# Towards development of a formulated diet for blue swimmer crab, *Portunus pelagicus* with emphasis on lipid nutrition

Thesis submitted by

Noordiyana Mat Noordin in June 2011

for the Degree of Doctor of Philosophy

in the School of Marine & Tropical Biology

James Cook University

## DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human (1999)* the Joint *NHMRC/AVCCStatement 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: A1285

Signature:

Name: Noordiyana Mat Noordin

Date: June 23rd, 2011

# Acknowledgements

Alhamdullilah,

Once, a professor told me that doing PhD is a process that involves mental, physical and emotional torture. I laughed. Few years later, here I am agreeing in every single word that he said. I could have not done this without help and support of people around me and I will be forever indebted to them. First and foremost, I would like to thank my supervisors, Dr Chaoshu Zeng and Professor Paul Southgate, for being a torch shining my path and guiding me all along. I could not ask for better supervisors. To Su Reilly of JCU histological lab and Ian Brooks of Department of Primary Industries and Fisheries, Queensland, thank you for teaching and helping me with my samples analysis. To Greg, Simon and John of MARFU, thank you for helping me with my lab set up. I also could not complete my PhD without the helped of my research mate especially Nick Romano, Jerome Genodepa and Wu Xugan, who at the end of my study has become more like my brothers than just my friends. To Maya, Malwine, Kiki, Jonathan, Michael, Matt and Laura, thank you for offering hands and motivation when I needed. Not to forget, to people who paint my life with colors in Australia; Azwita and family, Miin, Anne, Mandy, Alan and whole bunch of crazy people in Rotary International House 2006-2009, you guys are awesome.

Last but not least, to Nazipah, Mat Noordin, Azwa, Nirwandy, Hezly, Lin, Naem, Wafri, Darwish, Fatihah, Fara and Amir, no word will be enough to show my appreciation to all of you. You are my everything and thank you for always believing in me.

# Abstract

Market demands on the blue swimmer crab, *Portunus pelagicus*, have increased substantially worldwide over past decades and have expanded from traditional hard-shell crabs to soft-shell crabs as well as pasteurized crabmeat. Increasing fisheries pressure has led to declining wild population and aquaculture has been eyed as the alternative to supply the market. As an emerging aquaculture species, comprehensive and quantitative understanding of nutritional requirements of *P. pelagicus* is necessary to the development of formulated diets for the species to support sustainable growth of the industry.

As a crucial nutrient, lipid provides cultured crustaceans with concentrated energy, components of cell membrane and as precursors for various hormones. A series of experiments were hence conducted to evaluate the necessity as well as optimal levels of various lipid components for *P. pelagicus* early juveniles. Semi-purified diets were formulated to contain different levels (% diet dry weight) of a particular lipid component and the diets were fed to newly molted first stage crabs (C1) till the crabs reached C3 or C4 stage with survival, development and growth of crabs subjected to different dietary conditions closely monitored. To prevent cannibalism, all experimental crabs were kept individually for all experiments.

A starvation and re-feeding experiment was first conducted to investigate their effects on survival and development of newly settled C1 crabs as well as their fatty acid profiles. Meanwhile, hepatopancreas histology was undertaken to observe morphological changes

induced. Three treatments: 1) continuous starvation (S); 2) starved for 7 days and then re-fed (S-F); and 3) continuous feeding (F), were set up. In S treatment, crab survival declined rapidly (from 96.6% to 47.9%) between day 7 to 10, leading to the termination of the treatment on day 10. The $PNR_{50}$ (point-of-no return) for the C1 crabs is therefore between day 7 to 10. There was no significant differences on crab survival between S-F and F treatments, however, newly molted C2 and C3 of S-F treatment had significant lower dry weights ($p<0.05$). Interestingly, despite their significant lower dry weight, S-F crabs had a significant shorter mean intermolt period from C2 to C3 than that of F crabs ($p<0.05$). This suggests that following prolonged starvation, re-fed crabs prioritized development over growth. Fatty acid profiles of starved crabs showed a trend of decreased PUFA but increased HUFA (as % total fatty acids), suggesting high retention of HUFA. S-F crabs managed to recover their fatty acid profiles after being re-fed and were similar to F crabs as newly molted C2. Histology observation showed a continuous shrinking of hepatopancreas cells during starvation, however, as newly molted C2 and C3, no obvious differences was noticed between the re-fed and continuously fed crabs.

A series of 8 feeding experiments were then subsequently conducted to assess dietary cholesterol and phospholipid (PL) requirements, cholesterol and PL interactions, neutral lipids (triglyceride) requirements, fish oil to soybean oil ratio, arachidonic acid (ARA) requirements and polar lipids (PL) to neutral lipids (triglyceride) ratio for *P. pelagicus* early juveniles. The cholesterol experiment showed that out of 7 levels of dietary supplemental cholesterol (0, 2.5, 5.0, 7.5, 10, 12.5 and 15 g $kg^{-1}$) tested, 10 g $kg^{-1}$ consistently yield the best survival, development and growth with both the low and high end of cholesterol levels

gave inferior development and growth results. In addition, the highest incidents of molt death syndrome (MDS), 20.7% were found for crabs fed the diet with the highest level of cholesterol supplementation at 15 g $kg^{-1}$.

PL is known to play an important role in lipid and carbohydrate metabolism as well as enhancing absorption of ingested fats, including triglycerides and cholesterol. For second experiment, six iso-lipidic diets with PL supplemented at 0, 30, 60, 90, 120 and 150 g $kg^{-1}$ were tested and the results demonstrated that the diet without PL supplementation significantly impaired survival (with high incidents of post molt death), development and growth. PL supplemented at 120 and 150 g $kg^{-1}$ yielded the best survival, development and growth that were often differed significantly to other treatments. Salinity stress test (salinity dropped abruptly form 30 to 7‰ for 7 h) using C4 crabs also showed that while the majority of crabs from PL 120 and 150 g $kg^{-1}$ treatments survived to the end of stress test, all crabs from other treatments dead within 5 h.

As interactive effects between cholesterol and PL have been demonstrated in other crustaceans, two experiments were designed to test this for *P. pelagicus*. In the first experiment, 10 iso-energetic diets were formulated, including 9 diets in a 3x3 factorial design with various combinations of 3 levels of PL (30, 60 and 90 g $kg^{-1}$) and cholesterol (0, 5 and 10 g $kg^{-1}$), respectively and a basal diet (deficient in both PL and cholesterol). It was demonstrated that the addition of PL at 90 g $kg^{-1}$ could compensate lack of dietary cholesterol by substantially improved crab survival. As the first experiment showed that crabs fed the diet with the highest supplemented PL (90 g $kg^{-1}$) and cholesterol (10 g $kg^{-1}$)

yielded the best results, a second experiment was conducted to investigate if higher PL and cholesterol levels would lead to even better performance. Four diets with combinations of 2 cholesterol (10 and 15 g $kg^{-1}$) and PL levels (90 and 120 g $kg^{-1}$) were fed to C1 crabs and the results showed that the further increase in cholesterol and PL did not lead to significant improved survival, development and growth.

With optimal cholesterol and PL level established, 3 experiments on neutral lipids requirements were executed. Firstly, effects of different levels of total neutral lipids (triglyceride) were assessed with 6 triglyceride levels at 0, 20, 40, 60, 80 and 100 g $kg^{-1}$ (fish oil to corn oil ratio fixed at 2:1) being included in the diets. The results demonstrated that level of triglycerides did not significantly affect survival of the crabs ($p>0.05$), however, crabs fed the diet supplemented with 40 g $kg^{-1}$ triglycerides achieved significant better growth than all other treatment except the diet supplemented with 20 g $kg^{-1}$ triglycerides. The 40 g $kg^{-1}$ triglycerides treatment also had the highest survival among all diet treatments. A subsequent experiment set triglyceride at the optimal level of 40 g $kg^{-1}$ with varied fish oil (FO) to soybean oil (SO) ratio at 1:0, 3:1, 2:1, 1:1, 1:2, 1:3 and 0:1, respectively, to investigate their effects on performance of *P. pelagicus* early juveniles. The results showed that the diet without supplemented fish oil (SO:FO at 1:0) impaired survival of the crabs with the longest development time to C4 and the lowest growth. The greatest Specific Growth Rate (SGR) for dry weight and carapace width was achieved when crabs fed the diet with SO:FO ratio at 1:1, which was significantly better than that of the treatments with SO:FO ratio of 1:0 and 2:1. The results suggest fish oil can be partially replaced by plant oil and indicates the need for a balanced n-3 and n-6 fatty acid profile in *P. pelagicus* diets.

Fatty acid analysis on both fish and soybean oil showed low level of arachidonic acid (ARA), an important n-6 HUFA. A further experiment was then carried out to investigate whether additional ARA on top of the level provided by triglyceride supplementation (40 g $kg^{-1}$ at SO: FO ratio 1:1) might promote performance of *P. pelagicus* early juveniles. Six iso-lipidic diets were formulated to contain additional ARA levels at 0, 2.0, 4.0, 6.0, 8.0 and 10.0 g $kg^{-1}$, respectively. Another two diets were formulated to contain ARA at 5.0 and 10 g $kg^{-1}$, but with triglyceride supplementation of macadamia oil (contain mostly SFA and MUFA). Survival of the crabs ranged from 50.0% to 76.7% with no significant differences detected among treatments with similar result obtained for development. However, the crabs fed the diet without additional ARA supplementation (0 g $kg^{-1}$) had significantly higher dry weight than all other treatments with supplemented ARA ($p<0.01$), the diet also resulted in the shortest development time from C1 to C4 and the biggest carapace size. The results indicated that further provision of ARA other than that already provided by triglyceride supplementation of soybean oil and fish oil at a fix ratio of 1:1 is not necessary.

A final experiment investigated effects of supplementing *P. pelagicus* diets with different polar lipids (PL) and neutral lipids (triglyceride; SO: FO ratio at 1:1) ratios. Five iso-energetic and iso-lipidic diets were formulated to contain PL and triglyceride mixture (TG) at 1:0, 2:1, 1:1, 1:2 and 0:1, respectively. The results showed that the diet without PL supplemented with triglyceride only (PL:TG ratio 0:1) impaired crab survival. The treatment also had a significantly longer mean development time to C4 when compared to crabs fed diets with PL:TG ratios at 1:0, 2:1 and 1:1 ($p<0.01$). Meanwhile, crabs fed the diet with a PL:TG ratio of 1:0 had the greatest dry weight and carapace size, which were significantly

higher than crabs fed diets formulated with PL:TG ratios at 0:1 and 1:2. Salinity stress test (salinity dropped abruptly form 30 to 7‰ for 9 h) using C4 crabs demonstrated that crabs fed diets with PL to TG ratios (PL:TG = 1:1 and 1:2) suffered 100% mortality while > 50% crabs fed diets formulated with higher PL to TG ratios (PL:TG = 1:0 and 2:1) survived to the end of the stress test.

This study has covered the majority optimal lipid requirements for *P. pelagicus*, an emerging aquaculture species. The optimal level of lipids constituents in this study were determined using parameters set for aquaculture, including survival, development and growth. The use of practical ingredients makes the outcomes of the study more readily for industry implementation.

# Table of Contents

# List of Tables

.

# List of Figures

# CHAPTER 1

## An introduction to the blue swimmer crab, *Portunus pelagicus*

### 1.1 Taxonomy and bionomics

The taxonomy of the blue swimmer crab, *Portunus pelagicus*, can be summarized as follows (Kangas, 2000):

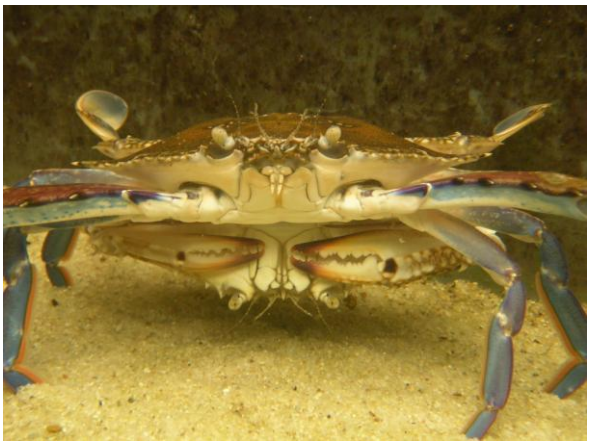
| | |
|---|---|
| Phylum | : Crustacea |
| Class | : Malacostraca |
| Subclass | : Eumalacostraca |
| Order | : Decapoda |
| Family | : Portunidae |
| Species | : *Portunus pelagicus* |
| Common names | : Blue swimmer crab, blue manna crab, sand crab, blue crab |

*P. pelagicus* is distributed throughout the tropical and sub-tropical regions of the Indo-Pacific, from east Africa to Japan and into the Pacific to Tahiti and the waters of northern New Zealand (Williams, 1982; Kangas, 2000; Williams, 2002; Chande and Mgaya, 2004). In temperate region, such as in South Australia, they are only able to grow and reproduce during warmer summer months (Smith, 1982). *P. pelagicus* lives in a wide range of the coastal margin and estuarine areas including sandy, muddy, algal and sea-grass habitats from the intertidal zone to at least a depth of 50 m (Williams, 1982; Edgar, 1990). Smaller crabs or juveniles are found in shallow waters, mainly in mangrove creeks and mud flats, while adults are usually found in deeper waters (Kumar et al., 2000). The differences in

their habitat are thought to be related to food abundance and relative sensitivity to changes in salinity (Meagher, 1971; Kangas, 2000).

### 1.2 Life cycle

Mating of blue swimmer crabs occurs anytime between sexually mature males and females provided the females are in a post-molt, soft-shell condition. The mating behavior of *P. pelagicus* was described in detail by Fielder and Eales (1972). In general, the male climbs onto the female and cradles her beneath him by hooking his second periopods under her body. In this courting position, the male is extremely aggressive and the courting behavior could take 4 to 10 days. After the females molt, copulation begins by the female shifting her position upward and facing the male with their abdominal flaps opened (Fig. 1.1 A and B). The male then extends his copulatory pleopods into the genital apertures of the female. The sperm, however, does not immediately fertilize the eggs but is stored in a sperm capsule within the female (Fielder and Eales, 1972). Males can mate with a number of females, however, large mature females only mate once a year since they are capable of multiple spawning from a single insemination (Kangas, 2000).



(A)

(B)

**Figure 1.1** A. Male (top) and female in a copulation position B. Male and female with opened abdominal flap for copulation.

Spawning of *P. pelagicus* occurs year around in tropical and subtropical waters but is restricted to warmer months in temperate regions (Smith, 1982; Potter et al., 1983). When spawning occurs, the female settles into the sand with her abdomen extended outwards and the eggs are extruded and attach to hairs on her abdomen (Kangas, 2000). The incubation period for *P. pelagicus* eggs has been reported to vary with water temperature: 8 days at 25$^{o}$C, 15 days at 24$^{o}$C and 18 days at 20$^{o}$C (Smith, 1982; Kangas, 2000). However, personal

observations during the course of this study showed an incubation period of 10 days from spawning to hatching for berried females maintained at 28$^{o}$C. The differences in incubation period of this species may be attributed by other environmental factors in culture system such as salinity, pH and light in addition to maturity and health status of the female crabs. Furthermore, in an unpublished genetic study of *P. pelagicus*, it was suggested that they are also likely different sub-species of blue swimmer crabs in different location (Chiu et al., 2005).

Larval development of *P. pelagicus* consists of four zoeal stages and a post larval megalopal stage (Shinkarenko, 1979), and laboratory reared larvae under optimal conditions requires approximately 12-14 days to reach the first crab stage (C1) (Romano and Zeng, 2006). The larvae readily accepted food as soon as they hatch from eggs (Castine et al., 2008), a beneficial characteristic for mass hatchery production. Like all crustaceans, *P. pelagicus* undergoes regular molting which involves morphological changes during larval development and increase in size during juvenile development. It has been reported that *P. pelagicus* requires approximately 12 and 14 molts from the first juvenile stage (C1) to attain sexual maturity for males and females, respectively (Jose and Menon, 2005).

**1.3 Feeding**

The larval phase of *P. pelagicus* is planktonic and the larvae eat various zooplankton in open waters. After they metamorphose as juvenile crabs, they become benthic and change their feeding behavior to opportunistic scavengers, cannibals and bottom feeding carnivores (Kangas, 2000). A study of the stomach contents of *P. pelagicus* in sub-tidal and inter-tidal

areas of Moreton Bay, Queensland, Australia suggests that their diets are largely dependent on local availability of prey species (Williams, 1982). Their diets consist of a wide range of sedentary or slow moving benthic invertebrates, including bivalves and other mollusks, small crustaceans and worms and some teleost and plant materials (Williams, 1982; Wassenberg and Hill, 1987; Lestang et al., 2003). Diet compositions of P. pelagicus are also influenced by the size of their prey, spawning and the molting period of the crabs (Williams, 1982; Edgar, 1990). Prior to molting, the feeding activity of crabs usually declines and they only start to consume food again when their exoskeleton is hardened enough to handle food (Phlippen et al., 2000). This feeding-fasting-feeding cycle also occurs during spawning and is a common behavior among crustaceans (Sanchez-Paz et al., 2006). During fasting or starvation periods, crustaceans store certain nutrients and utilize others to maintain essential processes and metabolic activity (Steffens, 1989). Thus starvation has been used as a research tool to determine the relative importance of various nutrients in energy production and storage and critical structural and metabolic roles (Ritar et al., 2003). For example, in starvation experiments with juvenile Chinese mitten crabs, *Eriocheir sinensis*, fatty acids were found to be the primary metabolic substrates in supplying concentrated energy (Wen et al., 2006). In contrast, polar lipids have been suggested to be preferentially conserved during starvation because of their important functions in cell membrane structure and function (Halver, 1989; Koven et al., 1989).

### 1.4 Portunus pelagicus: fisheries exploitation and aquaculture potentials

*P. pelagicus* supports substantial commercial fisheries in many countries and is an important component of recreational fisheries throughout the range of its natural distribution.

Global landings of *P. pelagicus* increased approximately 56% from 75 968 tonnes in 1990 to 173 509 tonnes in 2008, with the highest catches reported from China and the Philippines (FAO, 2010). *P. pelagicus* are collected from the wild using artisanal traps, trawls, beach seines, cylindrical wire traps, folding traps, pots, hop nets, drop nets and crab gill nets (FAO, 2010). In Australia, with the exception of Tasmania, commercial catches of blue swimmer crabs have grown rapidly in most states, including South Australia, Western Australia, Queensland and New South Wales (Kumar, 1997; Williams, 2002) with Queensland responsible for 25% of Australia's annual fisheries production (Williams, 2002).

In the past, *P. pelagicus* were primarily sold as either uncooked or cooked whole hard-shell crabs, but the trade has expanded to include live crabs and value added products such as soft-shell crabs, cooked meat, pasteurized crab meat and cocktail claws, reflecting increasing market demand for this species (Baker and Kumar, 1994; Saraswathy et al., 2006; Romano and Zeng, 2008; Wu et al., 2010a). In Southeast Asian countries, such as Indonesia and the Philippines, *P. pelagicus* are not only consumed as a sought-after seafood item locally but are also exported extensively to the US and European markets as pasteurized crab meat products (Romano and Zeng, 2008). Currently, production is largely based on wild-caught crabs, however, the sharp increase in the fishery landings of the crab over the past decade suggests that fisheries based on wild stock are likely unsustainable (FAO, 2010).

*Fisheries Management Act 1991* in Australia set the basis for legislations on statutory fishing rights, licenses and permits to sustain wild fisheries of many species, including *P. pelagicus*. Each state within Australia enforced the amendment in details, such as *Fisheries*

*Act 1994* in Queensland and *Fish Resources Management Act 1994* (FRMA) in Western Australia (D.o.F, 2005; DPI&F, 2008). Under the amendment of Queensland, removal of female *P. pelagicus* from the wild is prohibited, and there are minimum size limits, apparatus restriction, limits on the number of crabs that can be taken and spatial closures with which commercial and recreational fisheries must comply (DPI&F, 2008). The report on the status of *P. pelagicus* and *Ovalipes australiensis* (common sand crab) in New South Wales showed that commercial landings of these species significantly decreased from approximately 300 tonnes per years between 1995-1999 to approximately 150 tonnes in 2005/2006 (Scandol et al., 2008). In Cockburn Sound, which represents the second largest *P. pelagicus* fishery in Western Australia, the commercial and recreational fisheries for *P. pelagicus* ceased in 2006 due to a sharp decline in catches (Johnston et al., 2011). In contrast, in Queensland, it is suggested that wild stocks of *P. pelagicus* are currently resilient to fishery exploitation due to strict regulations imposed (DPI&F, 2008).

In countries with poorly managed fisheries, there is no legislation to help sustain wild stocks of *P. pelagicus*. For example, in Singapore, China, Malaysia and Indonesia, fishermen are allowed to catch juvenile and female crabs from the wild since females are considered a delicacy and are sold at substantially higher prices than male crabs (Wu et al., 2010a). In Thailand, the higher proportion of smaller sized *P. pelagicus* caught from the wild suggests over-exploitation of this species (Klinbunga et al., 2007). Considering the importance of sustaining wild stocks and allowing over-exploited wild populations to recover, there is growing interest in the aquaculture of *P. pelagicus* and is likely to become increasingly important in meeting future market demands.

Crab farming is a relatively new sector of aquaculture industry, however, it has grown rapidly over the past decades. In 2005, approximately 660 000 tonnes of farmed crabs were produced with a total value of USD$ 2.8 billion. However, the vast majority of the farmed crab production is for freshwater mitten crabs and mud crabs, with only limited *Portunus* sp. production (FAO, 2008). Limitations in the development of *Portunus* crab culture industries has been related to limited opportunities for farmers to implement 'best farming practice' as most research and development is done at laboratory-scale. Furthermore, in many countries, crab farmers have relied on wild-caught 'seed' crabs to on-grow to market size in ponds, lakes or reservoirs (Paterson, 2009). However, such practices are not feasible in Australia since collecting larvae or juveniles from the wild conflicts with management of wild crab fisheries *(Fisheries Act 1994*) and this puts severe limit on the development of crab aquaculture industry in Australia. However, recent success in hatchery production of *P. pelagicus* has opened new opportunities for aquaculture of this species. *P. pelagicus* has been shown to have a relatively short larval duration (approximately 14 days) and can be propagated using relatively simple culture methods. Larval survival is also relatively high which is highly beneficial for commercial *P. pelagicus* farming (Jose and Menon, 2005; Romano and Zeng, 2006; Castine et al., 2008). Studies on developing formulated diets for *P. pelagicus* larvae have also shown promising results with successful laboratory scale production on cost effective diets for megalopal larvae that were readily ingested by the larvae (Castine et al., 2008).

