b) and Owens (unpubl. data) categorised the development stage of the LOS into three distinct phases, the need for a further stage after the last (third) stage (in which the LOS became more vacuolated and necrotic), a transition phase before the LOS disappeared is important to explain what happened to the LOS after the third stage. Both groups of scientists have not described such a stage. Furthermore, in the present study, even though some spheroids were vacuolated, they were not as intensely vacuolated as other researchers had found (Owens *et al.*, 1991; Anggraeni, 1998; Hasson *et al.*, 1999b). This suggests *in situ* necrotic destruction is non-existent or rare.

It was interesting that during the experiments, the appearance of necrotic eosinophilic foci within the LO were observed sometimes and even more often in the last experiment (Chapter 7). It was apparent that the eosinophilic foci observed developing within the LOS and were never found within or developed from the normal lymphoid organ tubules (stromal matrix). Additionally, Anggraeni (1998) commented that the eosinophilic foci were LOS cells with fibrocyte encapsulation. The appearance of eosinophilic foci within the LO of penaeids was first reported by Spann *et al.* (1997) in GAV infected *P. monodon* and followed by Anggraeni (1998) in MCMS in the same species. Furthermore, Owens (pers. commun.) revealed that the eosinophilic foci were commonly found in the viral infected penaeid prawns associated with the LOS cells and it was not only found in the GAV infection as claimed by Spann *et al.* (1997). Once again, a similar question arose as to what happened next after the formation of these foci? All in all, evidence favoured the hypothesis that the LOS cells were disposed of during the lifespan of the animal.

How the spheroids were removed from the lymphoid organ, is the big question. During the experiments some prawns illustrated the appearance of ectopic spheroids within the antennal gland, tegmental gland, heart, hepatopancreas, gills, and connective tissue (Figure 8.1). Numerous ectopic spheroids have been previously observed in other organs such as gonads, muscle and appendages (Lightner *et al.*, 1987; Owens *et al.*, 1991; Turnbull *et al.*, 1994; Hasson *et al.*, 1999b; Littik, 2003). Whether the ectopic spheroids in those tissues migrated through the haemolyph or diapedised from the lymphoid organ or were produced *in situ* from spent haemocytes in those tissues is unclear. Lightner *et al.* (1987) assumed the ectopic spheroids were metastatic ectopic foci. However, Hasson *et al.* (1999b) found a lack of evidence of spheroid metastasis from the lymphoid organ to the other tissues. Furthermore, Anggraeni and Owens (2000) commented on the lack of *in situ* dividing cells in the lymphoid organ, making cellular division and proliferation unlikely.

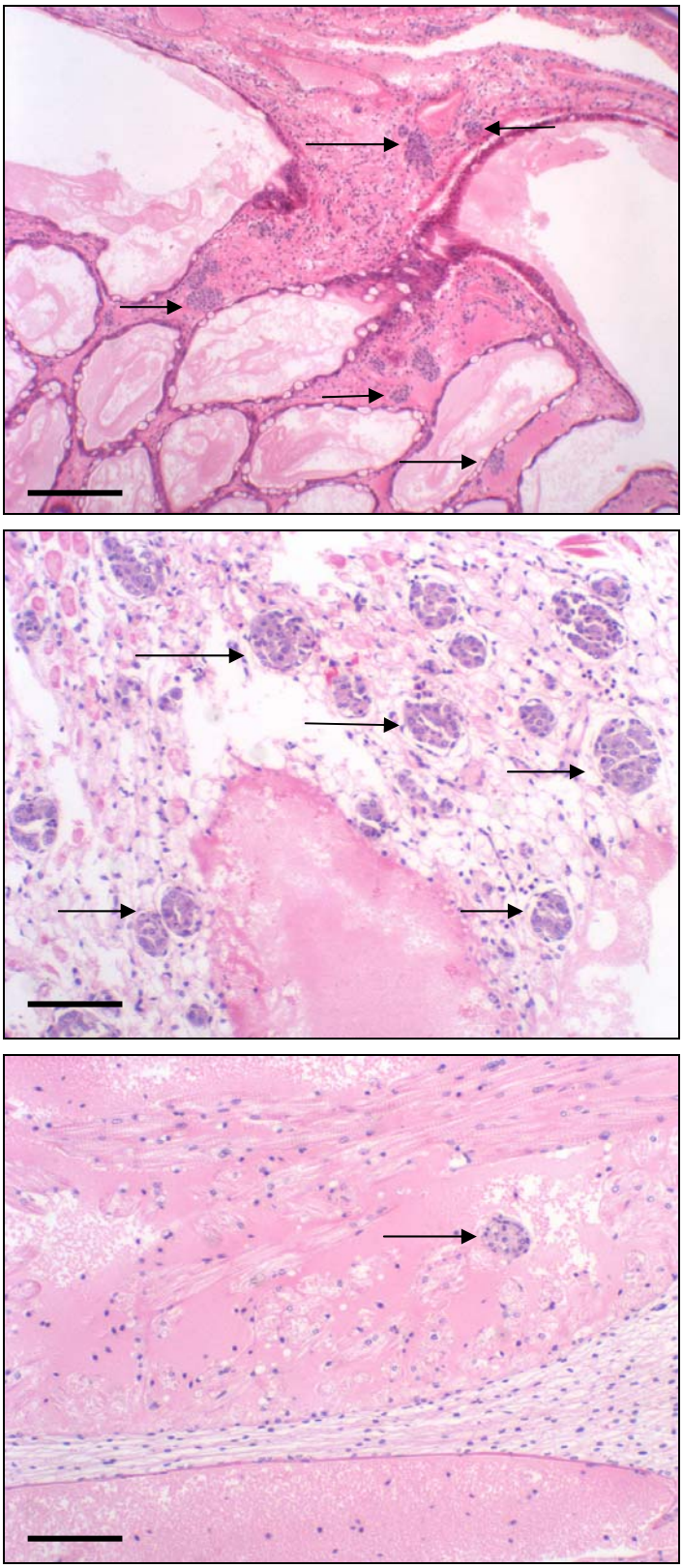

**Figure 8.1.** Light micrograph of longitudinal section of various tissue of *P. monodon* from one slide showing ectopic spheroids (arrow) in hepatopancreas (a), connective tissue (b) and the heart (c). H & E stain. Scale bar = 100 µm