To date, few reports that exist on the farming of *P. pelagicus* are related to soft shell crab production or fattening, which are largely cultured over the short-term. Soft shell crabs are sold in a post-ecdysial state as they command premium prices compared to hard-shelled crabs (Paterson et al., 2007). Farmed soft shell crabs are currently being marketed at the size range from 60 to 120 g while the hard shell crabs are marketed at larger size of around 300 g (DPI&F, 2005). *P. pelagicus* produced for the soft-shell trade are usually cultured within individual compartments in lined ponds and are then periodically observed and removed (Walker, 2006; O'Sullivan and Savage, 2008). In Australia, one of the major producers of soft shell *P. pelagicus,* Watermark Seafoods, has developed a robotic system to harvest newly molted soft shell crabs (DPI&F, 2005). Fattening of *P. pelagicus* has also been practiced in countries such as India and Thailand, where empty crabs are bought from fishermen for much lower prices and reared until meat texture improves (Chaiyawat et al., 2009).

Despite the success with the hatchery culture of *P. pelagicus* and recent developments in soft shell crab culture, limited attempts have been made to culture *P. pelagicus* from early juveniles to market size. Many challenges impede the progress of *P. pelagicus* culture, including cannibalism and lack of their nutritional requirements information. The massive expansion of penaeid shrimp culture since the 1970's is believed to be hugely benefited from the successful development of cost-effective formulated feeds based on extensive nutrition studies (Pedrazzoli et al., 1998), which highlights the importance of nutritional knowledge for successful crustacean culture. Research towards the development of nutritionally optimized diets for other crustacean species is been ongoing (Cuzon, 1998; Holme et al.,

2009), however, to date, no studies have yet attempted to establish the quantitative nutrient requirements for *P. pelagicus*, which form the basis for the formulation of species-specific diets for *P. pelagicus*.

### 1.5 Use of a formulated diet to investigate nutritional requirements

Aquaculture has expanded rapidly over past several decades, however the industry still heavily relies on processed fish meal and in many developing countries, trash fish as the major component of aquafeeds (New and Wijkstrom, 2002; NACA and FAO, 2009). In 2006, the aquafeed industry was estimated to have consumed 3 724 thousands tonnes of fish meal and 835 thousands tonnes of fish oil, equivalent to 16.6 million small pelagic forage fish (trash fish) (Tacon and Metian, 2008). Although trash fish are often favored by cultured species, the nutritional quality of trash fish varies between species and the quality declines rapidly during transportation if the fish are not stored under sanitary conditions (Edwards et al., 2004; Zhou et al., 2004). The use of trash fish as a food source in fish and crustacean culture may also contribute to the deterioration of water quality, and the introduction of pathogens to culture systems (Wu, 1995; Edwards et al., 2004). In response to problems associated with the use of trash fish, research on the development of formulated feeds has become increasingly important. Formulated feeds have been shown to require less labor and facilities to process, and they maintain better water quality in pond culture compared to using trash fish in aquaculture (Chen et al., 1989). Furthermore, formulated feeds can be manipulated to effectively deliver optimal amounts of essential nutrients, such as amino acids, fatty acids, vitamins and minerals, and can be easily manipulated to reduce production

costs through the inclusion of terrestrial-based ingredients, such as plant meals and vegetable oils.

Nutritional studies with crustaceans began in the 1960s, prompted by the need to develop a suitable formulated diet for cultured penaeid shrimp. The research was conducted on various penaeid species, but there were difficulties in comparing results between studies because of differences in experimental design, diet formulation, the purity of feed ingredients, palatability of diets and variations in nutrient leaching and growth performance measurements (D'Abramo and Castell, 1997). Lipid nutrition was a major focus of these studies with research involving various lipids components, including phospholipid, essential fatty acids, sterols and caratenoids (Gonzalez-Felix et al., 2002a) as these lipids constituents play major roles in crustacean physiology and energy provision (Oliva-Teres, 2000).

Lipids nutrition research has evolved from investigating individual dietary lipid constituents to their interactions and optimal ratios (Glencross and Smith, 2001a; Glencross et al., 2002d; Sheen and Wu, 2003; Limbourn and Nichols, 2009; Ward and Carter, 2009). However, although there is an abundance of data from lipid nutrition studies, many areas remain unclear, incomplete or even poorly known, such as the balance between dietary n-3 and n-6 fatty acids as well and their complex interactions. Despite the general acceptance that the lipid requirements of crustaceans are species-specific, there is relatively limited knowledge in this field for non-penaeid crustacean species. Research on *P. pelagicus* lipid nutrition should provide a better understanding of the function of lipids in crustaceans and will be vital for the development of the *P. pelagicus* aquaculture industry.

The aim of the following chapter is to provide comprehensive insight into crustacean lipid nutrition by surveying available literature relevant to the quantitative requirements of lipid constituents and the function of each lipid constituent for crustaceans. It will begin with a general introduction to lipid classification and the terms used in lipid nutritional studies, followed by a review on the important lipid classes for crustaceans and the interactions between them. Since the data chapters of this thesis were formatted for journal submission, repetition of information in the following sections and in the data chapters are inevitable but were reduced whenever possible.

### *1.5.1 Lipids: An introduction*

Lipids can be generally divided into two groups: i.e. polar lipids, which are composed principally of phospholipids, and neutral lipids, composed primarily of triglyceride (Tocher, 2003). These two lipid groups are classified based on their ability to associate with water, being either hydrophilic (polar lipids) or hydrophobic (neutral lipids) (Gurr and Harwood, 1991). In nutritional studies with crustaceans, dietary polar lipids are usually provided in the form of phospholipids while neutral lipids are supplied in the form of oil and/or fats (Tocher, 2003). Phospholipids and some neutral lipids (i.e. essential fatty acids and cholesterol) must be supplied in crustacean diets because crustaceans have limited or inability to synthesize these essential nutrients (Kanazawa et al., 1985; Sheen and Wu, 1999).

For triglyceride, three fatty acids are joined to a glycerol molecule by an ester linkage; a bond between the hydroxyl group and a carboxyl group (Campbell et al., 1999).

The fatty acids are differentiated on the basis of their chain length, degree of saturation (number of ethylenic or double bonds) and the position of the double bonds (Tocher, 2003). They are grouped into saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) or highly unsaturated fatty acids (HUFA). SFA are usually straight chain fatty acids and do not posses any double bonds. MUFA contain a single double bond, while PUFA and HUFA both posses more than one double bond and they are differentiated on the basis of the number of carbon atoms in the molecule and the number of double bonds (Gurr et al., 2002b). Cholesterol is type of sterol, which are lipids characterized by four fused rings; it is a significant component of animal cell membranes and also a precursor from which other steroids are synthesized (Campbell et al., 1999). While vertebrates are capable of synthesizing cholesterol to satisfy their metabolic requirements, invertebrates largely rely on their diets for cholesterol supply (Gurr et al., 2002a).

Phospholipid (PL) is a general term for phosphoglyceride, which can be grouped to various groups, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) based on their esterification with alcohol bases, such as choline, ethanolamine, serine and inositol (Tocher et al., 2008). As suggested by the term phosphoglyceride, they also contain fatty acids and a glycerol ‘back bone’, and the type of fatty acids esterified in phospholipid varies depending on their source e.g. SFA and MUFA in egg derived phospholipid, PUFA in phospholipid derived from soybean and HUFA in phospholipid come from marine fish (Kanazawa et al., 1985; Coutteau et al., 2000; Jiang et al., 2001). Phospholipids are added to the diets of crustaceans as sources

of energy, cell membrane components, and to enable emulsification of lipid during lipids digestion and absorption (Gong et al., 2001).

*1.5.2 Triglyceride*

The importance of an optimal amount of dietary lipids for maximizing the growth of decapod crustaceans has been suggested by various researchers and many papers and reviews have been dedicated to this (Table 1.1). In many past nutritional studies, supplemental dietary lipids often refer to the addition of neutral lipids (usually in the form of triglyceride) into the diets (e.g. fish oil and/or vegetable oil) (Castell and Covey, 1976; Davis and Robinson, 1986; Sheen and D'Abramo, 1991; Sheen et al., 1994a; Glencross and Smith, 1997; Sheen, 1997; Sheen and Wu, 1999). Sheen and Wu (1999) proposed that optimal dietary lipid level for crustaceans is generally between 2 to 10% of diet weight. Studies on juveniles of the giant freshwater prawn, *Macrobrachium rosenbergii*, and the black tiger prawn, *Penaeus monodon*, have shown that excessively high dietary lipid levels (12% and 13.5% of diet weight, respectively) adversely affects their lipid utilization, retards growth and lipid storage capacity (Sheen and D'Abramo, 1991; Glencross et al., 2002c). In juvenile of *P. monodon*, their digestibility rate was significantly lower when they were fed total lipids at 135 g $kg^{-1}$ (approximately 13.5%) compared to when they were fed total lipids at 45, 75 and 105 g $kg^{-1}$ (approximately 4.5, 7.5 and 10.5%, respectively) (Glencross et al., 2002b). In contrast, juvenile mud crab, *Scylla serrata* appear to be able to utilize relatively high levels of dietary lipid with high growth rates being recorded when they were fed a diet containing 13.8% lipid, since their growth was not significantly different from that of the crabs receiving 5.3% dietary lipid (Sheen and Wu, 1999).

While such substantially varied results obtained from different studies are likely the demonstration of the species-specific nature of the dietary lipid requirements of crustaceans, it could also be due to differences in the fatty acid composition of the lipids used in those studies (Merican and Shim, 1994; Kamarudin and Roustaian, 2002; Vasagam et al., 2005; Ward and Carter, 2009). The efficiency of lipid utilization has been shown to depend on the fatty acid composition of the lipid source. For example, the juveniles of Pacific white shrimp, *Litopenaeus vannamei,* showed better growth when they were fed with diets formulated with menhaden fish oil instead of a range of plant oils, including stearic acid, coconut oil, safflower oil, corn oil, soybean oil and linseed oil (Lim et al., 1997). This has also been the case for juveniles of black tiger prawn, *P. monodon* when fed with lipids from various source (Catacutan, 1991). Menhaden fish oil contains higher HUFA levels and is a good source of ecosapentaenoic acids (EPA) and docosahexaenoic acid (DHA) (Xu et al., 1994; Watanabe, 2002), while plant oils are usually rich in SFA, MUFA and PUFA but lack of HUFA (Vasagam et al., 2005). Meanwhile, juveniles of the mud crab, *S. serrata,* fed a lipid-free diet showed low molting frequency caused by a lack of eicosanoids, a molting hormones derived from arachidonic acid (ARA), another major HUFA (Sheen and Wu, 1999). However, utilizing marine fish oils in aquafeeds is limited by the fact that fish oils are non renewable resources which are often costly.

The demand for fish oils by the aquafeed industry has increased dramatically over past decades, which is clearly unsustainable (Kamarudin and Roustaian, 2002). As the result, research has been conducted in the attempt to substitute fish oils with plant oils in aquafeed

production. While various studies have shown success in reducing fish oil reliance by mixing fish oil with plant oils (Read, 1981; Sheen and D'Abramo, 1991; Merican and Shim, 1994; Kamarudin and Roustaian, 2002; Vasagam et al., 2005; Holme et al., 2007b; Peng et al., 2008), it is worth noting that inappropriate combinations of dietary plant oil with fish oil can impair survival and growth as the result of altered fatty acid compositions of the diets.

**Table 1.1** Optimal levels of dietary neutral lipids (triglyceride) (% of diet weight) for juveniles of various crustacean species.

| **Species** | **Lipid level (%)** | **Source** | **Author** |
|---|---|---|---|
| *Scylla serrata* | 5.3 -13.8 | cod liver oil:corn oil (2:1) | Sheen and Wu (1999) |
| *Macrobrachium rosenbergii* | 2-10 | cod liver oil:corn oil (2:1) | Sheen and D'Abramo (1991) |
| *Astacus astacus* | 7 | - | Ackefors et al. (1992) |
| *Cherax quadricarinatus* | 7.5 | fish oil:soy lecithin (1:1) | Cortes-Jacinto et al. (2005) |
| *Procambarus clarkii* | 6 | - | Jover et al. (1999) |
| *Procambarus acutus* | 0-6 | menhaden oil | Davis and Robinson (1986) |
| *Penaeus chinensis* | 3.6-9.6 | cod liver oil:corn oil (2:1) | Sheen (1997) |
| *Homarus americanus* | 5 | cod liver oil | Castell and Covey (1976) |
| *Penaeus monodon* | 4.5-10.5 | Mixture of marine oil, plant oil and purified fatty acids | Glencross et. al. (2002b) |

1.5.2.1 Saturated (SFA) and monounsaturated fatty acids (MUFA)

Early study has shown that *M. rosenbergii* was able to synthesize SFA and MUFA, including 14:0, 16:0, 16:1n-9 and 18:1n-9 fatty acids, using dietary carbohydrate and protein as a carbon source (Reigh and Stickney, 1989). It was then later suggested that crustaceans are able to convert SFA to MUFA using Δ-9-desaturase enzymes (D'Abramo, 1997). Due to this ability, only limited attention has been given to the dietary requirements for SFA and MUFA of crustaceans. Understanding the functions and effects of dietary SFA and MUFA are commonly inferred indirectly from studies that involved inclusion of plant oils, which contain high levels of SFA and MUFA, or from food deprivation studies (Merican and Shim, 1994; Vasagam et al., 2005; Wen et al., 2006). For example, inclusion of SFA and MUFA in diets for the black tiger prawns, *P. monodon,* juveniles, resulted in variable digestibility rates; SFA digestibility decreased with increased carbon chain length, ranging from 95% for 14:0 to 66% for 18:0. In the same study, in contrast, the digestibilitiy of MUFA increased with increasing carbon chain length (Merican and Shim, 1994). The digestibility of SFA was believed to be more efficient when it is in a free fatty acid form than when it is bound together with glycerol (Leger, 1985). This is perhaps because free fatty acids can be directly utilized by the animal whereas glycerol compounds must be hydrolyzed before they can be utilized by crustaceans.

SFA and MUFA are often catabolised to generate metabolic energy and they become major energy sources during periods of food deprivation (Sargent et al., 1999; Ritar et al., 2003; Limbourn et al., 2008). For instance, in Chinese mitten crab, *E. sinensis*, juveniles,

SFA and MUFA were preferentially used during starvation as reflected by a decrease in the levels of SFA and MUFA in body tissues while PUFA was conserved (Wen et al., 2006). For starved larvae of the western rock lobster, *Panulirus cygnus*, utilization of SFA and MUFA varied with developmental stages (Liddy et al., 2004). In addition, comparison between SFA and MUFA showed that during winter starvation, Kuruma prawn, *Marsupenaeus japonicus* adult increased tissue levels of oleic acid (18:1), a type of MUFA, at the expense of SFA, particularly palmitic acid (16:0) and stearic acid (18:0). The higher content of MUFA was suggested to be related to building of triglycerides as energy reserves (Guary et al., 1975).

Although crustaceans possess the ability to synthesize SFA and MUFA, providing sufficient amounts of dietary SFA and MUFA in the diets is still important in reducing lipid oxidation in the diets (Kanazawa et al., 1985). Fish oil, when diluted with palm oil that contains shorter-chain fatty acids, is very effective in lowering the dietary lipid unsaturation levels and prolongs lipid stability by reducing peroxidation during feed storage (Watanabe, 2002). Considering the function of SFA and MUFA in developing diets, perhaps more research should be done on SFA and MUFA.

1.5.2.2 Polyunsaturated fatty acids (PUFA)

A large body of research has been dedicated to determining the essential fatty acid requirements of crustaceans. PUFA that consist of linolenic acid (LNA; 18:3n-3) and linoleic acid (LOA; 18:2n-6), have been recognized as very important nutrients for crustaceans and diets deficient in PUFA, have been shown to detrimentally affect the growth and survival of crustaceans (Read, 1981; Merican and Shim, 1996). For example, the growth of the black

tiger prawn, *P. monodon*, juveniles, was depressed when LNA was deficient in their diet, at the same time, supplementing LNA at 1.5 to 3% of the diet significantly increased survival and growth (Merican and Shim, 1997). Although, the essentiality of LNA and LOA, and the importance of providing sufficient amounts of these fatty acids in crustacean diets are well known (D'Abramo and Sheen, 1993; Merican and Shim, 1997; Glencross and Smith, 1999; Glencross et al., 2002a; Gonzalez-Felix et al., 2003a), there are conflicting reports concerning relative nutritional superiority of LNA and LOA. Some studies have suggested that LNA as an n-3 PUFA has greater nutritional value compared to LOA, a PUFA from n-6 families (Xu et al., 1993; Xu et al., 1994; Lim et al., 1997; Gonzalez-Felix et al., 2003b). For example, feeding juvenile Chinese prawn, *Penaeus chinensis* with a diet supplemented with 1% LNA showed significantly superior growth rates, molting frequency and survival than those juveniles fed a diet supplemented with 1% LOA (Xu et al., 1993). Similar experiments with the freshwater prawn, *M. rosenbergii*, however, gave contrasting results since significantly higher weight gain was achieved when *M. rosenbergii* were fed the diet containing 1% LOA compare to the diet containing 1% LNA (Reigh and Stickney, 1989). Meanwhile, research with juveniles of *Penaeus indicus* and *L. vannamei* has demonstrated no significant differences on their weight gain when they were fed diets supplemented with either LOA or LNA at the same level (Read, 1981; Gonzalez-Felix et al., 2003a).

It is suggested that the relative supremacy of dietary LOA over LNA, or vice versa, reflects physiological adaptations to the natural environments of the animals tested. Freshwater crustaceans, for example, are thought to require predominantly n-6 fatty acids while marine species have a preference for n-3 fatty acids and this is reflected in the fatty

acid compositions of their body tissues (D'Abramo, 1997; Ackman, 1998). Thus, marine crustaceans generally utilize dietary fish oils that are rich in n-3 fatty acids better than plant oils that are generally high in n-6 fatty acids (Lim et al., 1997).

**Table 1.2** Optimal levels of dietary linolenic acid (LNA), linoleic acid (LOA), ecosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (% of diet weight) for juveniles of various crustacean species.

| Species | LNA | LOA | EPA | DHA | Author |
|---|---|---|---|---|---|
| *Penaeus indicus* | 2.0 | 2.0 | | | Read (1981) |
| *Penaeus chinensis* | 0.7-1.0 | | 1.0 | | Xu et al. (1994) |
| *Macrobrachium rosenbergii* | 0.6 | 0.6 | | | D'Abramo and Sheen (1993) |
| *Penaeus monodon* | 2.5 | - | | 1.0 | Merican and Shim (1997) |
| | - | 2.1 | | | Glencross and Smith (1999) |
| | | | 1.5 | 1.5 | Glencross et al. (2002b) |

1.5.2.3 Highly unsaturated fatty acids (HUFA)

The inability of crustaceans to elongate and desaturate 18 C chain fatty acids to more unsaturated forms makes it a necessity to provide HUFA in crustacean diets (Kanazawa et al., 1979b). Gonzalez-Baro and Porello (1998) suggested that the inability of crustaceans to convert PUFA to HUFA is because of inactive enzymatic system of Δ6 and Δ5 desaturase in crustaceans. Using radioactive α-LNA in *Macrobrachium borelli,* juveniles, it was found that when prawns were incubated for 8 h, 3% of radioactive LNA were elongated to 20:3n-3.

However, the synthesized 20:3n-3 only appeared in small amounts and was not further elongated. The inability of some aquatic animals to synthesize HUFA is thought to relate to a metabolic pattern reflecting the readily availability of HUFA in their environments (Ackman, 1967). For example, marine fish are often carnivorous/piscivorous and their diets are naturally rich in n-3 and n-6 HUFAs; this has therefore resulted in an evolutionary down-regulation of desaturase and /or elongase activities. In contrast, freshwater fish generally consume diets consisting of other freshwater fish, insect and plants that are rich in PUFA, which might have led to establishing a need to be able to synthesis HUFA from PUFA (Sargent et al., 2002).

Although HUFA and PUFA cannot be synthesize *de novo* by crustaceans (Merican and Shim, 1996; Glencross and Smith, 1999), comparisons of HUFA and PUFA in dietary studies clearly illustrates the general superiority of dietary HUFA for crustaceans. Sheen and Wu (2003) pointed out that mud crab, *S. serrata*, juveniles, fed diets containing 0.2% various HUFA (DHA, ARA and DHA + ARA, respectively) gave better performance compared to those crabs fed the diets containing LNA or LOA at the same level. In *P. monodon*, lower levels of dietary DHA can reach an equivalent weight gain as those shrimps fed a similar diet but contained LNA instead (Merican and Shim, 1997). In addition, a 1% supplementation of ARA in the diet fed to *P. chinensis* juveniles for 60 days gave a significantly increased growth rates (182.3%) and higher survival as compared to those fed the same level of supplemental dietary LOA, LNA or a combination of both (Xu et al., 1994). While dietary HUFA appears to be superior to PUFA for crustaceans, feeding *P. monodon* with a diet containing purified DHA, gave inferior weight gain compared to those fed a diet containing

cod liver oil that were rich in both PUFA and HUFA, suggesting that the optimal balance of dietary fatty acids is more important than individual fatty acid (Merican and Shim, 1996). Interactions between fatty acids and other lipid constituents may also play very significant roles.

Of n-3 HUFA, both of the two important fatty acids; eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), play important roles as components of membrane phospholipids and as precursors of biologically active eicosanoids (Bell et al., 1986; Mourente and Tocher, 1992; Ibeas et al., 1994). Past research have shown that optimal dietary n-3 HUFA improved growth and survival of crustaceans, however, both inadequate and excessive dietary HUFA usually results in severely reduced growth rates and high mortalities (Read, 1981; Merican and Shim, 1997; Gonzalez-Felix et al., 2003b; Gonzalez-Felix et al., 2003a; Sheen and Wu, 2003). Kanazawa et al. (1985) reported that the survival and growth of *M. japonicus* larvae increased with increasing dietary n-3 HUFA levels from 0 to 1% of diet dry weight but decreased at 2% of diet weight. Depressed growth rates at relatively high levels of dietary n-3 HUFA may be related to a metabolic response to excessive HUFA levels as unsaturated fatty acids have high tendency and susceptibility to oxidation (Yu and Sinnhuber, 1976; Gurr and Harwood, 1991).

Surprisingly, among the numerous studies on n-3 HUFA nutrition of crustaceans, relatively little attention has been given to address the quantitative requirements of EPA and DHA respectively, often DHA has been the major focus due to its perceived higher nutritional value (Read, 1981; Merican and Shim, 1996; Merican and Shim, 1997; Mourente

and Rodriguez, 1997; Sheen and Wu, 2003). For example, DHA is more efficacious than EPA in increasing carapace width and molting frequency of mud crab, *S. serrata*, larvae, which is also indicated by decreased content of DHA in larvae after molts (Suprayudi et al., 2004). Furthermore, DHA can also be retro-converted to EPA, which has been suggested for *M. rosenbergii*, (D'Abramo and Sheen, 1993), *P. monodon* (Merican and Shim, 1996) and *S. serrata* (Sheen and Wu, 2003). HUFA in general is believed to be saturated and broken down by β-oxidation processes to become LOA or LNA by peroxisomal enzymes (Smith et al., 1983; Van Veldhoven et al., 2001). However, in studies with *M. rosenbergii* and *P. monodon* juveniles, LOA and LNA were not found in significant amounts in their tissues, indicating that β-oxidation processes may have limitations (D'Abramo and Sheen, 1993; Merican and Shim, 1996).

Many studies tend to focus on the importance of n-3 HUFA in crustacean nutrition with far less consideration paid to the role of the n-6 HUFA, arachidonic acid (ARA; 20:4n-6) as an essential fatty acid. As a HUFA, ARA has greater nutritive value and is preffered over LNA or LOA as a dietary fatty acid (Xu et al., 1994; D'Abramo, 1997; Sheen and Wu, 2003). A lack of dietary n-6 fatty acids for crustaceans has been shown to induce negative growth responses (Reigh and Stickney, 1989; Glencross and Smith, 1999). In addition, the molting process in crustaceans is regulated by eicosanoids for which ARA is a precursor. Eicosanoids derived from ARA have been suggested to be more biologically active than those produced from other HUFA, such as EPA and DHA (Sargent et al., 1999; Gonzalez-Felix et al., 2003b). When the brown tiger prawn, *Penaeus esculentus* were injected with prostaglandin $E_2$, a type of eicosanoid derived from ARA, they displayed shorter molt cycles

and a better growth rate as compared to the controls (Koskela et al., 1992). In unfed freshwater crab *E. sinensis* juveniles, ARA was retained in their muscle, suggesting a metabolic priority and important role of ARA as a structural lipid (Wen et al., 2006).

However, when comparing the effectiveness of ARA to other HUFA in promoting growth and survival of crustaceans, ARA is often considered less significant. For example, when diets containing 1% dietary ARA was compared to the same level of dietary DHA, the former led to significantly reduced survival, molting frequency, growth rate and final weight gain of *P. chinensis,* juveniles (Xu et al., 1994). Feeding *P. monodon,* juvenile with ARA deficient diet that contained other essential fatty acids also demonstrated no noteworthy reduction on growth rate (Merican and Shim, 1996). In addition, the growth of *P. monodon,* juveniles, was found to be the poorest when they were fed a diet containing high ARA (20% of total fatty acids) with optimized levels of LOA, LNA, EPA and DHA (Glencross and Smith, 2001b). It was suggested that the inclusion of high dietary ARA may have increased essential fatty acids to excessive levels needed and subsequently led to oxidation as discussed above. In addition, the high level of n-6 HUFA in diets may imbalance the n-6 to n-3 ratio, which has been suggested to have considerable effects on crustacean growth (Glencross et al., 2002d; Gonzalez-Felix et al., 2003b).

### *1.5.3 Phospholipid*

For many crustaceans, there is a need to supply phospholipids (PL) exogenously as their synthesizing rates by the animals often fail to meet metabolic requirements (Teshima et al., 1986a). Many studies have shown that feeding crustaceans with PL supplemented diets

promoted good survival and growth, likely as a result of the important roles that PL play in lipid transportation, hence facilitating better utilization and absorption of triglyceride (Paibulkichakul et al., 1998; Gong et al., 2000b; Hien et al., 2005; Holme et al., 2007a). PL are also surface-active agents that could aid digestibility and improve the physical properties of the diet by increasing the water stability of food particles and acting as a source of antioxidants as well as feed attractant (Coutteau et al., 1997; Tocher et al., 2008).

The optimal level of dietary PL reported for crustaceans varies substantially between species, which is likely due to species-specific factors but may also reflect the differences in PL purity and type, experimental design and life stages of the animals tested in those experiments (Hilton et al., 1984; Coutteau et al., 1996; Thongrod and Boonyaratpalin, 1998; Coutteau et al., 2000; Gong et al., 2007; Holme et al., 2007a; Wu et al., 2007). For example, when diets containing 1.5% soybean phosphatidylcholine (PC) and 1.5% marine fish roe PC, were fed to Pacific white shrimp, *L. vannamei*, post-larvae for 35 days, respectively, those in the soybean PC diet treatment had significantly higher weight gain (Coutteau et al., 2000). Juvenile of banana shrimp, *Penaeus merguiensis,* has been shown to require 1-2% soybean lecithin in the diet for optimal growth (Thongrod and Boonyaratpalin, 1998). Similarly, for American lobster, *Homarus americanus,* juveniles, it has been demonstrated that refined soybean PC is more effective in improving survival than egg PC, soybean phosphatidylinositol (PI) or hydrolyzed soy-bean PC (D'Abramo et al., 1981). In addition, the requirement of dietary PC appeared to decrease from 30 g $kg^{-1}$ to 15 g $kg^{-1}$ with increasing age of Kuruma prawn, *M. japonicus*, post-larvae (Camara et al., 1997).

The effectiveness of dietary PL for crustaceans is thought being influenced by the class and fatty acid composition of the PL source (D'Abramo et al., 1981; Kanazawa et al., 1985; Coutteau et al., 1997). Among the components of PL, phosphatidylcholine (PC) has been found to be the most active constituent due to the importance of choline in feed utilization and biological function of crustaceans (Michael et al., 2007). Kanazawa et al. (1985) found that when diets formulated with soybean PC and soybean PI, were fed to *M. japonicus* larvae, better growth and survival resulted when compared to those formulated with bovine brain phosphatidylserine (PS), bonito egg PC and bovine brain phosphatidylethanolamine (PE). Meanwhile, comparisons of purified PC and de-oiled lecithin that contain PC, PE and PI, showed that purified PC alone was nutritionally inferior to de-oiled lecithin in improving the growth of Pacific white shrimp, *L. vannamei,* juveniles (Gong et al., 2000a). Due to the fact that PL also contain fatty acids, the addition of PL to the diets of crustaceans inevitably will also add fatty acids as well, and fatty acids in the polar lipid fraction have been suggested to be more beneficial for survival and growth of crustaceans (Coutteau et al., 1996; Camara et al., 1997; Wold et al., 2007). Among PL from terrestrial sources, PL from soy bean is preferred due to high levels of PUFA, particularly linoleic acid (LOA; 18:2n-6) and also linolenic acid (LNA; 18:3n-3) (Kanazawa et al., 1985; Piedad-Pascual, 1985).

**Table 1.3** Optimum levels of dietary phospholipid for juveniles of various crustacean species.

| **Species** | **Phospholipid (%)** | **Source** | **Author** |
|---|---|---|---|
| *Penaeus merguiensis* | 1-2.0 | Soybean lecithin | Thongrod and Boonyaratpalin (1998) |
| *Penaeus penicillatus* | 1.25 | Phosphatidylcholine | Chen and Jenn (1991) |
| *Marsupenaeus japonicus* | 1.5<br>6.5 | Phosphatidylcholine<br>De-oiled lecithin | Camara (1997) |
| *Penaeus monodon* | 1.25 | Phosphatidylcholine | Chen (1993b) |
| *Litopenaeus vannamei* | 5.0 | De-oiled lecithin | Gong et al. (2000b) |
| *Homarus americanus*<br>*H. americanus* vs *H. gomarus* | 6-8.0 | Soybean lecithin | Conklin et al. (1980) |

*1.5.4 Cholesterol*

Crustaceans are generally unable to synthesize cholesterol *de novo* although cholesterol can be found abundantly throughout crustacean tissues since this is important as parts of cell structure (Kean et al., 1985; Teshima et al., 1989; Olsen, 1998). Only a limited number of crustaceans are able to synthesize cholesterol by metabolic conversion of other dietary sterols, such as β-sitosterol, ergosterol, stigmasterol, desmosterol, brassicasterol and 24-methylcholesterol, to cholesterol, or through biosynthesis of cholesterol from smaller compounds such as acetate and mevalonate (D'Abramo et al., 1984; Teshima et al., 1997). Among the many sterols, only β-sitosterol was found to be as effective as cholesterol in diets

for lobster, *Homarus* sp., juveniles, when it was provided alone or in combination with cholesterol at an inclusion level of 0.6% of diet weight (D'Abramo et al., 1984).

Other than its function related to membrane permeability, fluidity and rigidity (Hac-Wydro et al., 2007), cholesterol also act as a crucial precursor for many important biologically active compounds, such as sex hormones, molting hormones, adrenal corticoids, bile acids and vitamins D (Sheen, 2000). One of the major molting hormones, ecdysteroids, are produced by conversion of dietary cholesterol to 7-dehydrocholesterol and other oxidation products (Grieneisen et al., 1993). The provision of dietary cholesterol at either excessive or inadequate levels has been shown to result in inferior survival, growth and molting frequency of many crustaceans (D'Abramo et al., 1984; Chen and Jenn, 1991; Sheen et al., 1994b; Duerr and Walsh, 1996; Sheen, 2000; Smith et al., 2001; Hernandez et al., 2004; Holme et al., 2006). For example, in redclaw crayfish, *Cherax quadricarinatus*, juveniles, tested with diets containing different cholesterol levels from 0 to 1.0% at 0.25% increments, showed the highest weight gain and best feed conversion ratios were achieved at a level of 0.5% (Hernandez et al., 2004).

**Table 1.4** Optimal levels of dietary cholesterol for juveniles of various crustacean species.

| Species | Cholesterol Level (%) | Author |
|---|---|---|
| *Homarus* sp. | 0.2-0.6 | D' Abramo (1984) |
| *Pacisfastacus leniuculus* | 0.4-1.0 | D'Abramo (1985) |
| *Macrobrachium rosenbergii* | No specific requirement | Briggs et al. (1988) |
| *Penaeus penicillatus* | 0.5-1.0 | Chen and Jenn (1991) |
| *Penaeus monodon* | 0.2-0.8 | Chen (1993) ; Sheen et al. (1994b) |
| *Cherax quadricarinatus* | 0.5 | Hernandez et al. (2004) |
| *Scylla serrata* | 0.51 | Sheen (2000) |
| *Marsupenaeus japonicus* | 0.1-1.4 | Shudo et al. (1971) |

Various optimal levels of dietary cholesterol reported in different studies might also reflect compositions variability of other ingredients in the diet (New, 1976). Cholesterol and other sterols may be indirectly introduced from 'non-cholesterol' ingredients (e.g. fish meal, fish oil) that could subsequently lead to changes in total dietary cholesterol content in experimental diets (Gong et al., 2000b). For instance, the highest weight gain of the banana shrimp, *Penaeus merguiensis*, was obtained when fed with a cholesterol free diet; however, the basal diet used already contained 0.6% sterols, and the further addition of dietary cholesterol only served to decrease growth (Thongrod and Boonyaratpalin, 1998). New (1976) pointed out that as crustaceans needs only a small amount of dietary cholesterol, optimal requirements could be easily achieved in multi-ingredient diets.

*1.5.5 Interaction between dietary lipid constituents*

Often varied results and difficulties in understanding lipid nutrition of crustaceans could partially be attributed to complex function of lipids as they did not act individually but

also interact and compensate for each other (Sargent et al., 1999). The following section will highlight key interactions between phospholipid and other lipid classes as well as interactions among essential fatty acids.

1.5.5.1 Phospholipid; transporter and emulsifier

Phospholipid (PL) plays important roles in lipid mobilization and is believed that PL facilitate transport of cholesterol and triglyceride by means of lipoproteins (Coutteau et al., 1997; Gong et al., 2000b). Lipoproteins, a combined form of apoproteins and PL, export absorbed lipids from the gut epithelium into the hemolymph and carry it to various tissues and organs (Teshima and Kanazawa, 1980; Coutteau et al., 1997; Yepiz-Plascencia et al., 2000). This mechanism was first suggested in a study with *M. japonicus* juveniles where cholesterol was found to be retained longer in the midgut glands and was slowly incorporated into hemolymph when fed PL deficient diets. The addition of 3% soybean lecithin (crude PL) accelerated the occurrence of radioactive cholesterol and cholesteryl esters within the hemolymph (Teshima and Kanazawa, 1986). The interactive effects of cholesterol and PL have also been reflected in nutritional studies where dietary cholesterol and PL levels were manipulated. For example, Pacific white shrimp, *L. vannamei*, juveniles, showed maximal growth when fed a diet containing 5% PL, however, when the diet was supplemented with 0.4% cholesterol, 3% PL was sufficient to achieve a similar result (Gong et al., 2000b). A similar compensative effect has also been observed in mud crab, *S. serrata*, megalopae (Holme et al., 2007a). In contrast, studies with *P. penicillatus, M. rosenbergii* and *H. americanus*, have reported that both PL and cholesterol are vital for crustaceans grow but no

significant interactive effects were observed (Kean et al., 1985; Briggs et al., 1988; Chen and Jenn, 1991).

Phospholipid has also been shown to improve digestion and utilization of neutral lipids, thus leading to increase growth rates (Kanazawa et al., 1985; Coutteau et al., 1996; Gong et al., 2001; Vasagam et al., 2005). For example, when soybean lecithin (crude PL) was present in diets that contained fish oil, coconut oil and palm oil, the apparent crude lipid digestibilities of the tiger prawn, *P. monodon,* showed marked increases (Vasagam et al., 2005). In addition, a study on black tiger prawn, *P. monodon,* juveniles, showed that dietary PL at 2.7% were optimal for the digestion of dietary neutral lipid at 8.5% (Glencross et al., 1998). The increase in assimilation and solubilisation of fatty acids has been suggested to be due to increased levels of acylsarcosyltaurine, an internal emulsifier secreted when dietary PL are fed to crustaceans (Lester et al., 1975).

1.5.5.2 Essential fatty acids and their ratios

Because of the different nutritional values of essential fatty acids (EFA), it has been proposed that the requirements for these should be considered simultaneously rather than only on each individual basis (Sargent et al., 1999). For example, an appropriate ratio of n-3 to n-6 fatty acids has been suggested to be very important for the survival and growth of crustaceans (D'Abramo and Sheen, 1993; Glencross et al., 2002d). As some crustaceans showed preferences for dietary n-6 fatty acids (Read, 1981; Reigh and Stickney, 1989; D'Abramo and Sheen, 1993) over n-3 fatty acids or vice verse (Xu et al., 1993; Xu et al., 1994; Merican and Shim, 1996; Lim et al., 1997; Gonzalez-Felix et al., 2003b; Sheen and

Wu, 2003), it is crucial to balance their ratio according to species-specific requirements. The n-3 and n-6 ratio is also a major consideration in studies attempting to replace dietary marine oils with plant oils since the former generally contain higher levels of n-3 fatty acids while the later contain higher levels of n-6 fatty acids (Merican and Shim, 1995; Zhou et al., 2007). The partial replacement of fish oil with plant oil could lead to detrimental effects (Bell et al., 2001; Kamarudin and Roustaian, 2002; Vasagam et al., 2005; Holme et al., 2007b; Piedecausa et al., 2007), however, very few publications in the area address the overall optimal ratio of n-3 to n-6 fatty acids, from both plant and fish oils. In the tiger prawn, *P. monodon* juveniles, it was suggested that the optimal ratio of dietary n-3 and n-6 fatty acids is about 2.5 to 1 (Glencross et al., 2002d).

In a study on dietary ratio of n-3 and n-6 PUFA with tiger prawn, *P. monodon*, juveniles, it was found that in the absence of LNA (n-3 PUFA), the growth improved dramatically when LOA (n-6 PUFA) was supplied at a level of 21% of total dietary fatty acids. However, when dietary LNA was available, interactive effects were detected and the same level of dietary LOA was not as beneficial. Furthermore, the best growth was achieved when LOA was supplemented at a lower level than LNA at 2:3 ratio in the diet (Glencross and Smith, 1999). There were also interactive effects suggested to be due to competition between n-3 HUFA (EPA, DHA) and n-6 HUFA (ARA) embedded within tissue phospholipid for the cylo-oxygenase and lipoxygenases cycles. These cycles produce a series of prostanoids and leukotrienes which are related to eicosanoid formation and action (Sargent et al., 1999). The competitive interactions of eicosanoids is known to be more apparent in fish, such as fingerlings of gilthead bream, *Sparus aurata* (Fountoulaki et al., 2003) and

juvenile turbot, *Scophthalmus maximus* (Henderson et al., 1985). However, as eicosanoids have been suggested to play important roles in the molting process of crustaceans (Koskela et al., 1992), the ratio of dietary n-3 to n-6 HUFA may have more significant impacts than the similar ratio for PUFA in crustaceans. In addition, it has also been suggested that the capacity of essential fatty acids (i.e. LNA, LOA, EPA, DHA and ARA) to interact may increase as the carbon chain length and level of unsaturation increase (Glencross et al., 2002d).

Another important dietary fatty acid ratio is within the n-3 HUFA family, i.e. DHA to EPA ratio. There are relatively few studies on optimal dietary n-3 HUFA ratio for crustaceans and much of knowledge comes from fish nutritional studies (Rodríguez et al., 1997; Rodríguez et al., 1998; Glencross et al., 2002a; Ando et al., 2004; Sui et al., 2007). In black tiger prawn, *P. monodon*, juveniles, adverse effects on weight gain were observed when the ratio was imbalanced (Glencross et al., 2002c). It was showed that the best growth rates were obtained when the same amount of dietary EPA and DHA were supplied (1.5g $kg^{-1}$) along with 6.5 g $kg^{-1}$ LOA and 7.5 g $kg^{-1}$ LNA. In Chinese mitten crab, *E. sinensis*, larvae, however, it was found that a higher DHA:EPA ratio in enriched dietary *Artemia* improved survival and dry weight of the larvae, indicating a greater importance of DHA to EPA (Sui et al., 2007).

### 1.6. Conclusions

Despite the abundance of literature on lipid nutrition of crustaceans, the vast majority relates to penaeids. Our knowledge of lipid requirements of *P. pelagicus* is almost nonexistent and this is a major bottleneck in developing species-specific diets for *P.*

*pelagicus* to support the development of the *P. pelagicus* aquaculture industry. This present PhD study aimed at addressing this bottleneck and the results described within this thesis will provide a basis for the development of nutritionally optimized diets for this species.

### 1.7 Aims of study

The overall objective of this PhD project is to quantify the optimal dietary levels of lipids and their constituents that are needed for early juveniles of *P. pelagicus.* A series of experiments were designed and carried out using mostly a quantitative approach and practical ingredients in order to maximize implementation potentials in aquafeed industry. The specific aims of this study were;

1. Review information available on lipids nutrition of crustaceans, with particular focus on juveniles to provide knowledge basis for subsequent lipid nutrition studies on early *P. pelagicus* juveniles (Chapter 1).
2. To assess the effect of starvation and re-feeding on *P. pelagicus* early juveniles and their effects on fatty acid compositions (Chapter 2).
3. To establish dietary cholesterol requirements for *P. pelagicus* early juveniles (Chapter 3).
4. To determine the optimal level of dietary phospholipid for *P. pelagicus* early juveniles (Chapter 4).
5. To evaluate potential interactions of dietary phospholipid and cholesterol for *P. pelagicus* early juveniles (Chapter 5).

6. To determine the optimal level of dietary triglyceride at a fixed fish oil to plant ratio for *P. pelagicus* early juveniles (Chapter 6).
7. To evaluated the possibility of reducing the used of fish oil by totally and partial replacing fish oil with soybean oil in the diets of *P. pelagicus* early juveniles (Chapter 7).
8. To assess the effect of supplementing n-6 HUFA, arachidonic acid (20:4n-6), in diet of *P. pelagicus* early juveniles (Chapter 8)
9. To develop a better understanding on the effect of different ratio of polar lipids (phospholipid) to neutral lipids (triglyceride) on *P. pelagicus* early juveniles (Chapter 9).
10. Summary, overall discussion on the results and future directions (Chapter 10).

# CHAPTER 2

## Survival, development, growth and fatty acid compositions of *Portunus pelagicus* early juveniles, subject to starvation and re-feeding

---

### 2.1 Introduction

It is common to discontinue feeding in aquaculture practices whenever there are issues such as suboptimal water quality and disease, or prior to extended transportation (Wu and Dong, 2002a). Full recovery of the animal following starvation occurs once feeding recommences as long as the point-of-no-return (PNR) is not passed. PNR is defined as the point when the ability of an animal to recover from starvation is lost, eventually leading to the death despite a return to normal feeding (Anger, 2001). The ability of animals to recover from periods of starvation, and in many cases, showing compensatory growth following re-feeding, is viewed as a beneficial characteristic in aquaculture. Compensatory growth is defined as a phase of accelerated growth, commonly seen when favorable conditions are restored after a period of growth depression (Ali et al., 2003). In fish culture, after a short period of starvation, subsequent feeding has been shown to lead to increase growth rates, better food conversion and better fillet yield which is advantageous from an economic perspective (Heide et al., 2006). While most reports on compensatory growth concern fish, compensatory growth has also been reported for some crustaceans (Bosworth and Wolters, 1995; Singh and Balange, 2007; Li et al., 2009; Zhang et al., 2009). For crustaceans, periods of fasting occur naturally prior to molting or spawning (Sanchez-Paz et al., 2006). The extent of compensatory growth in crustaceans has been further reported to vary depending on a

number of factors, including the duration of food deprivation, the amount of food supplied during re-alimentation and culture temperature (Wu et al., 2001; Wu and Dong, 2002b; Singh and Balange, 2007). In addition, starvation experiments have often been conducted to assess the effects of food deprivation on nutrient utilization in crustaceans (Barclay et al., 1983; Clifford and Brick, 1983; Dall and Smith, 1986; Wen et al., 2006). Changes in tissue compositions during periods of food deprivation provide important information about the various roles of specific tissue components (Limbourn et al., 2008).

Lipids are an important source of energy, precursors of molting hormones and structural components of cell membranes for crustaceans (Lim et al., 1997; Oliva-Teres, 2000). They are also known to play an important role in sustaining survival of crustaceans during starvation (Stuck et al., 1996; Ritar et al., 2003; Limbourn et al., 2008; Holme et al., 2009). For juvenile crustaceans, the continuous supply of dietary lipids is likely to be more critical than for adults because of their substantially higher molting frequency and growth rate, but lower capacity for lipid storage. Hence starvation is likely to exert more severe and significant impacts. For example, in Chinese mitten crab, *Eriocheir sinensis*, juveniles, the molting rate as well as hepatopancreatic lipid content was reported to drop significantly with increased starvation period (Wen et al., 2006). In addition, tissue levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) such as myristic (14:0) and oleic acid (18:1n-9) were depleted during period of starvation while ecosapentaenoic acid, (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), the highly unsaturated fatty acids (HUFA) were increased or retained in tissues and hepatopancreas of the crab (Wen et al., 2006). In contrast, EPA and DHA in the digestive gland and abdominal muscle of brown

tiger prawn, *Penaeus esculentus*, were reported to decline more than 50% following starvation for 21 days (Dall et al., 1992). Therefore, this experiment was conducted to gain insight into the tolerance to food deprivation, the potential for compensatory growth following re-feeding and to better understand fatty acids metabolism of first stage *P. pelagicus* juvenile during starvation.