Marking or tagging the lymphoid organ spheroids was an alternative method to examine the origin of the ectopic spheroids. Using carmine and carbon black to mark the lymphoid organ spheroids (unpubl. data) failed. Even though the LO can be easily recognised by red colouration of carmine when cut in half longitudinally, the problem came when the slides were redehydrated with alcohol during staining. Carmine and carbon black dissolved slightly in the alcohol. Furthermore, some carmine was still left in the tissue and could be recognised after the staining process under a light microscope but it did not end up in the LOS. Another highly specific method or marker for LOS cells of penaeid prawns needs to be found.

The presence of melanised nodules was noticed within the LO, hepatopancreas and gills caused by bacterial infection in the experimental animals (predominantly in Chapter 7). These granulomas were formed as protective reaction to bacterial infection (Egusa *et al.*, 1988; Jiravanichpaisal & Miyazaki, 1994). Particularly in the lymphoid organ, this indicated that this organ was also a target for bacterial infection in penaeid prawns. Moreover, these findings suggest that the spheroid cells are not produced as a reactive form due to bacterial infection as indicated by the previous work of Alday-Sanz *et al*. (2002) and Van de Braak *et al*. (2002c) but by viral infection (Anggraeni & Owens, 2000).

The high variability in STT between individual prawns in each group, in factor measurements and the relatively small sample sizes in each category might have compromised the statistical power and might have resulted in the lack of statistical significance. Therefore, ideally using a bigger sample size might offer a good solution to overcome this problem. However, it should be pointed out that STT analysis takes a long time for processing and viewing which limits the number of animals that can be assessed in a given time. Furthermore, if at all possible, application of a specific marker to the spheroid cells of penaeid prawns is important in order to investigate the elimination mechanism of these cells. Additionally, utilisation of SPF species or stock would be preferable in experimentally infected animals.

Analysis of histopathological changes in the lymphoid organ of penaeid prawns could lead to a better understanding on immune reactivity of the prawns against

invading pathogens, especially viruses. This present investigation will contribute to future research on the immunological state of penaeid prawns. In turn, such research could be used effectively to enhance immune capability in prawns to deal with foreign invaders in the aquaculture industry particularly since immunostimulation and vaccination do not clearly provide benefits in these animals.

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# APPENDICES

## Appendix 1. Fixative, Staining, Preservative and Injection Solution

### 1.1. Davidson's fixative

| | |
|---|---|
| Acetic acid | 115 mL |
| Absolute ethanol | 313 mL |
| Formaldehyde | 220 mL |
| Distilled water | 352 mL |

### 1.2. Haematoxylin and eosin (H & E) stain

#### 1.2.1. Mayer's haematoxylin

| | |
|---|---|
| Haematoxylin | 2.0 g |
| Sodium iodate | 0.4 g |
| Aluminium ammonium sulphate ($Al\ NH_4(SO_4)_2.12H_2O$) | 100.0 g |
| Citric acid | 2.0 g |
| Chloral hydrate | 100.0 g |
| Distilled water | 2.0 L |

#### 1.2.2. Scott's tap water

| | |
|---|---|
| $NaHCO_3$ | 8.75 g |
| $MgSO_4.7H_2O$ | 50.00 g |
| Distilled water | 2.50 L |

#### 1.2.3. Young's eosin

| | |
|---|---|
| Eosin | 15 g |
| Erythrosin | 5 g |
| Calcium chloride | 5 g |
| Distilled water | 2 L |

## 1.3. Gram stain

### 1.3.1. 2% Crystal violet

| | |
|---|---|
| Crystal violet | 2.0 g |
| Ammonium oxalate | 0.8 g |
| 95% Alcohol | 20.0 mL |
| Distilled water | 8.0 mL |

### 1.3.2. Lugol's iodine

| | |
|---|---|
| Iodine crystal | 1 g |
| Potassium iodide | 2 g |
| Distilled water | 100 mL |

### 1.3.3. Twort stain

| | |
|---|---|
| 0.2% Alcohol neutral red | 100.0 mL |
| 0.2% Alcohol fast green | 11.3 mL |

## 1.4. Phloxine and tartrazine stain

### 1.4.1. Phloxine solution

| | |
|---|---|
| Calcium chloride (dissolved first) | 0.5 g |
| Phloxine | 0.5 g |
| Distilled water | 100 mL |

### 1.4.2. Tartrazine in cellosolve

| | |
|---|---|
| Tartrazine | 2.5 g |
| Cellosolve | 100 mL |

### 1.5. Artificial seawater

| | |
|---|---|
| NaCl | 17.50 g |
| KCl | 0.75 g |
| $MgS0_4.7H_2O$ | 6.16 g |
| $MgCl_2.6H_2O$ | 5.08 g |
| $CaCl_2.2H_2O$ | 1.47 g |
| Distilled water | 1.00 L |

### 1.6. 1 M phosphate buffer saline (PBS)

| | |
|---|---|
| NaCl | 8.00 g |
| KCl | 0.20 g |
| Na2HPO4 | 1.44 g |
| KH2PO4 | 0.24 g |
| Distilled water | 1.00 L |