## 2.2 Materials and methods

### *2.2.1 Source of crabs*

Broodstock crabs were caught from estuarine areas of Ross River in Townsville, north Queensland, Australia using baited pots. They were transported to a laboratory in the Marine Aquaculture Research and Facility Unit (MARFU) at James Cook University within a few hours of collection. The crabs were placed in a quarantine bath of seawater containing formalin at a concentration of 75 µL $L^{-1}$ for 6 h. The crabs were then transferred to, and maintained in, 1000 L outdoor tanks with re-circulating sea water supply equipped with filtered and UV irradiated seawater. The holding tanks had a sandy bottom and PVC pipe or half clay pots were provided as shelters. Broodstock crabs were fed a diet of squid, prawn and mussel once daily at a rate of approximately 5-8% body weight and their copulation behavior and spawning were checked regularly. Berried females were retained in outdoor holding tanks until eggs had developed eyespots when they were again disinfected in a seawater and formalin bath (at a lower concentration of 50 µL $L^{-1}$) before being relocated to indoor 300 L hatching tanks. The indoor hatching tanks were supplied with 1 µm filtered and UV treated re-circulating seawater at a rate of approximately 1.5 L $min^{-1}$. Water temperature and salinity were maintained between 26-29$^{o}$C and 30-35‰, respectively. The berried

females were not fed once transferred to hatching tanks and the tanks were siphoned every morning to remove feces and any discarded eggs.

On the day of hatching, active newly hatched larvae were collected and transferred to flat bottomed, 300 L indoor tanks. They were cultured on the basis of a protocol developed previously in this laboratory (Castine et al., 2008). Briefly, newly stocked larvae were fed rotifers, *Brachionus* sp., at a density of 20 to 40 individuals $mL^{-1}$, which is maintained by daily addition of microalgae, *Nannochloropsis* sp.. Newly hatched *Artemia* nauplii were introduced to larval culture tanks at a density 0.5 individuals $mL^{-1}$ when larvae molted to zoea II stage. As larvae developed into zoea III, enriched *Artemia* metanauplii (enriched with Selco DC DHA, INVE) were added daily at a density of 1-2 individuals $mL^{-1}$ and this was gradual increased to 2-4 individuals $mL^{-1}$ toward the end of the larval culture phase. During the first 3 days of culture, new seawater was added to the tanks at about 10 to 15% of tanks volume daily and on day 4, water exchange began. Water exchange was initially 10 to 20% of the tank volume but increased to 30 to 50% towards the end of larvae culture and all tanks were siphoned daily. The culture water was treated with antibiotic (10 mg $L^{-1}$ Streptomycin sulfate, Sigma Aldrich, S6501) once, upon initial stocking, and temperature was maintained at 28 ± $1^{o}$C throughout. Salinity was initially set at 25‰ but gradually increased to 28‰ during larval development.

*2.2.2 Experimental design and set-up*

Towards the end of larval culture, when mass metamorphosis from megalopae to the first stage crabs (C1) was expected to occur on the following day (i.e. 4$^{th}$ day of megalopae stage, when megalopae settled on the tank bottom), the tanks were meticulously searched for C1 crabs (normally in small numbers) which were removed from the tanks. This was done to ensure that all crabs used for the experiment were newly settled within 12 h. In the morning of the following day, C1 crabs that molted overnight (average wet weight; 6.34 ± 0.56 mg, carapace length and width; 2.03 ± 0.29 mm and 3.08 ± 0.17 mm, respectively) were collected with a strainer and briefly rinsed with seawater before being placed individually into 750 mL circular culture vessels. Because of the cannibalistic nature of *P. pelagicus,* it was necessary to keep all experimental crabs separately to avoid any compounding effects of cannibalism.

Three treatments tested as follows;

1. Continuous feeding (F) as a control (189 crabs were cultured until C3 stage);
2. Crabs starved for first 7 days and the re-fed (S-F) (262 crabs were cultured until C3 stage); and
3. Continuous starvation (S) (205 crabs were cultured until 10$^{th}$ day).

The uneven numbers of crabs used for each treatment was based on sampling requirements. Throughout the experiment, 100% water changes were performed each morning for each of the 656 replicate culture units when mortality and molts were recorded. Crabs in treatment F and treatment S-F (after day 7) were fed to satiation with commercial prawn pellets designed for juvenile *Penaeus monodon* (Ridley Aquafeed ® Starter Crumble #1; 43% crude protein, 6% crude fat and 3% crude fiber). Any crabs that had molted

overnight during daily checking were sampled for weight and carapace measurement. The body weight and carapace length/width of the crabs was measured using a digital balance (Sartorius; 0.0001 g) and image managing software (Leica IM50 V.120), respectively. For crabs from the continuous starvation (S) treatment, where no molts occurred, samples were taken on the 3$^{rd}$, 7$^{th}$ and 10$^{th}$ days of starvation for measurement of wet weight, carapace length and fatty acid compositions. Due to high mortality, the continuous starvation treatment had to be terminated on day 10 when all remaining crabs were sampled for measurement and analysis. For the continuous feeding (F) and starvation and re-fed (S-F) treatments, the experiment was run until all crabs had either died or had molted to the C3 juvenile crab stages (underwent two molts). Throughout the experiment, water temperature was kept at 26.5 ± 1.5$^{o}$C, salinity at 30 ± 2‰ and pH between 7.5 to 8.1, while photoperiod was set at 14:10 h (light: dark).

*2.2.3 Fatty acid analysis*

Crabs sampled for fatty acid analysis were washed briefly with distilled water to remove salt. They were then snap frozen using liquid nitrogen and stored at -80$^{o}$C (to prevent fatty acids degradation) until analysis. Lipid was first extracted from whole body of early *P. pelagicus* using chloroform and methanol, and lipid content was determined gravimetrically by drying the extraction for 4 hours at 80$^{o}$C to remove solvent (Folch et al., 1957). The lipid was transesterified to fatty acids methyl esters (FAME) using 14% boron trifluoride-methanol (Wijngaarden, 1967). FAME were separated using gas chromatograph (Agilent Technologies 6890) equipped with split injection with helium as carrier gas, flame ionization detector and fused silica capillary column (30 mm x 0.25 mm and 0.25 µm coating) (Agilent

Technologies, USA). Column oven temperature was set to plateau at 210°C with a starting point of 140°C for 5 minutes and further increases of at 3°C minute$^{-1}$. The peak areas of FAME were identified by comparing their retention times with genuine sample standards (Sigma-Aldrich Co, USA). They were then were quantified by comparison with internal standard (heneicosanoic acid).

*2.2.4 Data collection and analysis*

The intermolt period was counted from the day each crab molted to the day of the consecutive molting stage. Independent T-test was used to determine whether differences existed between dry weight, carapace measurement and intermolt period of crabs from continuously fed (F) and starved and then re-fed (S-F) treatments at either the C2 or C3 stages. One way analysis of variance (ANOVA) was used to compare the differences in variances between data pooled for crabs starved for 3, 7 and 10 days and crabs fed for 3 days, after confirmation of normality and homogeneity of variance. Log or arcsine transformation of the data was performed before further analysis when required. Crabs that were subjected to one way ANOVA were all in the same stage (C1). Significant differences were determined using Tukey's test and the least significance difference was set at $p<0.05$.

**2.3 Result**

*2.3.1 Survival, development and growth*

In the starvation treatment (S), survival of first stage (C1) crabs remained very high at 96.6% until day 7. However, mass mortality occurred over the next 3 days and on day 10, survival was only 47.9% (Table 2.1); this prompted termination of the treatment in order to

collect enough crabs for measurement, fatty acids analysis and histology. In contrast, both continuously fed crabs (F) and those starved for 7 day and then re-fed (S-F), showed excellent survival of 98.9% to the second crabs stage C2. However, survival to the C3 stage was lower (85.3%) for the S-F treatment compared to those from the F treatment (93.9%) (Table 2.1).

Compared to the continuous feeding treatment (F), seven days of starvation of C1 stage for crabs in the S-F treatment significantly affected the intermolt duration at both C1 and C2 stages (Table 2.1). Interestingly, while the mean C1 development duration of the S-F crabs were, as expected, significantly longer than that recorded for the F treatment ($p<0.05$), the mean time taken by C2 crabs from the S-F treatment (7.8 ± 1.7 days) to develop to the C3 stage was significantly shorter ($p<0.05$) than that of C2 crabs from the F treatment (9.5 ± 2.4 days) (Table 2.1).

No significant differences in dry weight were detected ($p>0.05$) among C1 crabs that were starved for up to 10 days (Table 2.1) although with the increased starvation period, a decreasing trend in dry weight was shown for the C1 crabs of the S treatment. However, for dry weight of newly molted C2 and C3 crabs, S-F crabs (C2: 1.55 ± 0.34 mg and C3: 2.51 ± 0.57 mg) showed significant lower dry weight when compared to crabs that were continuously fed (C2: 2.34 ± 0.39 mg and C3: 3.15 ± 0.30 mg) ($p<0.05$). Interestingly, carapace length and width of S-F crabs were only significantly smaller than those of F crabs at the C2 stage ($p<0.05$) but not at the C3 stage ($p>0.05$) (Table 2.1). Percentage moisture of crabs starved for more than 3 days was always above 80% while for C1 crabs fed for 3 day,

the moisture content was lower at 70.62%. However, these differences were not statistically significant ($p>0.05$). Similarly, the mean percentage water content of newly molted C2 and C3 crabs from the S-F treatment were both slightly higher than those in the F treatment and no significant differences were detected ($p<0.05$). For newly molted C2 crabs, the moisture content ranged from 81.16% to 84.47% for F and S-F crabs, respectively while for C3, it was 83.69 and 85.32%, respectively.

**Table 2.1** Survival (%) and mean (± SE) of dry weight (DW), carapace length (CL), carapace width (CW), moisture content and intermolt period of early *P. pelagicus* when starved (S), continuously fed (F) and starved and re-fed (S-F).

| | Survival (%) | DW (mg) | CL (mm) | CW (mm) | Intermolt period (days) | Moisture content (%) |
|---|---|---|---|---|---|---|
| Day 0 | 100.0 | 1.44±0.27[a] | 2.30 ± 0.29[a] | 3.08 ± 0.16[a] | - | 76.03 ± 5.56[a] |
| S: Day 3 | 98.3 | 1.29±0.14[a] | 2.23 ± 0.07[a] | 3.17 ± 0.09[a] | - | 80.19 ± 2.45[a] |
| S: Day 7 | 96.6 | 1.07±0.09[a] | 2.18 ± 0.05[a] | 3.10 ± 0.10[a] | - | 82.45 ± 1.79[a] |
| S: Day 10 | 47.9 | 1.13±0.08[a] | 2.21 ± 0.07[a] | 3.16 ± 0.14[a] | - | 82.32 ± 1.38[a] |
| F: Day 3 | 100.0 | 1.75±0.14[a] | 2.18 ± 0.09[a] | 2.99 ± 0.13[a] | - | 70.62 ± 5.69[a] |
| F: C2 | 98.9 | 2.34±0.39[A] | 3.07 ± 0.11[A] | 5.03 ± 0.27[A] | 6.2 ± 0.9[A] | 81.16 ± 2.22[A] |
| S-F: C2 | 98.9 | 1.55±0.34[B] | 2.79 ± 0.08[B] | 4.50 ± 0.11[B] | 13.6 ± 2.4[B] | 84.47 ± 3.37[A] |
| S-F: C2 *( after fed at 7th day)* | | | | | 7.1 ± 1.2[A] | |
| F: C3 | 93.9 | 3.15±0.30[1] | 3.59 ± 0.15[1] | 6.38 ± 0.24[1] | 9.5 ± 2.4[1] | 83.69 ± 0.90[1] |
| S-F: C3 | 85.3 | 2.51±0.57[2] | 3.43 ± 0.19[1] | 5.75 ± 0.46[1] | 7.8 ± 1.7[2] | 85.32 ± 1.29[1] |

[ab AB 12] different superscripts indicate significant differences ($p<0.05$).
Note that statistical comparison was based on crabs from the same juvenile stage.

*2.3.2 Fatty acid compositions*

The effects of starvation on whole body fatty acid compositions of blue swimmer crabs are shown in Tables 2.2 and 2.3. Generally, as a proportion of total fatty acids, total saturated fatty acids (∑SFA) increased steadily from 26.29 g 100 $g^{-1}$ in newly settled C1, to 43.53 g 100 $g^{-1}$ on day 7 of starvation, and then declined back slightly to 40.38 g 100 $g^{-1}$ on day 10 of starvation (Table 2.2). The most abundant SFA over the starvation period was palmitic acid (16:0) and stearic acid (18:0). For S-F crabs, after re-feeding commenced on day 7, the proportion of total SFA decreased from 43.53 g 100 $g^{-1}$ to 34.84 g 100 $g^{-1}$ for newly molted C2 with stearic acid showing the greatest decline (Table 2.2). Total monounsaturated fatty acid (∑MUFA) on the other hand, decreased relatively slowly for the first 7 days of starvation, but dropped dramatically from 28.72 g 100 $g^{-1}$ on day 7 of starvation to only 18.50 g 100 $g^{-1}$ on day 10. Meanwhile, a steady decreased in the polyunsaturated fatty acid (∑PUFA) content of tissues was seen up to day 7 of starvation, which saw ∑PUFA drop sharply from 22.25 g 100 $g^{-1}$ to 4.73 g 100 $g^{-1}$ on day 7 of starvation; but levels stabilized afterward. Among the main PUFAs of linoleic acid (LOA; 18:2n-6), linolenic acid (LNA; 18:3n-3) and stearidonic acid (STA; 18:4n-3), LNA and STA decreased throughout the first 7 days of starvation while LOA was retained and noticeable decreases only occurred after day 3 of starvation (Table 2.3).

Of the major highly unsaturated fatty acids (HUFA), a decreasing trend was found for the proportion of eicosapentaenoic acid (EPA; 20:5n-3), particularly after day 3 of starvation. However, docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (ARA; 20:4n-6) appeared to be retained as the proportion increased over the 10 day starvation period (Table

2.3). The total proportion of HUFA (∑HUFA) also generally increased over the starvation period (Table 2.2). Comparing newly molted C2 and C3 crabs from the S-F and F treatments, the 7 days of starvation had limited effects on their fatty acid profiles as they are generally similar, particularly for the new molted C3 crabs (Table 2.3). This suggests that the subsequent period of re-feeding had very much made up the differences resulting from starvation.

**Table 2.2** Fatty acid groups (g 100 $g^{-1}$) of whole *P. pelagicus* from starved (S), continuously fed (F) and starved and re-fed (S-F) treatments.

| | ∑SA | ∑MUFA | ∑PUFA | ∑HUFA |
|---|---|---|---|---|
| In: Day 0 | 26.29 | 30.92 | 22.25 | 19.64 |
| S: Day 3 | 31.60 | 26.64 | 15.34 | 26.60 |
| S: Day 7 | 43.53 | 28.72 | 4.73 | 23.00 |
| S: Day 10 | 40.38 | 18.50 | 5.63 | 35.57 |
| F: Day 3 | 30.99 | 26.50 | 14.64 | 27.49 |
| F: C2 | 34.84 | 23.60 | 9.47 | 30.31 |
| S-F: C2 | 33.51 | 18.54 | 11.12 | 37.30 |
| F: C3 | 32.49 | 16.68 | 9.66 | 41.20 |
| S-F: C3 | 32.22 | 15.88 | 10.84 | 42.60 |

**Table 2.3** Fatty acid profiles (g 100 $g^{-1}$) of whole *P. pelagicus* from starved (S), continuously fed (F) and starved and re-fed (S-F) treatments.

| Major fatty acids | Day 0 | S: Day 3 | S: Day 7 | S: Day 10 | F: Day 3 | F: C2 | S-F: C2 | F: C3 | S-F: C3 |
|---|---|---|---|---|---|---|---|---|---|
| 14:0 | 1.07 | 1.17 | 0.74 | 1.66 | 1.48 | 0.97 | 0.80 | 1.21 | 0.84 |
| 15:0 | 0.39 | 0.57 | 0.36 | 0.81 | 0.54 | 0.45 | 0.51 | 0.56 | 0.54 |
| 16:0 | 14.12 | 16.42 | 21.23 | 19.76 | 17.94 | 18.90 | 17.64 | 18.88 | 17.52 |
| 16:1n-7 | 2.32 | 1.18 | 0.66 | 0.95 | 1.66 | 0.93 | 0.61 | 0.91 | 0.50 |
| 17:0 | 1.16 | 1.24 | 0.82 | 1.79 | 1.04 | 1.00 | 1.07 | 1.04 | 1.02 |
| 18:0 | 8.70 | 10.92 | 19.52 | 16.36 | 8.96 | 12.88 | 12.47 | 11.81 | 12.31 |
| 18:1n-9 | 21.05 | 18.69 | 20.72 | 11.27 | 17.10 | 15.46 | 10.42 | 12.37 | 10.42 |
| 18:1n-7 | 6.11 | 5.20 | 4.22 | 3.69 | 4.98 | 4.58 | 4.01 | 3.62 | 3.43 |
| 18:2n-6 | 7.26 | 7.55 | 3.94 | 3.59 | 9.29 | 7.46 | 8.79 | 10.04 | 10.11 |
| 18:3n-3 | 13.57 | 7.41 | 0.79 | 2.04 | 4.98 | 2.01 | 0.87 | 1.08 | 0.73 |
| 18:4n-3 | 1.43 | 0.38 | - | - | 0.37 | - | - | - | - |
| 20:0 | 0.31 | 0.37 | 0.32 | - | 0.31 | 0.30 | - | - | - |
| 20:1n-9 | 1.05 | 1.09 | 1.62 | 1.13 | 1.26 | 1.40 | 1.17 | 1.18 | 1.10 |
| 20:1n-7 | 0.19 | - | 0.38 | - | 0.29 | 0.31 | - | - | - |
| 20:2n-6 | 0.91 | 1.31 | 1.14 | 1.53 | 1.37 | 1.78 | 2.37 | 2.14 | 2.63 |
| 20:4n-6 | 1.62 | 2.17 | 4.12 | 3.31 | 1.72 | 2.65 | 2.56 | 2.29 | 2.75 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 20:3n-3 | 1.86 | 2.15 | 0.77 | 2.02 | 1.57 | 1.19 | 0.86 | 1.04 | 0.69 |
| 20:5n-3 | 6.72 | 9.70 | 4.09 | 1.60 | 9.91 | 10.86 | 17.24 | 14.60 | 16.30 |
| 22:0 | 0.54 | 0.58 | 0.29 | - | 0.46 | 0.34 | - | - | - |
| 22:1n-9 | - | 0.47 | 0.78 | 1.46 | 0.63 | 0.65 | 0.46 | 0.47 | 0.43 |
| 22:4n-6 | - | - | 1.70 | - | - | 0.58 | - | - | - |
| 22:5n-6 | 0.50 | 0.46 | 0.30 | - | 0.46 | 0.40 | 0.39 | 0.39 | 0.41 |
| 24:0 | - | 0.34 | 0.25 | - | 0.25 | - | - | - | - |
| 22:5n-3 | 0.66 | 0.55 | 0.93 | - | 0.90 | 0.88 | 0.83 | 0.89 | 0.81 |
| 22:6n-3 | 8.28 | 10.07 | 9.98 | 12.62 | 11.93 | 13.75 | 16.93 | 15.47 | 17.46 |
| n3/n6 | 3.3 | 2.7 | 1.5 | 2.3 | 2.3 | 2.2 | 2.7 | 2.2 | 2.3 |
| DHA/EPA | 1.2 | 1.0 | 2.4 | 7.9 | 1.2 | 1.3 | 1.1 | 0.98 | 1.1 |

## 2.4 Discussion

Newly settled first stage *P. pelagicus* appear to have high tolerant to starvation as their survival remained above 95% on day 8 of starvation, considering their small size with average wet weight of 6.34 mg, and mean carapace length and widths of 2.30 mm and 3.08 mm, respectively. The point of no return at 52% mortality ($PNR_{52}$) for early *P. pelagicus* was estimated to occur on day 10 of starvation. PNR is a critical point for the measurement of animal ability to withstand starvation and is usually marked by the time that 50% of starved animals would survive (i.e. $PNR_{50}$) when being re-fed (Anger, 2001; Limbourn et al., 2008). In other decapoda crustaceans, $PNR_{50}$ has been determined experimentally for substantially larger juveniles, for example, for 750 mg (wet weight) juveniles of the Chinese prawn, *Fenneropenaeus chinensis,* $PNR_{50}$ was 7.86 days (Zhang et al., 2009) and for 1070 mg (wet weight) juvenile of Pacific white shrimp, *Litopenaeus vannamei*, juveniles $PNR_{55}$ was 15 days (Comoglio et al., 2004).

In this experiment, no molting was recorded and no significant reduction in dry weight was found during 10 days of starvation. Similarly, no significant weight losses were reported for juvenile penaeids (*L. vannamei* and *F. chinensis*) during starvation experiments (Comoglio et al., 2004; Zhang et al., 2009). During starvation, tissue reserves that are catabolised to provide energy are suggested to be replaced with water to retain their 'necessary body volume and internal turgidity' (Dall, 1974); this is especially important for crustacean whose volume is fixed by their rigid exoskeleton during intermolt (Barclay et al., 1983; Stuck et al., 1996). For example, Chinese mitten crab, *E. chinensis,* adults lost more than 58% of their body weight after 70 days of starvation and this was accompanied by

significant increase in moisture content (Wen et al., 2006). However, in the present study, although dry weight of the first stage crabs showed a clear decline with concomitant increase in moisture content during starvation, no significant differences were detected. This is probably due to the high proportion of the dry weight contributed by inorganic components (calcium and chitin) of the carapace and exoskeleton of the crabs which were not affected by starvation.

The C1 crabs re-fed after 7 days of starvation had significantly lower dry weight, carapace length and carapace width as newly molted C2 when compared to C2 crabs from the normally fed (F) treatment. However, for the newly molted C3 crabs, significant differences were only detected for dry weight and the relative difference in dry weight between treatments was substantially reduced at this stage. It appears likely that a longer post-starvation feeding period could fully compensate growth as has been reported for *F. chinensis* (Wu et al., 2001; Wu and Dong, 2002b).

Interestingly, although the molting cycle appeared suspended during continuous starvation as none of C1 crabs from the starvation treatment molted to C2, subsequent re-feeding of starved crabs on day 7 resulted in very high survival (98.9%) to the C2 stage, which was the same as C1 crabs that were continuously fed. Furthermore, the mean feeding duration required for these crabs to molt to C2 (i.e. from the time when re-feeding started to C2) was not significantly different from that of crabs that had been continuously fed (F). Similarly, Abrunhosa and Kittaka (1997) also reported that starved and re-fed larvae of lobsters, *Jasus verreauxi* and *Homarus americanus,* had similar intermolt periods to those

that were continually fed when they were subjected to shorter period of starvation of 3 to 4 days (Abruhonsa and Kittaka, 1997). More intriguingly, the intermolt period for the next stage (from C2 to C3) was significantly shorter for the re-fed crabs when compared to that of crabs that had been fed continuously. This indicates a clear preference for development (i.e. molting) over growth for these crabs. This interesting phenomenon warrant further research on its underlining mechanisms.

In general, as a percentage of total fatty acid (g 100 $g^{-1}$), most HUFA, including DHA and ARA, remained relatively stable or substantially increased during starvation. The relative high retention of these essential fatty acids suggests important biological functions. Similar reports on relative conservation of essential fatty acids during starvation have been reported in other crustaceans e.g. phyllosoma of *J. edwardsii* and *P. cygnus* and juvenile of *E. chinensis* (Ritar et al., 2003; Liddy et al., 2004; Wen et al., 2006). After being re-fed following 7 days of starvation, the fatty acid profiles of newly molted C2 and particularly C3 *P. pelagicus* from the S-F treatment, largely recovered as they were similar to those of same stage crabs from the continuously fed treatment. This suggests that a relatively short period of re-feeding could quickly reverse the changes resulting from starvation in young *P. pelagicus.*

# CHAPTER 3

## Survival, development and growth of *Portunus pelagicus* early juveniles, fed diets containing various levels of cholesterol

---

### 3.1 Introduction

Cholesterol is found abundantly throughout cellular membranes of animals and it functions to assist in membrane permeability, fluidity and rigidity (Hac-Wydro et al., 2007). For crustaceans, cholesterol also act as a crucial precursor for many important biologically active compounds such as sex hormones, molting hormones, adrenal corticoids, bile acids and vitamins D (Sheen, 2000). However, crustaceans species are generally incapable of biosynthesizing cholesterol either from simple compounds such as acetate and mevalonate (Teshima et al., 1997), or from metabolical conversion of other sterols e.g. β-sitosterol, stigmasterol, band 24-methylcholesterol to cholesterol (D'Abramo et al., 1984). This is because, unlike mammals, most crustacean may not posses one or more of the enzymes, such as squalene synthase/monooxygenase, HMG-CoA synthase reductase and lanosterol synthase/dimethylase that are required to synthesize cholesterol (Brown and Goldstein, 1978). Hence, dietary supply of cholesterol is crucial for the wellbeing of crustaceans, particularly for optimal survival and growth of commercially cultured species. As a consequence, research over past decades has been conducted on various decapod crustaceans to investigate their dietary cholesterol requirements.

The results of previous research have shown that the optimal level of dietary cholesterol for crustaceans varies among species and according to their age and developmental stage. For example, the acceptable or optimal dietary cholesterol level was reported to be from 0.1% to 1.4% for juvenile Kuruma prawn, *Marsupenaeus japonicus* (Shudo et al., 1971), 0.2% to 0.8% for juvenile black tiger prawn, *Penaeus monodon* (Sheen et al., 1994b), 0.19% to 0.59% for juvenile clawed lobster, *Homarus* sp. (D'Abramo et al., 1984), 0.5% for juvenile redclaw crayfish, *Cherax quadricarinatus* (Hernandez et al., 2004) and 0.51% and 0.8% for juvenile (Sheen, 2000) and megalopae larvae (Holme et al., 2006) of the mud crab, *Scylla serrata*, respectively. However it is worth noting that the cholesterol requirements identified in these studies are not necessarily absolute requirements but often the level required to supplement existing dietary cholesterol in other diet ingredients, such as fish meal and fish oil. In juveniles of the banana shrimp, *Penaeus merguiensis*, Thongrod and Boonyaratpalin (1998) found that 0.6% of the sterols contained in the basal diet were sufficient for optimal growth of the prawn. In addition, mastication of the diet by crustaceans may also resulting in loss of nutrients (Teshima et al., 1997) and this can lead to over-estimation of optimal dietary cholesterol levels reported in the above studies.

Information on optimal dietary cholesterol level for a crustacean is crucial in aquaculture as negative growth responses have been observed when dietary cholesterol level was inadequate or excessive. For example, for juvenile redclaw crayfish, *C. quadricarinatus*, fed diets with cholesterol levels of 0, 0.25, 0.5, 0.75 and 1.0% (of the dry diet), the highest weight gain and the best food conversion ratio were found at a level of 0.5% with both higher and lower levels of inclusion resulting in significantly lower weight gain (Hernandez et al.,

2004). Similar results have also been demonstrated for juveniles and megalopae of the mud crab, *S. serrata* (Sheen, 2000; Holme et al., 2006), sub-adults of the black tiger prawn, *P. monodon* (Smith et al., 2001) and juvenile of Pacific white shrimp, *Litopenaeus vannamei* (Duerr and Walsh, 1996). Moreover, suboptimal and excessive dietary cholesterol fed to crustaceans have also been shown to cause inferior survival (D'Abramo et al., 1984; Chen and Jenn, 1991; Sheen, 2000).

Crustaceans need to shed their exoskeleton i.e. molt, to be able to grow. When diets deficient in cholesterol were fed to juvenile mud crab, *S. serrata* (Sheen, 2000) and juvenile freshwater crayfish, *Pascifastacus leniuculus* (D'Abramo et al., 1985), their molting frequency decreased significantly. Sheen (2000) suggested that low molting frequency may have resulted from reduced biosynthesis of ecdysone, a steroid hormones that is responsible for initiating molting in crustaceans (Koolman, 1982). Such speculation is supported by the fact that biosynthesis of ecdysone, begins with the conversion of dietary cholesterol to 7-dehydrocholesterol and other oxidation products (Grieneisen et al., 1993). Molt death-syndrome, a condition whereby crustaceans are unable to extricate themselves successfully from the old exoskeleton during the molt, has been commonly linked to inappropriate nutrition such as inappropriate levels of phospholipid that is responsible for MDS found in cultured lobsters, *Homarus* sp. (Conklin et al., 1980; Bowser and Rosemark, 1981; Baum et al., 1990). It is generally accepted that phospholipids enhance the mobilization of cholesterol (Teshima and Kanazawa, 1980) and dietary cholesterol may therefore also be directly linked with molt-death syndrome in crustaceans. This study examines the effects of different levels

of dietary cholesterol on survival, development and growth of early juvenile *P. pelagicus* using iso-caloric, gelatinized semi-purified diets.

## 3.2 Materials and methods

### *3.2.1 Source of crabs*

Broodstock capture and husbandry and larval culture protocol as were described in Chapter 2 (Section 2.2.1).

### *3.2.2 Diet preparation*

Seven iso-caloric diets were formulated to contain cholesterol levels at 0 g $kg^{-1}$ (control), 2.5, 5.0, 7.5, 10.0, 12.5 and 15 g $kg^{-1}$ of the diet dry weight, respectively (Table 3.1). The cholesterol used in this study was obtained from Sigma Aldrich Pty-Ltd (C8267) and had purity of more than 98%; in powder form and could be readily mixed with other diet ingredients. Iso-caloricity of the diets was achieved by adjusting corn starch and cellulose contents of the diets. The energy content of the diets were calculated using standard energy equivalents of 23.01, 38.07 and 17.15 MJ $kg^{1}$ for protein, lipid and carbohydrate, respectively (Anderson and Silva, 2003).

Prior to diet preparation, fishmeal was pulverized and defatted three times by solvent extraction using chloroform and methanol (2:1, v/v) for one hour (Folch et al., 1957). All dry and wet ingredients were then weighed individually using a Sartorius TE2145 electronic balance (0.0001 g), mixed with an electric mixer before being combined together and to become a homogenous blend. Subsequently, agar, the diet binder used in this study, was

prepared by dissolving it in hot water at above 80$^{o}$C (Sheen and Wu, 1999); as the solution became clear, it was allowed to cool to 40$^{o}$C when the ingredient mixture was added slowly to the agar solution and thoroughly mixed. The agar quickly gelatinized when the temperature dropped to 37$^{o}$C. The diets were finally cut to small pieces of approximately 2 mm$^{3}$ and stored in a freezer at -20$^{o}$C freezer until used.

**Table 3.1** Formulation (g kg$^{-1}$ dry diet) of the experimental diets. The analyzed cholesterol and total lipid contents in all diets are also presented in the lower part of the Table. Diet formulation was modified from Holme (2006), Sheen and Wu (1999) and Genodepa (2004).

| Ingredient | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 | Diet 6 | Diet 7 |
|---|---|---|---|---|---|---|---|
| Fish meal (defatted)[1] | 500 | 500 | 500 | 500 | 500 | 500 | 500 |
| Fish oil[2a]:corn oil[2b]; 2:1 | 60 | 60 | 60 | 60 | 60 | 60 | 60 |
| Cholesterol[2c] | 0 | 2.5 | 5.0 | 7.5 | 10.0 | 12.5 | 15.0 |
| Phospholipid[2d] | 60 | 60 | 60 | 60 | 60 | 60 | 60 |
| Vitamin mixture[3a] | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| Mineral Mixture[3b] | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| Choline chloride[2e] | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| DCP[2f] | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Agar[2g] | 120 | 120 | 120 | 120 | 120 | 120 | 120 |
| Starch[2h] | 113 | 108 | 102 | 97 | 91 | 86 | 80 |
| Cellulose[2i] | 51 | 54 | 57 | 60 | 63 | 66 | 69 |
| *Analyzed* | | | | | | | |
| Cholesterol | 0.3 | 2.6 | 4.5 | 6.7 | 10.0 | 11.7 | 14.3 |
| Total Lipid | 134 | 131 | 145 | 137 | 147 | 151 | 151 |

[1] Skretting Tasmania

[2] Sigma-Aldrich Pty Ltd [a]from menhaden F8020 [b]C8267 [c]C8667 [d]P3644 [e]98% powder C7527 [f]dibasic calcium phosphate C4131 [g]A7002 [h]S4126 (corn) [i]C8002 alpha

[3] Rabar Pty Ltd [a]ZZ600 DPI, each 1kg contains: vitamin A 2miu, vitamin D3 0.8miu, vitamin E 40g, vitamin K 2.02g, inositol 50g, vitamin B3 30.40g, vitamin B5 9.18g, vitamin B9 2.56g, vitamin B2 4.48g, vitamin B12 0.004g, biotin 0.1g, vitamin B6 4g, vitamin B1 3.4g, vitamin C 44.4g, para amino benzoic acid 20g, tixosil 5g, antioxidant 30g [b]ZZ603 DO 067 DPI, each 1kg contains: copper 1g, cobalt 100mg, magnesium 59.4mg, manganese 5g. iodine 800mg, selenium 20mg, iron 8mg, zinc 20g, aluminium 100mg, chromium 100mg

*3.2.3 Experimental design and set-up*

Newly metamorphosed C1 crabs were obtained as described in Chapter 2 (Section 2.2.2) and were placed individually into 750 mL circular culture vessels. Thirty healthy C1 crabs with average dry weight of 0.57 ± 0.03 mg, carapace length and carapace width of 2.34 ± 0.02 and 2.87 ± 0.05 mm, respectively, were used for each diet treatment and each culture unit was regarded as a replicate, hence a total of 210 crabs were used for the experiment. The individual housing of all *P. pelagicus* juvenile eliminated compounding effects of cannibalism and allowed precise recording of consecutive intermolt periods of each crab. The culture units were randomly placed in water-bath with submerged heaters to maintain temperature at 28 ± 2$^{o}$C. A 100% water exchange was carried out daily in the morning for all culture units before feeding. This was done by transferring crabs individually to new culture units while the previous units were sanitized with hypochlorite solution and dried overnight to be used the next morning. Molting and mortality were recorded during water exchange and crabs were then fed to satiation. Salinity and pH was maintained at 30 ± 2‰ and 7.5 to 8.1, respectively, throughout the experiment while photoperiod was maintain at 14 h:10 h (light:dark) at all times.

At the end of the experiment, any crabs that had molted to the crab stage 3 were removed in the afternoon for measurement of carapace length and carapace width and dry

weight determination. Sampling of crabs in the afternoon allowed sufficient time for newly molted crabs to harden their exoskeleton even if they molted later during the night. The carapace length and width of the C3 crabs were measured using a digital caliper (Mitutoyo: 0.01 mm). The distance from the longest rostral spike to the abdominal line was designated as carapace length and the distance between the tips of two lateral spines was designated as carapace width (Romano and Zeng, 2006). Following measurement of carapace size, the crabs were rinsed with distilled water to remove salt and then dried individually in an oven at 60$^{o}$C for 24 h before being weighed on a digital balance (Sartorius; 0.0001 g) to determine dry weight. The experiment was terminated when all crabs had either molted to the C3 stage or had died.

*3.2.4 Diet cholesterol and lipid analysis*

All experimental diets were analyzed to confirm their cholesterol and total lipid contents. This was done following extraction of lipid from samples of experimental diets based on the method of Folch et al. (1957), and total lipid was determined gravimetrically from an aliquot of the extract. Cholesterol level in the diets was quantified against an internal standard, 5α-cholestane (Sigma-Aldrich Co, Milwaukee, USA).

*3.2.5 Data and statistical analysis*

To assess growth over time, specific growth rate (SGR) based on dry weight, carapace width and length of the newly molted C3 crabs from each dietary treatment were calculated using the following equation;

$$SGR = (\ln F - \ln I)/ T * 100$$

where *F* is the final weight or carapace size, *I* is the initial weight or carapace size and *T* is development time (days) taken for crabs to molt from the C1 stage to C3 stage.

Due to the highly cannibalistic nature of *P. pelagicus*, all crabs were cultured individually to avoid compounding effects of cannibalism. The workload involved in daily handling of hundreds of culture units to obtain a single survival value, made it impractical to have more replicated for statistic analysis. However, development and growth data of early juvenile crabs were analyzed using one-way ANOVA after confirmation of normality and homogeneity of variance. Log or arcsine transformation of the data was performed before further analysis whenever needed. Significant differences between treatments were determined using Tukey's test and level of significant differences was set at $p<0.05$. All statistical analysis were performed using the SPSS statistic software version 16.

## 3.3 Results

### *3.3.1 Cholesterol and total lipid contents of diets*

The analyzed cholesterol and total lipids contents in all seven diets used in this study are shown in Table 3.1. The diet without cholesterol supplementation contained a trace quantity of cholesterol (0.3 g $kg^{-1}$ of dry diet weight), which originated from other diet ingredients. Diets supplemented with 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 g $kg^{-1}$ cholesterol actually contained 2.6, 4.5, 6.7, 10.0, 11.7 and 14.3 g $kg^{-1}$ cholesterol, respectively. The

analyzed total lipid levels for all diets were within a range of 131.0 to 151.0 g $kg^{-1}$ of the dry diet weight (Table 3.1).

*3.3.2 Survival, development time and growth*

Survival of early juvenile *P. pelagicus* was relatively low for all diet treatments and ranged from 20.0 to 47.5%. The highest survival to the C3 stage was recorded for crabs fed the diet containing 10.0 g $kg^{-1}$ cholesterol while the lowest survival was recorded for the diet without added cholesterol (Fig 3.1). Survival, in general, decreased substantially as the level of dietary cholesterol reduced below or increased above 10.0 g $kg^{-1}$. The occurrence of molt-death syndrome (MDS) also appeared to increase as crabs were fed diets containing either cholesterol levels lower than 5.0 g $kg^{-1}$ or higher than 10.0 g $kg^{-1}$, with the crabs fed the highest level of dietary cholesterol (15.0 g $kg^{-1}$) recording the highest percentage of death resulting from MDS (20.7%) (Fig 3.1). Figure 3.2 (A and B) shows successfully molted *P. pelagicus* and *P. pelagicus* that died because of MDS.

No significant difference was found for mean development time from the C1 to C2 stage among all treatments ($p>0.05$) (Table 3.2). However, the mean cumulative development time from C1 to C3 were significantly shorter for *P. pelagicus* fed the diets with 2.5 and 10.0 g $kg^{-1}$ supplemental cholesterol when compared to crabs fed diet deficient in cholesterol ($p<0.05$). No significant difference in mean development time from C1 to C3 was detected among other treatments ($p>0.05$) (Table 3.2).

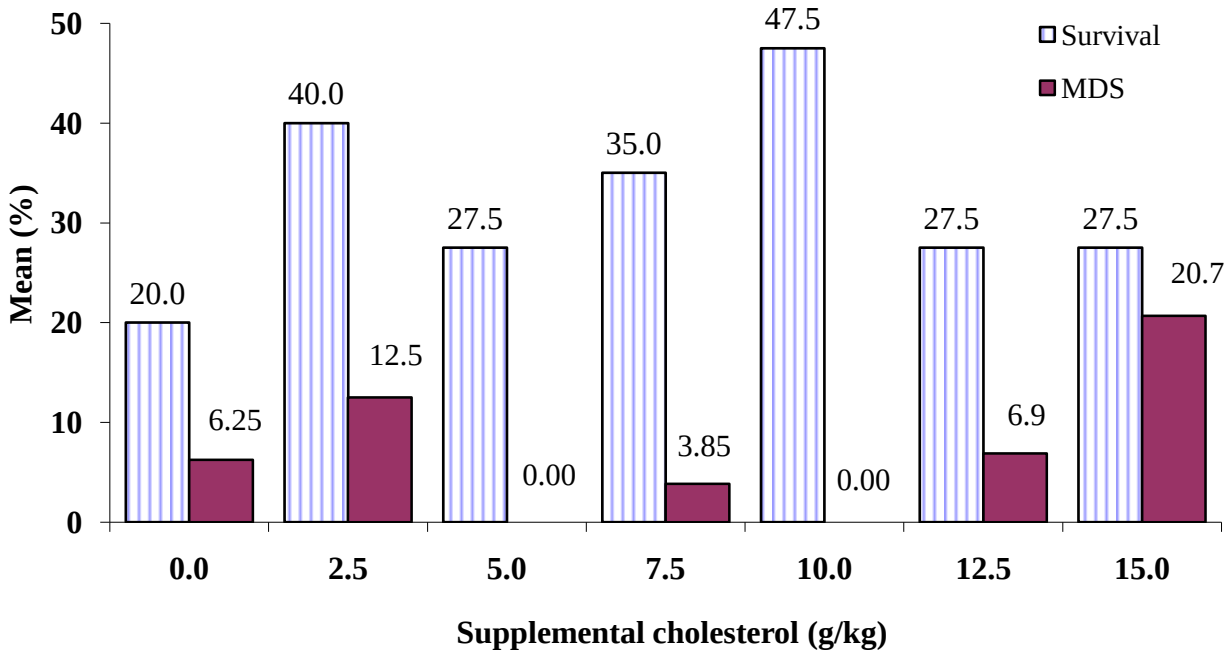
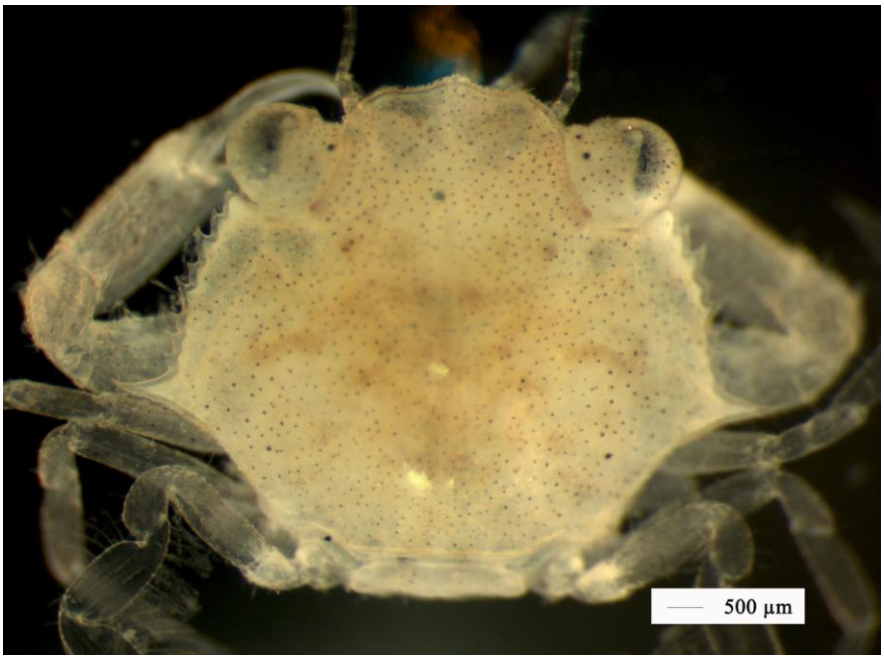



**Figure 3.1** Mean survival and mortality due to molt death syndrome (MDS) (%) of the blue swimmer crab, *Portunus pelagicus.* The crabs were fed diets containing different levels of cholesterol throughout the culture period.

A




B

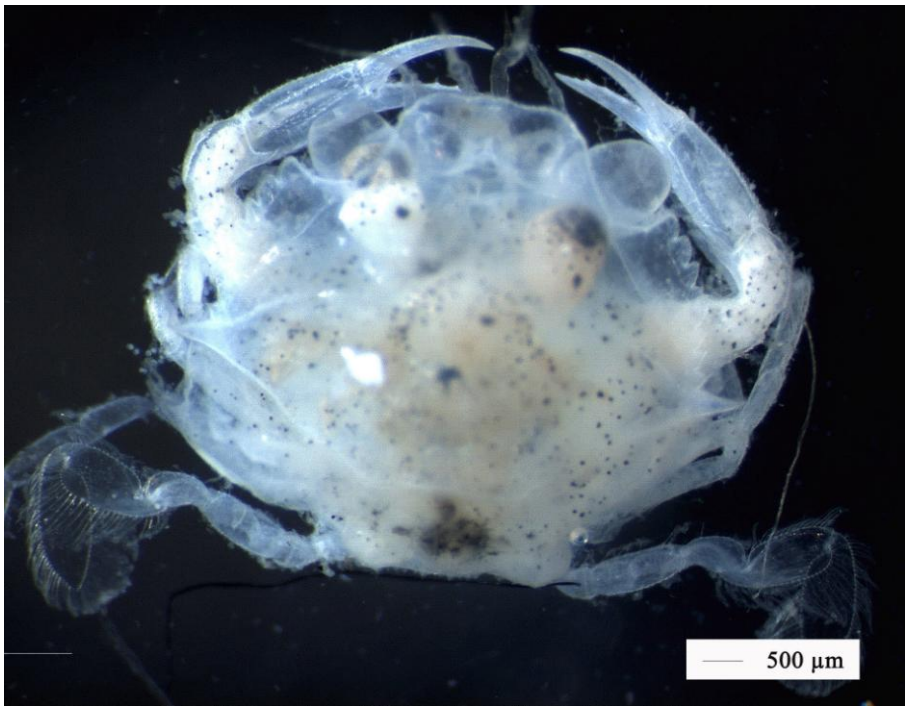



**Figure 3.2** Molted *P. pelagicus.* A. successfully molted from old carapace; and B. trapped in old carapace during molting (molt death syndrome, MDS).

**Table 3.2** The mean (± SE) intermolt period for each molt stages and cumulative development time from the C1 to C3 stage (in days) for early *Portunus pelagicus* juveniles fed semi-purified diets containing various levels of cholesterol.

| Supplemented levels (analyzed level) g $kg^{-1}$ | C1 to C2 | C2 to C3 | Cumulative development time from C1 to C3 |
|---|---|---|---|
| 0.0 (0.3) | 6.00 ± 0.36 | 9.18 ± 0.58[a] | 14.73 ± 0.83[a] |
| 2.5 (2.6) | 5.66 ± 0.29 | 7.06 ± 0.29[b] | 12.06 ± 0.48[b] |
| 5.0 (4.5) | 5.46 ± 0.25 | 8.18 ± 0.55[a] | 12.91 ± 0.73[ab] |
| 7.5 (6.7) | 4.75 ± 0.27 | 7.60 ± 0.48[a] | 11.90 ± 0.57[ab] |
| 10.0 (10.0) | 5.20 ± 0.40 | 7.42 ± 0.37[a] | 11.53 ± 0.52[b] |
| 12.5 (11.7) | 5.56 ± 0.25 | 8.36 ± 0.45[a] | 12.82 ± 0.0.55[ab] |
| 15.0 (14.3) | 5.00 ± 0.34 | 7.64 ± 0.39[a] | 12.29 ± 0.62[ab] |

[abc] Different superscripts within a column indicate significant differences ($p < 0.05$)

The dry weight and carapace size of early *P. pelagicus* juveniles were improved by increasing the level of dietary cholesterol to 10.0 g $kg^{-1}$ (Table 3.3). The highest growth, in term of dry weight, carapace width and length, were shown by crabs fed the diet supplemented with 10.0 g $kg^{-1}$ although it was not significantly different from other treatment ($p>0.05$). The specific growth rate (SGR), for dry weight was the highest (10.00 ± 1.00 % $day^{-1}$) when crabs were fed the diet containing 10.0 g $kg^{-1}$ cholesterol while the lowest SGR (dry weight) (5.21 ± 0.84 % $day^{-1}$) was found for crabs fed the diet deficient in cholesterol. While the differences in SGR (dry weight) among all diet treatments was not statistically significant ($p>0.05$), there was an overall trend of increasing SGR (dry weight) with

increasing dietary cholesterol level and this peaked at 10.0 g $kg^{-1}$ cholesterol, and then decreasing as cholesterol level increased further (Table 3.3). The SGR for carapace width and SGR for carapace length followed a similar trend as SGR based on dry weight, with the highest values found for crabs fed the diet containing 10.0 g $kg^{-1}$ cholesterol and the lowest values obtained for crabs fed the diet without added cholesterol. The differences between the highest and the lowest SGR (carapace width) and SGR (carapace length) were both statistically significant ($p<0.05$) (Table 3.3).

**Table 3.3** Mean (± SE) final dry weight, carapace width and length and mean (± SE) specific growth rate (SGR) of newly molted 3rd stage juvenile *Portunus pelagicus* fed diets containing different levels of cholesterol.

| Supplemented levels (analyzed level) g $kg^{-1}$ | Newly molted C4 stage | | | Specific growth rate (SGR) | | |
|---|---|---|---|---|---|---|
| | Dry weight (mg) | Carapace width (mm) | Carapace length (mm) | Dry weight (% $day^{-1}$) | Carapace width (% $day^{-1}$) | Carapace length (% $day^{-1}$) |
| 0.0 (0.3) | 1.26 ± 0.14 | 4.69 ± 0.13 | 3.12 ± 0.07 | 5.21 ± 0.84 | $3.32 \pm 0.19^{a}$ | $1.94 \pm 0.14^{a}$ |
| 2.5 (2.6) | 1.77 ± 0.19 | 4.81 ± 0.10 | 3.18 ± 0.05 | 8.21 ± 0.94 | $4.27 \pm 0.17^{bc}$ | $2.38 \pm 0.20^{ab}$ |
| 5.0 (4.5) | 1.72 ± 0.25 | 4.89 ± 0.11 | 3.19 ± 0.06 | 7.96 ± 1.11 | $4.12 \pm 0.17^{abc}$ | $2.39 \pm 0.13^{ab}$ |
| 7.5 (6.7) | 1.90 ± 0.35 | 4.77 ± 0.13 | 3.15 ± 0.06 | 9.02 ± 1.40 | $4.26 \pm 0.23^{abc}$ | $2.47 \pm 0.15^{ab}$ |
| 10.0 (10.0) | 1.94 ± 0.24 | 4.98 ± 0.08 | 3.26 ± 0.05 | 10.00 ± 1.00 | $4.78 \pm 0.13^{c}$ | $2.86 \pm 0.12^{b}$ |
| 12.5 (11.7) | 1.49 ± 0.29 | 4.78 ± 0.14 | 3.18 ± 0.06 | 6.55 ± 1.21 | $3.96 \pm 0.71^{ab}$ | $2.38 \pm 0.14^{ab}$ |
| 15.0 (14.3) | 1.57 ± 0.31 | 4.70 ± 0.09 | 3.16 ± 0.07 | 7.25 ± 1.36 | $4.02 \pm 0.15^{abc}$ | $2.44 \pm 0.17^{ab}$ |

*Different superscripts within a column indicate significant differences ($p<0.05$)

### 3.4 Discussion

The value of dietary cholesterol to early juvenile blue swimmer crabs *P. pelagicus* was clearly demonstrated in the present study and it showed that the best survival, development and growth of the young crabs were achieved when they were fed a diet containing a cholesterol level of 10.0 g $kg^{-1}$ of the dry weight. Earlier studies demonstrating the importance of cholesterol for crustaceans, particularly aquaculture species, date back several decades. For example, when comparing effects of various dietary sterols for larvae of the Kuruma prawn, *M. japonicus*, Teshima and Kanazawa (1983) reported that among the sterols tested, cholesterol had the highest dietary value in promoting larval growth and survival. They also found that two other sterols, ergosterol and 24-methylenecholesterol could be converted to cholesterol by the prawn larvae and therefore had a similar dietary value to cholesterol.

Survival data suggests that feeding *P. pelagicus* juveniles with diets that were either deficient or overly high in cholesterol leads to high mortality. When crabs fed the diet supplemented with the highest level of cholesterol (15.0 g $kg^{-1}$), high mortality (20.7%) was found because crabs unable to completely dislodge from their old exoskeleton during the molt (MDS). The occurrence of MDS in juvenile freshwater crayfish, *P. leniuculus*, has also been linked to disorders in sterol metabolism (D'Abramo et al., 1985). The cholesterol deficient diet also led to significant prolonged development time of *P. pelagicus* when compared to crabs fed the diet containing 10.0 g $kg^{-1}$ cholesterol. Past studies on freshwater crayfish, *Procambarus clarkii,* clawed lobster, *H. americanus* and shore crab, *Carcinus maenas,* have shown that during the molting cycle, their ecdysteroids level are generally low

throughout the intermolt stage and only increase during the pre-molt stage (Chang, 1985; Lachaise et al., 1989; Nakatsuji et al., 2009). Ecdysteroids is synthesized by the steroidogenic glands i.e. the Y-organ (Skinner, 1985) and the uptake of cholesterol by the Y-organ is maximal when the molting process is initiated (Spaziani and Kater, 1973). A low level of dietary cholesterol may inhibit this process and thus prolong the intermolt period of *P. pelagicus*. It is worth noting that no significant adverse effect on development of juvenile *P. pelagicus* was detected when they were fed diets containing cholesterol levels above 10.0 g $kg^{-1}$, and a similar result was found for the molting frequency of juvenile mud crab, *S. serrata*, when they were fed diets containing cholesterol at above optimal levels (Sheen, 2000).

Specific growth rate (SGR) based on dry weight, carapace width and length, all showed a similar trend of increased with increasing dietary cholesterol level up to 10.0 g $kg^{-1}$ and further inclusion of cholesterol reduced the SGR. Such a response curve is often a characteristic in nutritional studies as after dietary content of a certain nutrient reaches its optimal level; further increase leads to excessive supply which is not beneficial (Mercer, 1982). An inverse relationship has been found between molting frequency and size of crustaceans (Barnes, 1987; West and Costlow, 1987). Similarly in this study, early *P. pelagicus* fed the diet containing 10.0 g $kg^{-1}$ cholesterol had both the highest mean development rate and largest size, further suggesting the important of supplying the correct level of dietary cholesterol for this species.

Overall, the optimal dietary cholesterol level for *P. pelagicus* early juveniles found in this study is higher than that reported for most other crustaceans. For example, the suitable dietary cholesterol level was reported to be within range of 0.2 to 0.8% (2 to 8 g $kg^{-1}$) of dry diet weight for the mud crab, *S. serrata*, another commercially important portunid crab (Sheen, 2000). For non-brachyuran crustaceans, the optimal dietary cholesterol level is generally in the vicinity of 0.5% (5 g $kg^{-1}$) or less (Kanazawa et al., 1971; D'Abramo et al., 1984; Chen and Jenn, 1991; Chen, 1993; Sheen et al., 1994b). In contrast to the research mentioned above, some studies have shown that the provision of supplemental cholesterol or sterols in the diet of the giant freshwater prawn, *Macrobrachium rosenbergii* (Briggs et al., 1988) and banana shrimp, *P. merguiensis*, juveniles (Thongrod and Boonyaratpalin, 1998) did not improve survival and growth when compared to a basal diet without cholesterol supplementation. While it is worth noting that there was a low level of cholesterol (1.2 g $kg^{-1}$ in the case of *M. rosenbergii)* or sterols (6 g $kg^{-1}$ in the case of *P. merguiensis*) in the basal diets originating from other diet ingredients, it is clear that the dietary cholesterol or sterols requirements of these two species is lower than *P. pelagicus*. These very different results from past studies clearly demonstrate a highly species-specific nature of dietary cholesterol requirements in crustaceans and verify the need for similar research for any aquaculture candidate species.

Cholesterol absorption in crustaceans has also been closely related to dietary phospholipid content. It is known that for crustaceans, cholesterol is transported from the hepatopancreas to the hemolymph mainly via lipoprotein, a transporter with phospholipid as an essential part (Teshima and Kanazawa, 1980). It has been reported that when clawed

lobsters, *Homarus* sp., fed a phosphatidylcholine deficient diet, their cholesterol transport was negatively affected (D'Abramo et al., 1982) and a similar situation was also reported for the Kuruma prawn, *M. japonicus* (Teshima and Kanazawa, 1986). Mankura et al. (1980) compared the level of cholesterol esterification in crustaceans, fish, rats and human beings, and suggested that crustaceans had the lowest lecithin:cholesterol acyltransferase activity, which is responsible for cellular cholesterol metabolism, particularly cholesterol contained in plasma lipoproteins. In the present study, a same level of phospholipid was present in all diets; whether this level of phospholipid limited the cholesterol utilization and/or assimilation at high cholesterol levels needs further investigation. In fact, in several crustaceans including megalopae of the mud crab, *S. serrata* (Holme et al., 2007a), a compensatory relationship of dietary phospholipid and cholesterol was demonstrated. This issue will be the next step in our research toward development of a nutritionally optimized formulated diet for *P. pelagicus* juveniles and are reported in Chapter 4 and 5.

# CHAPTER 4

## The influence of dietary phospholipid on survival, development, growth and stress resistance of *Portunus pelagicus* early juveniles

### 4.1 Introduction

Phospholipids (PL) are one of the most important lipid components for aquatic animals. They play important roles in lipid and carbohydrate metabolism and enhance absorption of ingested fats, such as triglyceride (Paibulkichakul et al., 1998; Hien et al., 2005). Better mobilization of lipids results in increased lipid deposition in tissues and energy available for growth (Teshima et al., 1986a; Chen and Jenn, 1991; Kontara et al., 1997). Crustaceans are able to synthesize PL *de novo* via the similar pathway of esterification used by mammals, however, their synthesizing rates often cannot meet their metabolic requirements (Shieh, 1969; New, 1976; Teshima et al., 1986a) thus dietary supply is vital (Bowser and Rosemark, 1981; Baum et al., 1990; Coutteau et al., 1996; Gong et al., 2001; Roy et al., 2006; Holme et al., 2007a). This is evident in past studies which showed that PL deficient diets caused poor survival and growth in several cultured crustacean e.g. American lobster, *Homarus americanus*, juveniles (Kean et al., 1985), black tiger prawn, *Penaeus monodon*, larvae and postlarvae (Paibulkichakul et al., 1998), Pacific white shrimp, *Litopenaeus vannamei*, juveniles (Gong et al., 2000b) and mud crab, *Scylla serrata*, megalopae (Holme et al., 2007a). The inclusion of PL in formulated diets has further been suggested to improve the water stability of food particles, and provide a source of antioxidant and feed attractant (Coutteau et al., 1997).

Variations in the purity and sources of PL used in nutritional studies, experimental conditions, and the life stages of the animals used makes it difficult to compare results of past studies relating to optimal PL requirement of crustaceans (Hilton et al., 1984; Coutteau et al., 1996; Thongrod and Boonyaratpalin, 1998; Coutteau et al., 2000; Gong et al., 2007; Holme et al., 2007a). For example, it has been reported that optimal dietary PL levels in the form of soybean lecithin ranged from 1 to 2% of the diet for banana shrimp, *Penaeus merguiensis*, juveniles (Thongrod and Boonyaratpalin, 1998), while it was 1.5% as phosphatidylcholine and 6.5% as de-oiled lecithin for Kuruma prawn, *Marsupenaeus japonicus*, post larval (Camara et al., 1997). For clawed lobster, *Homarus* sp. juveniles it was 6 to 8% of the diet weight in the form of refined soybean lecithin (Conklin et al., 1980). In contrast, dietary soybean lecithin was found to have no significant effects on growth and survival of giant freshwater prawn, *Macrobrachium rosenbergii*, post larvae (Hilton et al., 1984) or juveniles (Briggs et al., 1988).

Phospholipids are characterized by phosphatidic acids as a backbone esterified to two fatty acids and a base such as choline, ethanol, inositol or serine becoming either phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) (Tocher et al., 2008). For larvae of *M. japonicus*, PL from soybean is preferred compared to those from other terrestrial source e.g. chicken egg phosphatidylcholine (PC), bovine brain phosphatidylethanolamine (PE) and bovine brain phosphatidylserine (PS) (Kanazawa et al., 1985). Soybean lecithin is normally preferred as an ingredient for aquaculture feeds because it contains high levels of polyunsaturated fatty acids (PUFA), dominantly linoleic acid (LOA; 18:2n-6) and also linolenic acid (LNA;

18:3n-3) (Kanazawa et al., 1985; Piedad-Pascual, 1985). Soybean lecithin also contains high levels of phosphatidylcholine (PC) which has been suggested as an active component of phospholipid and the main growth promoting fraction for crustaceans (D'Abramo et al., 1981; Coutteau et al., 2000). Several past studies have used highly purified PC in laboratory experiments with several aquacultured crustaceans and obtained good survival and growth (Chen and Jenn, 1991; Camara et al., 1997; Kontara et al., 1997; Holme et al., 2009). However, highly purified PC is very costly and therefore not very practical for use in commercial production of aquaculture feeds (Geurden et *al.*, 1998). Gong et al. (2000a) suggested that other than PC, dietary supplementations of other components of PL such as phosphatidylinositol (PI) and phosphatidylethanolamine (PE) also enhanced growth and increased the beneficial effects of dietary PC on lipid mobilization, utilization and synthesis.

Aiming at practical application of the results for *P. pelagicus* food formulation, this study was conducted to assess survival, development and growth of *P. pelagicus* early juveniles fed semi-purified diets supplemented with various level of crude PL (PL content > 90% and 55% purified PC and 20% PE, respectively). At the end of the experiment, a stress test in the form of osmotic shock was performed on surviving crabs to evaluate their stress resistance capacity and overall physiological status.

## 4.2 Materials and methods

### *4.2.1 Source of crabs*

Broodstock capture and husbandry and larval culture protocol were described in Chapter 2 (Section 2.2.1).

*4.2.2 Diet preparation*

Six iso-lipidic diets were formulated to contain phospholipid (PL) at 0 (basal diet), 30, 60, 90, 120 and 150 g $kg^{-1}$ (Table 4.1). Iso-lipidicy was achieved by replacement of soybean derived PL with soybean oil resulting in diets that contained equivalent levels of lipids and hence were iso-energetic. Phospholipid (Type IV-S, P 3644) used in this study was obtained from Sigma-Aldrich (Australia). Information provided by the manufacturer showed that the PL were derived from soybean with purity more than 90% and contained approximately 55% phosphatidylcholine (PC) and 20% phosphatidylethanolamine (PE). The PL also contained 65% >18:2 PUFA (of total fatty acids). Soybean oil used in this study was supplied by Cootamundra Oilseeds Pty. Ltd. Australia, and was high quality, fine grade cold press soybean oil produced under low heat to maintain the nutritional value.

To prepare the diets, fish meal was pulverized and sieved through 100 µm mesh before being added to a chloroform/methanol solution (2:1, v:v) (Folch et al., 1957). The solution was then filtered with the aid of a vacuum pump and this defatting process was repeated three times to ensure maximum removal of lipid from the fish meal. All dry and wet ingredients of the diets were then mixed well in separate mixing bowls before being combined and throughout blended using an electric mixer. Subsequently, agar the binder used in this study was dissolved in 100 mL distilled water heated to $\geq 80^{o}$C. As the agar solution turned clear, it was allowed to cool to $40^{o}$C when the diet mixture was added to the agar solution. The diet mixture quickly became gelatinized when temperature reduced to $37^{o}$C and the gelatinized diets were then cut into small pieces of approximately 2 $mm^3$ and stored in a freezer at $-20^{o}$C until used.

**Table 4.1** Formulation of the experimental diets (g $kg^{-1}$) supplemented with different levels of phospholipid. Diet formulation was modified from Sheen and Wu (1999), Genodepa (2004) and Holme (2007b). The analyzed actual contents of phospholipid in the diets are also shown at the lower part of the Table.

| Ingredient | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|---|---|
| Defatted fish meal[1] | 500 | 500 | 500 | 500 | 500 | 500 |
| Triglyceride (fish oil [2a]:corn oil[2b] ; 2:1) | 40 | 40 | 40 | 40.0 | 40 | 40 |
| Cholesterol[2c] | 10 | 10 | 10 | 10 | 10 | 10 |
| Phospholipid[2d] | 0 | 30 | 60 | 90 | 120 | 150 |
| Soybean Oil[4] | 150 | 120 | 90 | 60 | 30 | 0 |
| Vitamin mixture[3a] | 40 | 40 | 40 | 40 | 40 | 40 |
| Mineral Mixture[3b] | 40 | 40 | 40 | 40 | 40 | 40 |
| Choline chloride[2e] | 10 | 10 | 10 | 10 | 10 | 10 |
| DCP[2f] | 6 | 6 | 6 | 6 | 6 | 6. |
| Agar[2g] | 120 | 120 | 120 | 120 | 120 | 120 |
| Starch[2h] | 50 | 50 | 50 | 50 | 50 | 50 |
| Cellulose[2i] | 34 | 34 | 34 | 34 | 34 | 34 |
| *Analyzed* | | | | | | |
| Phospholipid | 4 | 30 | 54 | 79 | 104 | 131 |

[1] Skretting Tasmania

[2] Sigma-Aldrich Pty Ltd [a]from menhaden F8020 [b]C8267 [c]C8667 [d]P3644 [e98]% powder C7527 [f]dibasic calcium phosphate C4131 [g]A7002 [h]S4126 (corn) [i]C8002 alpha

[3] Rabar Pty Ltd [a]ZZ600 DPI, each 1kg contains: vitamin A 2miu, vitamin D3 0.8miu, vitamin E 40g, vitamin K 2.02g, inositol 50g, vitamin B3 30.40g, vitamin B5 9.18g, vitamin B9 2.56g, vitamin B2 4.48g, vitamin B12 0.004g, biotin 0.1g, vitamin B6 4g, vitamin B1 3.4g, vitamin C 44.4g, para amino benzoic acid 20g, tixosil 5g, antioxidant 30g [b]ZZ603 DO 067 DPI, each 1kg contains: copper 1g, cobalt 100mg, magnesium 59.4mg, manganese 5g. iodine 800mg, selenium 20mg, iron 8mg, zinc 20g, aluminium 100mg, chromium 100mg

[4] Cootamundra Oilseeds Pty. Ltd, Australia

*4.2.3 Experimental design and set-up*

Newly metamorphosed C1 crabs were obtained as described in Chapter 2 (Section 2.2.2). They were placed individually into 750 mL circular culture vessels to start the experiment. Three replicates set up for each dietary treatment with 15 individually cultured C1 crabs per replicate bringing the total culture units to 270. Throughout the experiment, a 100% water exchange was carried out every morning for all culture units when crabs were transferred individually to new culture vessels while the old vessels were sanitized in a hypochlorite solution and dried overnight to be used the next morning. Molting and mortality were recorded during water exchange and all crabs were fed to satiation. Throughout the experiment, temperature, salinity and photoperiod were monitored daily and kept at 28 ± 1$^{o}$C, 30 ± 2‰ and 14:10 (light: dark), respectively. The mean (± SE) initial wet weight and dry weight was 4.60 ± 0.54 mg and 1.14 ± 0.22 mg, respectively, while the mean carapace width and length was 3.12 ± 0.27 mm and 2.50 ± 0.12 mm, respectively.

Towards the end of experiment, any crabs that had molted to the 4$^{th}$ stage crabs (C4) were removed from the culture units in the afternoon for measurement of carapace size and dry weight determination. Sampling of crabs in the afternoon allowed sufficient time for newly molted crabs to harden their exoskeleton. The carapace length and width and dry weight of the newly molted C4 were determined as described in Chapter 3 (Section 3.2.3). The experiment was terminated when all crabs were either molted to the C4 stage or had died.

To test if different dietary PL levels resulted in variations in overall physiological condition and stress resistance, fifteen randomly selected C4 crabs from each dietary treatment were subject to sudden osmotic shock. All crabs used for this test were newly molted but fully calcified C4 crabs and they were transferred directly from a salinity of 30‰ to 7‰ and remained at 7‰ for 420 minutes (7 hours). The choice of 7‰ for the osmotic shock test was based on a salinity study with the same species by Romano and Zeng (2006). Following introduction to the lower salinity, mortalities of the crabs were checked every 10 minutes for the first hour and then every 20 minutes during the following 6 hours. Crabs were considered dead if they did not respond to gentle probes. The stress test was not conducted for crabs fed the basal diet because of their low survival at the end of feeding experiment. No replication was possible for osmotic shock stress test due to limited availability of crabs at the end of the experiment.

*4.2.4 Phospholipid and fatty acid contents of experimental diets*

To obtain polar lipid (phospholipid) and fatty acid contents (g $kg^{-1}$) of experimental diets, total lipid was first extracted using chloroform: methanol (2:1, v/v) based on the method of Folch et al. (1957). Phospholipid in the lipid aliquot was separated from neutral lipids using petroleum: methanol (1:1, v/v) and the solvents were then volatized in a stream of nitrogen to gravimetrically determine the residue from the petroleum fraction.

Fatty acid compositions of the diets were analyzed following transesterification lipid with 0.4 M KOH-methanol to produce fatty acid methyl esters (FAME). FAME were injected into an Agilent 6890 gas chromatograph fitted with an HP-5.5% Phenyl Methyl

Siloam capillary column (30.0 mm x 25 mm, Agilent 19091J-413, USA) and substantiated by flame ionization detection (FID). The injector, detector and column temperature were set as described by Wu et al (2007). Peaks were identified by comparing the retention times with fatty acids standards (Sigma Chemical Co, St Louis, MO, USA) and individual fatty acid was quantified by the reference to the internal standard (19:0).

*4.2.5 Data and statistical analysis*

To assess growth over time, specific growth rate (SGR) based on dry weight, carapace width and length of the newly molted C4 crabs from each diet treatment were calculated using the equation described in Chapter 3 (Section 3.2.5). Survival, development time, final data for dry weight, carapace length, carapace width and SGR data were analyzed using one-way ANOVA after confirmation of normality and homogeneity of variances. Survival data were arcsine transformed before analysis. However, whenever the variance remained heterocedastic after transformation due to outliers, analysis of variances was performed nonetheless with ANOVA which is robust, operating well even within heterogeneity of variances as long as all n are equal or nearly equal (Zar, 1999) as is the case in this study. Significant differences between treatments were determined using Tukey's test and the level of significance difference was set at $p<0.05$. All statistical analyses were performed using the SPSS statistic software version 16.

## 4.3 Results

### *4.3.1 Diet phospholipid and fatty acid contents*

The analyzed phospholipid and fatty acid compositions of the experimental diets are shown in Tables 4.1 and 4.2. The results showed that the basal diet without PL supplementation actually contained 4 g $kg^{-1}$ PL while diets formulated to contain 30, 60, 90, 120 and 150 g $kg^{-1}$ PL supplementation actually contained 30, 54, 79, 104 and 131 g $kg^{-1}$ PL, respectively.

With the exception of oleic acid (18:1n-9), linoleic acid (LOA: 18:2n-6) and linolenic acid (LNA: 18:3n-3), the fatty acid profiles, including the major essential fatty acids (EFA), i.e. arachidonic acid (ARA: 20:4n-6), ecosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA: 22:6n-3), were very similar among the experimental diets (Table 4.2). For oleic acid, LOA and LNA, generally, the basal diet without PL supplementation and diets with low levels of PL supplementation contained higher levels of these fatty acids and there was a clear trend of proportional reduction with increasing level of PL supplementation (Table 4.2). The differences in these three fatty acids also largely altered the contents (g $kg^{-1}$) of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), however, the HUFA level as well as the ratio of n-3 to n-6 fatty acids remained relatively stable among diets (Table 4.2).

**Table 4.2** Fatty acid profiles (g $kg^{-1}$ of diet dry weight) of the experimental diets with different levels of supplemental phospholipid (analyzed level; g $kg^{-1}$).

| Major fatty acids | 0 (4) PL | 30 (30) PL | 60 (54) PL | 90 (79) PL | 120 (104) PL | 150 (130) PL |
|---|---|---|---|---|---|---|
| 14:0 | 2.51 | 2.83 | 2.53 | 2.38 | 2.74 | 2.63 |
| 16:0 | 20.48 | 22.19 | 20.75 | 19.73 | 22.38 | 21.36 |
| 16:1n-7 | 3.58 | 3.98 | 3.57 | 3.4 | 3.91 | 3.68 |
| 18:0 | 7.98 | 7.78 | 6.75 | 5.70 | 5.69 | 4.79 |
| 18:1n-9 | 31.11 | 29.15 | 23.21 | 18.53 | 16.60 | 11.84 |
| 18:1n-7 | 2.86 | 3.01 | 2.69 | 2.45 | 2.68 | 2.46 |
| 18:2n-6 | 79.42 | 78.38 | 66.37 | 58.17 | 59.11 | 50.43 |
| 18:3n-3 | 12.95 | 12.35 | 9.92 | 8.29 | 7.87 | 6.19 |
| 18:4n-3 | 1.15 | 1.25 | 1.10 | 1.05 | 1.20 | 1.12 |
| 20:0 | 0.63 | 0.58 | 0.46 | 0.35 | 0.30 | 0.20 |
| 20:1n-9 | 0.84 | 0.88 | 0.78 | 0.70 | 0.77 | 0.68 |
| 20:4n-6 | 0.33 | 0.36 | 0.35 | 0.29 | 0.36 | 0.34 |
| 20:5n-3 | 4.44 | 4.90 | 4.37 | 4.14 | 4.77 | 4.45 |
| 22:0 | 0.63 | 0.59 | 0.48 | 0.39 | 0.37 | 0.28 |
| 22:5n-6 | 0.15 | 0.17 | 0.15 | 0.13 | 0.15 | 0.25 |
| 22:5n-3 | 0.89 | 0.98 | 0.89 | 0.84 | 0.98 | 0.92 |
| 22:6n-3 | 4.89 | 5.42 | 4.91 | 4.60 | 5.34 | 4.99 |

| | | | | | | |
|---|---|---|---|---|---|---|
| ∑ SFA | 33.11 | 34.91 | 31.82 | 29.34 | 32.36 | 30.08 |
| ∑ MUFA | 35.09 | 37.47 | 30.67 | 25.33 | 20.50 | 19.17 |
| ∑ PUFA (≥ 18:2n) | 93.69 | 92.17 | 77.56 | 67.67 | 68.34 | 57.90 |
| ∑ HUFA (≥ 20:3n) | 10.70 | 11.83 | 10.68 | 10.00 | 11.60 | 10.94 |
| | | | | | | |
| ∑ n-3 | 24.31 | 24.90 | 21.19 | 18.92 | 20.16 | 17.66 |
| ∑ n-6 | 80.08 | 79.10 | 67.05 | 58.75 | 59.79 | 51.15 |
| n-3/n-6 | 0.30 | 0.31 | 0.32 | 0.32 | 0.34 | 0.35 |
| DHA/EPA | 5.51 | 5.51 | 5.52 | 5.48 | 5.42 | 5.46 |
| TOTAL | 176.17 | 176.38 | 150.73 | 132.34 | 136.71 | 118.09 |

*(∑) include minor fatty acids that are not shown in the Table

*4.3.2 Survival, development time and growth*

The overall survival of *P. pelagicus* from newly settled C1 to the C4 stage was relatively high (≥ 77.8) for all diet treatments with PL supplementation, however, crabs fed the basal diet without PL supplementation (analyzed content; 4 g $kg^{-1}$) showed substantially lower survival (40%) which is significantly lower than that in all other treatments ($p<0.01$). The highest survival of 93.3% was obtained for crabs fed diets supplemented with the highest levels of PL at 120 and 150 g $kg^{-1}$ (analyzed content; 104 and 131 g $kg^{-1}$, respectively) although no significant differences were detected for survival between diet treatments with PL supplementation (Fig. 4.1). It worth noting that for all diet treatments, mortality due to molt-death-syndrome (MDS) was only occasionally observed, however, for crabs fed the basal diet, a high percentage of the observed mortality (26.7%) occurred within 48 h of a molt and was significantly higher than that in other treatments ($p<0.01$) (Fig 4.1).

Development of early *P. pelagicus* juveniles showed a clear trend of accelerating with the increasing level of dietary PL supplementation (Table 4.3). The first occurrence of C4 crabs was again found among *P. pelagicus* fed diets supplemented with the highest level of PL at 120 and 150 g $kg^{-1}$, and the mean cumulative development time from C1 to C4 for these two treatments were the shortest at 14.9 ± 0.2 and 14.6 ± 0.2 days, respectively, significantly shorter than those for other treatments ($p<0.05$) (Table 4.3). Crabs fed diets supplemented with 60 and 90 g $kg^{-1}$ PL (analyzed content; 54 and 79 g $kg^{-1}$, respectively) on average took 17.0 ± 0.3 and 16.6 ± 0.3 days to develop to the C4 stage, respectively, and were significantly faster than for crabs fed diet s with 30 g $kg^{-1}$ PL supplementation (analyzed content; 30 g $kg^{-1}$) or the basal diet treatment ($p<0.05$). Crabs fed the basal diet took the

longest time (20.9 ± 0.5 days) to reach C4; significantly longer than in all other treatments ($p<0.01$) and more than 6 days longer than crabs fed diets with 120 and 150 g $kg^{-1}$ PL supplementation (Table 4.3).

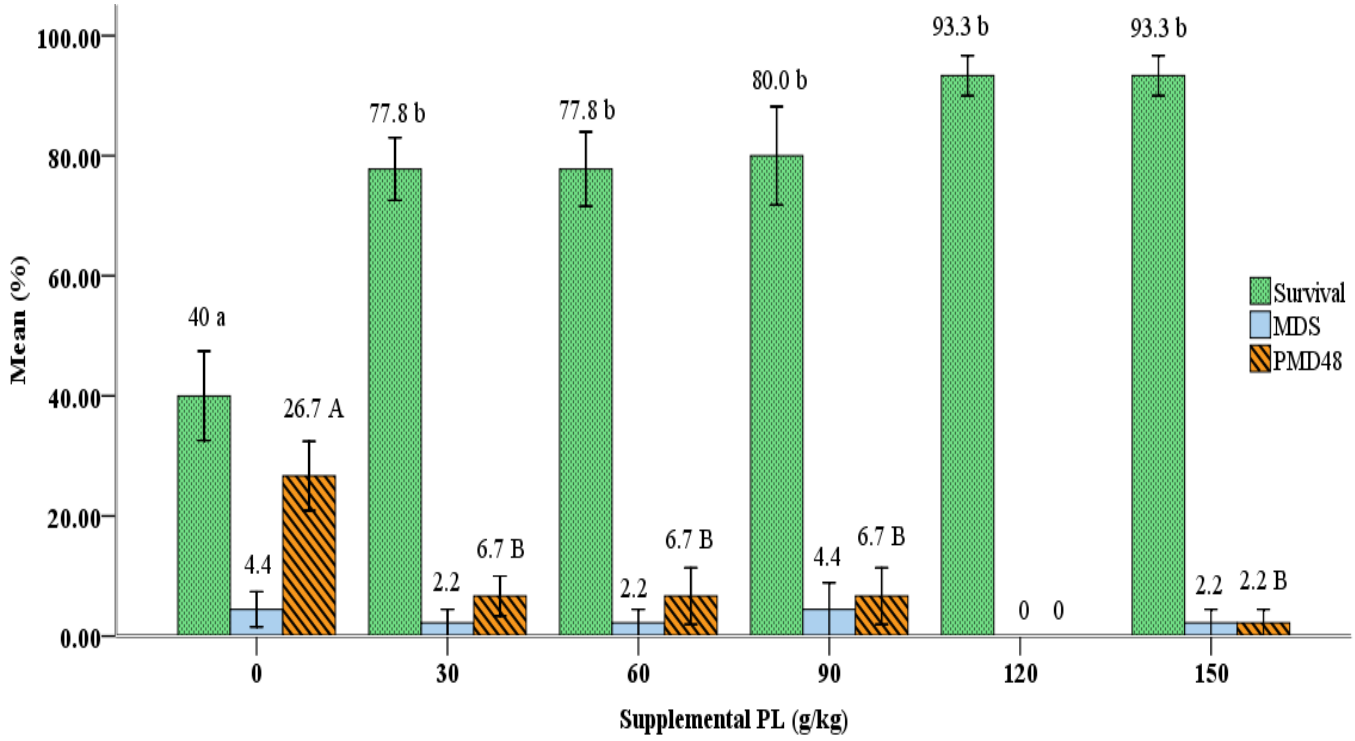



**Figure 4.1** Mean survival, mortality due to molt death syndrome (MDS) and mortality occurring 48 h of molt (%) during the culture period from the newly settled first stage crab (C1) to crab stage 4 (C4) of the blue swimmer crab *Portunus pelagicus*. The crabs were fed diets containing different level of phospholipid throughout the culture period. Different letters within same parameters indicate significant differences ($p<0.05$). No significant difference was detected for occurrence of MDS between treatments.

**Table 4.3** Mean (± SE) intermolt period for each molt stages and cumulative development time from C1 to C4 (in days) for early *Portunus pelagicus* juveniles fed semi-purified diets containing different level of phospholipid.

| Supplemented PL level (analyzed content) g $kg^{-1}$ | C1 to C2 | C2 to C3 | C3 to C4 | Cumulative development time (C1- C4) |
|---|---|---|---|---|
| 0 (4) | $5.7 \pm 0.2^{ab}$ | $7.7 \pm 0.3^{a}$ | $7.8 \pm 0.3^{a}$ | $20.9 \pm 0.50^{a}$ |
| 30 (30) | $5.1 \pm 0.1^{c}$ | $6.5 \pm 0.2^{b}$ | $7.0 \pm 0.2^{b}$ | $18.5 \pm 0.4^{b}$ |
| 60 (54) | $5.2 \pm 0.1^{bc}$ | $5.8 \pm 0.2^{bc}$ | $6.3 \pm 0.1^{bc}$ | $17.0 \pm 0.3^{c}$ |
| 90 (79) | $5.3 \pm 0.2^{a}$ | $5.3 \pm 0.1^{c}$ | $6.6 \pm 0.2^{cd}$ | $16.6 \pm 0.3^{c}$ |
| 120 (104) | $4.8 \pm 0.1^{a}$ | $4.7 \pm 0.1^{e}$ | $5.5 \pm 0.1^{de}$ | $14.9 \pm 0.2^{d}$ |
| 150 (131) | $4.8 \pm 0.1^{a}$ | $4.4 \pm 0.1^{e}$ | $5.3 \pm 0.1^{e}$ | $14.6 \pm 0.2^{d}$ |

$^{abcd}$ Different superscripts within a column indicate significant differences ($p<0.05$)

The dry weight and carapace size of early *P. pelagicus* juveniles was effectively improved by increasing the level of PL in their diets (Table 4.4). The highest growth, in term of dry weight, carapace width and length, were shown by crabs fed diets supplemented with 150, 120 and 90 g $kg^{-1}$ PL; those value were often significantly higher than those of crabs in other treatments although no significant difference were detected among them ($p>0.05$). No significant differences in dry weight or carapace size was detected between crabs fed diets supplemented with lower levels of PL at 30 and 60 g $kg^{-1}$ ($p>0.05$). However, the growth of the crabs fed the basal diet, without PL supplementation, was significantly lower than crabs in all other treatments ($p<0.01$). The average dry weight of newly molted C4 crabs from the basal diet treatment was only 3.61 ± 0.26 mg; less than half of the highest dry weight (7.98 ±

0.31 mg) recorded for crabs fed the diet with the highest PL supplementation (150 g $kg^{-1}$) (Table 4.4).

Specific growth rates (SGR) calculated using crab dry weights were highest (13.32 ± 0.29 % $day^{-1}$) for crabs fed the diet containing 150 g $kg^{-1}$ PL supplementation while the lowest SGR (5.33 ± 0.37 % $day^{-1}$) was found for crabs fed the basal diet (Table 4.4). The SGR (dry weight) of crabs fed the basal diet was significantly lower than those of crabs in other treatments and these differences were often highly significant ($p<0.01$). No significant differences of SGR (dry weight) was found among crabs that were fed diets supplemented with 30 and 60 g $kg^{-1}$ PL ($p>0.05$) and among crabs that were fed diets supplemented with 120 g $kg^{-1}$ PL and 150 g $kg^{-1}$ PL ($p>0.05$). SGR values based on carapace width were significantly higher at increased level of dietary PL ($p<0.01$) with the exception of PL supplementation above 120 g $kg^{-1}$. SGR value based on carapace length followed a similar trend to SGR values calculated using carapace width, however significant differences were not as apparent (Table 4.4).

**Table 4.4** Mean (± SE) final dry weight, carapace width and length and mean (± SE) specific growth rate (SGR) of newly molted 4th stage juvenile *Portunus pelagicus* fed diets containing different levels of phospholipid.

| Supplemented PL level (analyzed content) | Newly molted C4 stage | | | Specific growth rate (SGR) | | |
|---|---|---|---|---|---|---|
| | Dry weight (mg) | Carapace width (mm) | Carapace length (mm) | Dry weight (% $day^{-1}$) | Carapace width (% $day^{-1}$) | Carapace length (% $day^{-1}$) |
| 0 (4) g $kg^{-1}$ | $3.61 \pm 0.26^{a}$ | $6.96 \pm 0.15^{a}$ | $4.15 \pm 0.09^{a}$ | $5.33 \pm 0.37^{a}$ | $3.81 \pm 0.11^{a}$ | $2.41 \pm 0.10^{a}$ |
| 30 (30) g $kg^{-1}$ | $5.83 \pm 0.33^{b}$ | $7.77 \pm 0.15^{b}$ | $4.52 \pm 0.05^{b}$ | $8.71 \pm 0.33^{b}$ | $4.92 \pm 0.11^{b}$ | $3.21 \pm 0.06^{b}$ |
| 60 (54) g $kg^{-1}$ | $6.05 \pm 0.23^{bc}$ | $7.99 \pm 0.15^{b}$ | $4.57 \pm 0.05^{bc}$ | $9.75 \pm 0.27^{b}$ | $5.52 \pm 0.11^{c}$ | $3.56 \pm 0.06^{bc}$ |
| 90 (79) g $kg^{-1}$ | $7.21 \pm 0.33^{cd}$ | $8.43 \pm 0.12^{bc}$ | $4.77 \pm 0.06^{cd}$ | $11.05 \pm 0.29^{c}$ | $5.99 \pm 0.09^{d}$ | $3.90 \pm 0.07^{c}$ |
| 120 (104) g $kg^{-1}$ | $7.61 \pm 0.25^{d}$ | $8.42 \pm 0.06^{bc}$ | $4.84 \pm 0.05^{cd}$ | $12.70 \pm 0.24^{d}$ | $6.66 \pm 0.05^{e}$ | $4.44 \pm 0.06^{d}$ |
| 150 (131) g $kg^{-1}$ | $7.98 \pm 0.31^{d}$ | $8.61 \pm 0.11^{c}$ | $5.02 \pm 0.13^{d}$ | $13.32 \pm 0.29^{d}$ | $6.97 \pm 0.09^{e}$ | $4.78 \pm 0.15^{d}$ |

[abcd] Different superscripts within a column indicate significant differences ($p<0.05$)

*4.3.3 Crab resistance to osmotic shock*

When subjected to sudden osmotic shock, it was clear that crabs fed diets with the highest PL supplementation (120 and 150 g $kg^{-1}$) had considerably greater resistance as > 60% C4 crabs survived to the end of the 7 h stress test and 100% of the crabs from both treatments survived to 280 minutes. In contrast, within 300 minutes (5 h) of osmotic shock, total mortality was observed for crabs fed the diet with the lowest PL supplementation (30 g $kg^{-1}$). While for crabs fed diets supplemented with 60 and 90 g $kg^{-1}$ PL, a sharp drop in survival occurred after 140 and 160 minutes onwards, respectively (Fig 4.2).

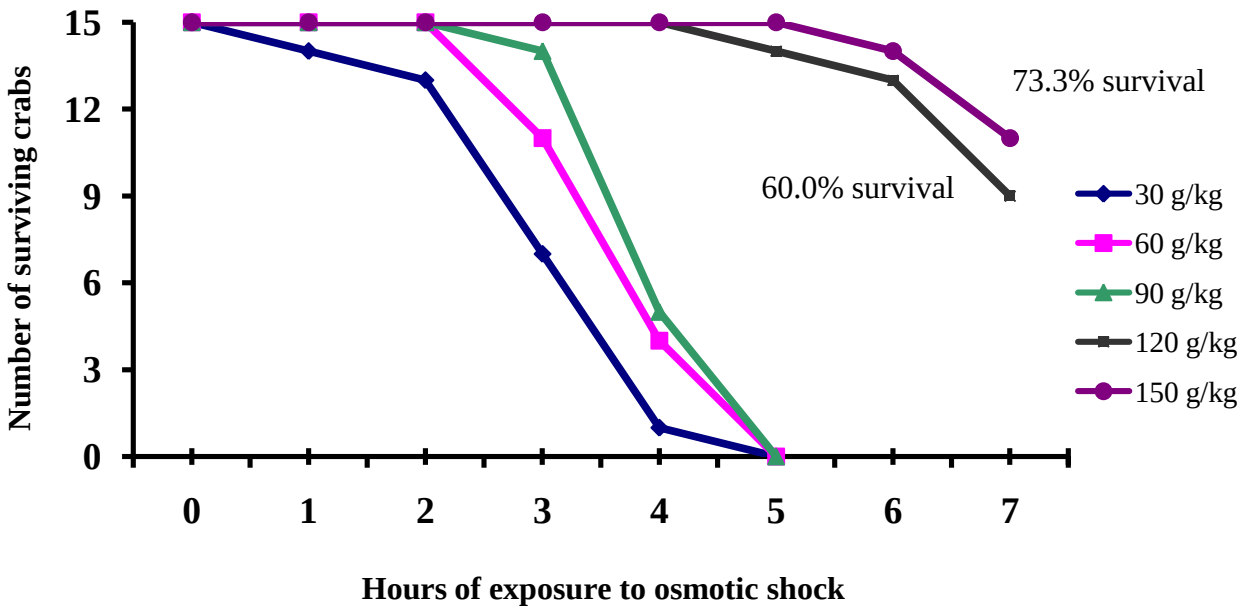



**Figure 4.2** Survival of newly molted 4th stage *Portunus pelagicus* juveniles subject to sudden salinity change from 30‰ to 7‰ over a 7 h period. Juveniles crabs were fed diets containing different levels of phospholipid indicated in the key.

## 4.4 Discussion

This experiment demonstrated the essentiality of phospholipid (PL) in diets for early juveniles blue swimmer crab, *P. pelagicus*, and clear beneficial effects in providing an appropriate level of dietary PL to enhancing survival, development and growth as well as resistance to osmotic stress. Addition of dietary PL has been reported to have similar beneficial effects in a number of cultured crustaceans, such as improving survival of megalopae of mud crab, *S. serrata* (Holme et al., 2007a), growth of post larval Pacific white shrimp, *L. vannamei* (Coutteau et al., 1996), and sensitivity to osmotic shock of Chinese mitten crab, *Eriocheir sinensis,* zoea (Wu et al., 2007). Based on the results of this study, *P. pelagicus* early juveniles fed diets containing 104 g $kg^{-1}$ and 131 g $kg^{-1}$ PL had the best survival, development, growth and capacity to withhold osmotic shock.

Survival of newly settled C1 *P. pelagicus* fed the basal diet was obviously inferior to other treatments, yielding only 40% survival to C4, which was about half, or less, that of diets with PL supplementation. Dietary PL supplementation has long been suggested to improve survival of crustaceans (New, 1976), however, very different results have been reported by some previous investigations. While many studies have demonstrated PL supplementation notably improves survival in crustaceans, such as lobster, *H. americanus*, juveniles (Conklin et al., 1980; D'Abramo et al., 1981), Kuruma prawn, *M. japonicus*, larvae (Kanazawa et al., 1985), mud crab, *S. serrata*, megalopae (Holme et al., 2007a) and larvae and post larvae of the black tiger prawn, *Penaeus monodon* (Paibulkichakul et al., 1998), others showed no significant effects on survival e.g. post larvae of giant freshwater prawn, *Macrobrachium rosenbergii* (Hilton et al., 1984), red tail prawn, *Penaeus penicillatus*,

juveniles (Chen and Jenn, 1991), as well as post larvae (Coutteau et al., 1996) and juveniles of the Pacific white shrimp, *L. vannamei* (Gong et al., 2001; Gonzalez-Felix et al., 2002c). Coutteau et al. (1996) suggested that the insignificant effects of dietary PL supplementation on survival with these species is possibly related to the larger size crustaceans used in the experiments (e.g. average wet weight between 80 mg to 1 g) except for the experiment on post larvae of *L. vannamei* which weighted 2 mg (Coutteau et al., 1996). In larvae of seabream, *Sparus aurata*, the PL requirements generally decrease with age (Koven et al., 1993). Likewise, in Atlantic salmon, *Salmo salar*, PL requirement decreases as the size of the fish increased (Poston, 1990). A similar situation may also exist for crustaceans, as during the early juvenile stages, crustaceans molt much more frequently than at later stages. In the present study, newly settled first stage *P. pelagicus* with a mean dry weight of only 4.6 mg were used thus the clear beneficial effects of dietary PL supplementation may partially be attributed to their small size and early developmental stage.

Molting in crustaceans involves extensive physiological activity, including the formation of a new integument under the old one, shedding of the old cuticle, calcification of new exoskeleton and subsequent tissues growth (Passano, 1960). Dietary phospholipid not only provide young crustaceans with the necessary inorganic phosphate, choline, inositol and ethanolamine but also essential fatty acids that serve as a substrate for formation of eicosanoids, an important mediator required for the molting process (Koskela et al., 1992; Coutteau et al., 1997; Tocher et al., 2008). Furthermore, addition of PL in the diet may help to reduced water leaching macro-nutrients such as minerals and vitamins from semi-purified diets (Coutteau et al., 1997). Crustaceans lose considerable amounts of minerals during the

molting process (Kanazawa, 1980) and diets lacking vitamins (i.e. riboflavin) when fed to juvenile black tiger prawn, *P. monodon,* promoted deficiency symptoms such as light coloration, irritability and abnormalities of exoskeleton in the prawns (Chen and Hwang, 1992). Considering the importance of PL in the molting of crustaceans, the absence of dietary PL has also been suggested to cause molt death syndrome (MDS) in juvenile lobster, *Homarus* sp (Conklin et al., 1980). Interestingly, in the present study, only a few crabs died due to MDS and this occurred across all treatments. In contrast, larger proportions of mortalities occurred within 48 h of molt particularly for crabs fed the basal diet. Completion of the molting process but subsequent deformity and death are also considered to be clinical sign of molt death syndrome (Bowser and Rosemark, 1981). Although deformity was not clearly observed in the present study, the high incidents of post-molt death occurring in crabs fed the basal diet suggests that it is also linked to PL deficiency in their diet.

In crustaceans, weight gain is a disjunctive, non-continuous process and highly dependent on the molting process (D'Abramo and Castell, 1997). In the present study, although no significant differences were found between dry weight and carapace measurement of crabs were fed diets supplemented with 90, 120 and 150 g $kg^{-1}$ PL, significantly highest SGR values of the same parameters were obtain when crabs fed diets supplemented with 120 g $kg^{-1}$ PL and 150 g $kg^{-1}$ PL only. The highest growth rates are mainly contributed by the shortest cumulative development time to C4 stage. In crustaceans, other than fatty acids, cholesterol is an important metabolic precursor for molting hormones, ecdysteroid, by conversion of dietary cholesterol to 7-dehydrocholesterol and other oxidation products in the ecdysial glands or Y-organs (Lachaise et al., 1989; Grieneisen et al., 1993).

Phospholipid helps lipid transportation (including cholesterol) in crustaceans by forming lipoproteins, the transporter for insoluble lipids (Yepiz-Plascencia et al., 2000). Lipoprotein, a micelle complex of protein and lipids, exports absorbed lipid from the gut epithelium into hemolymph and then to various tissues and organs (Teshima and Kanazawa, 1980; Coutteau et al., 1997; Yepiz-Plascencia et al., 2000). In juvenile Kuruma prawn, *M. japonicus*, it has been demonstrated that dietary PL improved mobilization of cholesterol and triglyceride from the gut to the hepatopancreas, hemolymph and muscle (Teshima et al., 1986b). The capacity of PL to increase absorption of cholesterol most likely depends on the degree of saturation of the fatty acids esterified together with the backbone of phospholipid, phosphate. Tested on Sprague-Dawley rats, soy bean phosphatidylcholine (PC) containing 61% 18:2 fatty acids significantly increase cholesterol absorption in the intestine of the rats compared to egg PC and hydrogenated PC that contained ≤ 18% 18:2 fatty acids and 0% 18:2 fatty acids, respectively (Jiang et al., 2001). Development of early juveniles of *P. pelagicus* was substantially accelerated with increased dietary PL level, which is likely a result of improved efficiency in absorbing and utilizing cholesterol, the precursor of molting hormones, aided by a high level of 18:2 fatty acids (65% of total fatty acids) in the phospholipid used for diet formulation.

This however should not be confused with the levels of fatty acids in the experimental diets. Diets with a lower level of PL in this study actually contained substantial higher level of ≥ 18:2 fatty acids or PUFA mainly in the forms of linoleic acid (18:2n-6) and linolenic acid (18:3n-3). The higher levels resulted from addition of cold pressed soybean oil to these diets to make them iso-lipidic with other diets. PUFA are essential nutrients for survival and

growth of various crustaceans (Read, 1981; Merican and Shim, 1996; Sheen and Wu, 2003), however, PUFA from soybean oil used in the present experiment were in neutral lipid form while PUFA derived from PL were in polar form. Fatty acids in the polar lipid fraction are believed to be more beneficial in promoting survival and growth due to better digestion and utilization (Coutteau et al., 1996; Camara et al., 1997; Salhi et al., 1999; Wold et al., 2007).

It is clear that supplementation of PL also markedly improved growth of juvenile *P. pelagicus* and optimal growth was achieved when *P. pelagicus* were fed diets containing a PL level at of 79 g $kg^{-1}$ PL upwards. Appropriate levels of dietary PL are likely to provide *P. pelagicus* with a suitable level of PC, a fraction of PL that has been suggested as the main growth promoting factor of PL (D'Abramo et al., 1981). In addition, choline deficiencies have been reported to cause retarded growth in some fish and shellfish species due to impaired lipid metabolism, fatty acid infiltration and disruption of the cell membrane (NRC, 1983). For examples, when different forms of choline were fed to juvenile clawed lobster, *Homarus sp*. (Conklin et al., 1980), and post larval Kuruma prawn, *M. japonicus* (Michael et al., 2007), choline from PL that was esterified together with phosphate was found to be more effective in improving their grow out.

Salinity stress tests or osmotic shock tests have often been used to evaluate the quality of seedlings produced in aquaculture hatcheries with the assumption better physiological condition permit higher tolerance to stress (Palacios, 2007). Earlier findings on crustacean have shown that crustaceans displayed better resistance to osmotic shock when given lipids enhanced diets (Tackaert et al., 1992; Rees et al., 1994; Gong et al., 2004; Palacios et al.,

2004; Sui et al., 2007). Similarly, in current study, newly molted but fully calcified stage 4 *P. pelagicus* from the two diet treatments with the highest level of PL supplementations, showed dramatically improved resistance to sudden salinity changes. In addition to generally improved physiological condition of the animals, better tolerance to osmotic shock is also likely correlated to improve osmoregulatory ability of the animals (Palacios, 2007). Appropriate level of dietary PL in the form of lecithin as well as cholesterol has been suggested to improve osmoregulatory capacity of Pacific white shrimp, *L. vannamei* (Gong et al., 2004). However, the exact underlying mechanisms are yet to be elucidated experimentally as better osmotic tolerance of *P. pelagicus* at higher inclusion level of PL may also be due to faster development of the juvenile crabs.

# CHAPTER 5

## Evaluation of dietary phospholipid and cholesterol requirements for *Portunus pelagicus* early juveniles

---

### 5.1 Introduction

Among major nutrients, both cholesterol and phospholipid (PL) cannot be synthesized *de novo* by many crustaceans (Coutteau et al., 1997) and therefore must be provided in their diets. Phospholipid has a number of important metabolic roles in crustaceans; they provide a source of energy, essential fatty acids and cell membrane components (Conklin et al., 1980; Coutteau et al., 1997; Gong et al., 2007). Inclusion of PL in the diet of crustaceans not only assists in lipid and carbohydrate metabolism and enhances the absorption of ingested fats such as triglyceride (Paibulkichakul et al., 1998; Hien et al., 2005), but also improve water stability of food particles and/or act as antioxidants and feed attractants (Coutteau et al., 1997). Past studies on phospholipid requirements in crustaceans have often used phosphatidylcholine (PC), (also known as soybean lecithin) which varied in their purity level (Thongrod and Boonyaratpalin, 1998; Gong et al., 2000b; L.Gonzalez-Felix et al., 2002). Soybean derivatives e.g. soybean oil, soybean meal and soybean phospholipid, contain high levels of polyunsaturated fatty acids, dominated by linoleic acid (LOA; 18:2n-6) and also linolenic acid (LNA; 18:3n-3). Both LOA and LNA have been recognized as important nutrients for crustaceans (Sheen and Wu, 2003). For example, in juvenile giant freshwater prawn, *Macrobrachium rosenbergii*, it has been reported that the inclusion of

appropriate levels of dietary LOA in the diets resulted in positive weight increment and improved survival (D'Abramo and Sheen, 1993).

Cholesterol is an important component of cell membrane and a metabolic precursor of vitamins and molting hormones in crustaceans (New, 1976; Gong et al., 2000b; Hernandez et al., 2004). Under controlled laboratory conditions, optimal dietary cholesterol levels for various crustaceans has been reported to be around 0.5% to 1.0% of the diet dry weight (Sheen et al., 1994b; Paibulkichakul et al., 1998; Thongrod and Boonyaratpalin, 1998; Sheen, 2000). PL is believed to facilitate transportation of cholesterol in crustacean (Gong et al., 2000b) and compensative interactions between the two have been reported for crustaceans (Teshima and Kanazawa, 1983; Teshima et al., 1986a) such as megalopae of *Scylla serrata* (Holme et al., 2007a). However, in other studies, although both PL and cholesterol have been confirmed as vital nutrients for crustaceans, no clear interactions have been shown (Kean et al., 1985; Briggs et al., 1988; Chen and Jenn, 1991; Chen, 1993; Paibulkichakul et al., 1998).

Chapter 3 and 4 have shown the positive effects of dietary cholesterol and PL for early juvenile *P. pelagicus* and indicated appropriate dietary levels. The aims of this study were to examine the effects of different levels of dietary PL and cholesterol on the survival, development and growth of early *P. pelagicus* juveniles and determine if there are any potential interaction effect between dietary PL and cholesterol, which may influence optimal dietary levels of both nutrients. Such information will be vital in formulation of cost-effective formulated diets for this species.

## 5.2 Materials and Methods

### *5.2.1 Source of crabs*

Broodstock capture and husbandry and larval culture protocol were described in Chapter 2 (Section 2.2.1).

### *5.2.2 Diet preparation*

For the first experiment, 9 semi-purified diets were formulated to contain 9 different combinations of three supplemented PL levels of 30, 60 and 90 g $kg^{-1}$ of diet weight and three supplemental cholesterol levels of 0, 5 and 10 g $kg^{-1}$ of diet weight. A basal diet deficient in both PL and cholesterol was also formulated as a control. Both phospholipid (Type IV-S, P 3644) and cholesterol (P 7688) used in this study were obtained from Sigma-Aldrich (Australia). Information provided by the manufacturer showed that the PL were derived from soybean with a purity >90% and contained 55% purified phosphatidylcholine (PC) and 20% phosphatidylethanolamine (PE) while cholesterol purity was >98%. Cholesterol was in powder form which facilitated mixing with other ingredients while PL were wax-like and was warmed for at least 20 minutes before being mixed with other diet ingredients. As capacity of PL to increase absorption of cholesterol in hemolymph has been shown to be dependent on the degrees saturation of the fatty acids incorporated in the PL (Jiang et al., 2001), the diets used in this study were not made iso-lipidic as in Chapter 4, but were made iso-energetic instead by using corn starch.

Prior to diet preparation, pulverized fish meal was defatted three times using chloroform and methanol (2:1, v/v) to remove lipids (Folch et al., 1957). The fish meal was then filtered to remove remaining volatile substances and aired in fume hood for 24 h before being sieved to remove particles larger than 100 µm. Corn starch and α-cellulose levels in the diets were adjusted accordingly to maintain iso-caloricity among diets (Table 5.1). Dry and wet ingredients were mixed separately before being combined and mixed with an electric mixer. The final addition of agar-agar as the binder was based on the technique described by Sheen (2000). Using a magnetic stirrer, agar was dissolved in 100 mL of water at ≥ 80$^{o}$C. When the liquid turned clear, heat was turn off and the agar was kept stirred until the temperature decrease to 40$^{o}$C when the homogenous diet mixtures prepared earlier was poured in and stirred at high speed before the stirrer was turn off as temperature decreased to 37$^{o}$C. The diet mixture soon gelled and the semi-hardened diets were cut into small pieces (approximately 2 mm$^{3}$) and stored at -20$^{o}$C until used.

The results of the first experiment showed that the performance of *P. pelagicus* early juveniles was best when fed diets containing the highest level of PL and cholesterol (i.e. PL 90 g kg$^{-1}$ and cholesterol 10 g kg$^{-1}$), thus, a second experiment using a second batch of early juveniles was designed to investigate whether higher dietary levels of PL and cholesterol might further enhance these results. In the second experiment, 4 diets were formulated in a similar factorial design with two levels of PL (90 and 120 g kg$^{-1}$) and two levels of cholesterol (10 and 12 g kg$^{-1}$). The formulation of the diets is shown in Table 5.1 and all diets were prepared in the same way as described for the first experiment.

**Table 5.1** Formulation (g $kg^{-1}$ dry diet) of the experimental diets used in the two experiments evaluating effects of dietary cholesterol and phospholipid on the performance of *P. pelagicus* early juveniles. The analyzed cholesterol and phospholipid contents in the diets are also presented. Diet formulation was based on Holme (2007b), Sheen and Wu (1999) and Genodepa (2004).

| | Experiment 1 | | | | | | | | | | | Experiment 2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ingredient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Defatted fish meal[1] | 500 | 500 | 500 | 500 | 500 | 500 | 500 | 500 | 500 | 500 | 500 | 500 | 500 | 500 |
| Fish oil[2a]:corn oil[2b]; 2:1 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 |
| Cholesterol[2c] | 0 | 0 | 0 | 0 | 50 | 5 | 5 | 10 | 10 | 10 | 10 | 10 | 12 | 12 |
| Phospholipid[2d] | 0 | 30 | 60 | 90 | 30 | 60 | 90 | 30 | 60 | 90 | 90 | 120 | 90 | 120 |
| Vitamin mix[3a] | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| Mineral Mix[3b] | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| Choline[2e] | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| DCP[2f] | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Agar[2g] | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 |
| Starch[2h] | 222 | 155 | 89 | 22 | 144 | 78 | 11 | 133 | 66 | 0 | 71 | 4.4 | 66.6 | 0 |
| Cellulose[2i] | 2 | 39 | 75 | 112 | 45 | 81 | 118 | 51 | 88 | 124 | 53 | 89.6 | 55.4 | 92 |
| *Analyzed* | | | | | | | | | | | | | | |
| Cholesterol | 0 | 0 | 0 | 0 | 4.7 | 4.8 | 5 | 7 | 9 | 8 | 9 | 9 | 14 | 10 |
| Phospholipid | 3 | 39 | 62 | 103 | 45 | 75 | 105 | 34 | 68 | 71 | 101 | 127 | 100 | 126 |

[1] Skretting Tasmania

[2] Sigma-Aldrich Pty Ltd [a]from menhaden F8020 [b]C8267 [c]C8667 [d]P3644 [e]98% powder C7527 [f]dibasic calcium phosphate C4131 [g]A7002 [h]S4126 (corn) [i]C8002 alpha

[3] Rabar Pty Ltd [a]ZZ600 DPI, each 1kg contains: vitamin A 2miu, vitamin D3 0.8miu, vitamin E 40g, vitamin K 2.02g, inositol 50g, vitamin B3 30.40g, vitamin B5 9.18g, vitamin B9 2.56g, vitamin B2 4.48g, vitamin B12 0.004g, biotin 0.1g, vitamin B6 4g, vitamin B1 3.4g, vitamin C 44.4g, para amino benzoic acid 20g, tixosil 5g, antioxidant 30g [b]ZZ603 DO 067 DPI, each 1kg contains: copper 1g, cobalt 100mg, magnesium 59.4mg, manganese 5g. iodine 800mg, selenium 20mg, iron 8mg, zinc 20g, aluminium 100mg, chromium 100mg

*5.2.3 Experimental design and set-up*

Newly metamorphosed C1 crabs were obtained as described in Chapter 2 (Section 2.2.2). They were placed individually into 750 mL circular culture unit. Individual culture of *P. pelagicus* juveniles required to eliminate cannibalism and allow precise record of consecutive intermolt periods for each crab. For each dietary treatment, 30 individually cultured crabs were kept in static water and each culture unit was setup as a replicate hence a total of 300 crabs used in the first experiment and 120 units in second experiments. During the experiments, a 100% water exchange was carried out by individually transferring the crabs to new culture units while the previous culture units were sanitized in hypochlorite solutions and dried overnight to be used the next morning. Any mortality and molting found during the daily water exchange were recorded and the crabs were then fed to satiation with designated diets. Toward the end of the experiments, crabs found molted to fourth crab stage (C4) in the morning were sampled for the measurement of carapace length and width and dry weight determination in the afternoon. Carapace measurement and dry weight determination were carried out as described in Chapter 3 (Section 3.2.3). The experiments were terminated when all crabs had either molted to the C4 stage or died.

Throughout the experiment, salinity, temperature and pH were maintained at 30 ± 2‰, 28 ± 1$^{o}$C and pH 7.5 to 8.1, respectively, and photoperiod was set at 14 h:10 h (light : dark). Prior to each experiment, newly settled C1 crabs were randomly sampled for the measurement of their initial carapace length and width as well as dry weight. For the first experiment, the mean initial dry weight was 1.22 ± 0.23 mg, carapace length was 2.62 ± 0.13 mm and carapace width was 3.35 ± 0.17 mm, while for the second experiment, the initial mean dry weight was 1.33 ± 0.28 mg and carapace length and carapace width was 2.62 ± 0.12 mm and 3.46 ± 0.14 mm, respectively.

*5.2.4 Diet cholesterol, phospholipid and fatty acids analysis*

All experimental diets were analyzed to confirm their actual contents of phospholipid, cholesterol and fatty acids. Following extraction of lipid from samples of experimental diets based on the method of Folch et al. (1957), the lipid extract was dissolved in petroleum and methanol (1:1, v/v) to separate the neutral and polar lipid components which were then volatized using a stream of nitrogen to determine their weight. Fatty acid compositions were determined from samples following transesterification of lipid with 0.4 M KOH-methanol to produce fatty acid methyl esters (FAME). FAME were injected into an Agilent 6890 gas chromatograph fitted with an HP-5.5% Phenyl Methyl Siloam capillary column (30.0 mm x 25 mm, Agilent 19091J-413, USA) and substantiated by flame ionization detection (FID). The injector, detector and column temperature were set as described by Wu et al. (2007). Peaks were identified by comparing the retention times with fatty acids standards (Sigma Chemical Co, St Louis, MO, USA) and individual fatty acids were quantified by the

reference to the internal standard (19:0). Cholesterol contents of the diets were quantified against an internal standard (Sigma-Aldrich Co, Milwaukee, USA).

### *5.2.5 Data and statistical analysis*

Specific growth rate (SGR) based on dry weight, carapace width and carapace length of the newly molted C4 crabs from each diet treatment were calculated using the equation described in Chapter 3 (Section 3.2.5). Survival, development, final dry weight, carapace length, carapace width and SGR data were analyzed using one-way ANOVA after confirmation of normality and homogeneity of variances. Significant differences between treatments were determined using Tukey's test and the level of significance difference was set at $p<0.05$. Interactive effects between dietary cholesterol and PL were determined using two-way ANOVA. All statistical analysis was performed using the SPSS statistic software version 17.

## 5.3 Results

### *5.3.1 Diet cholesterol, PL and fatty acid compositions*

The analyzed contents of cholesterol and PL of the experimental diets used in this study are shown in Table 5.1. The data show that for the first experiment, in diets without cholesterol supplementation (Diet 1 to 4), cholesterol was not detected. However, for the diet without supplemental PL (Diet 1), a low level of 3 g $kg^{-1}$ PL was detected. In diets formulated with different supplemental levels of cholesterol, as expected, some deviations were also shown between the theoretical and actual analyzed value. For example, the diet formulated with 5 g $kg^{-1}$ supplemental cholesterol actually contained cholesterol ranging

from 4.7 to 5 g $kg^{-1}$ while the diets with 30 g $kg^{-1}$ supplemental PL inclusion actually contained PL ranging from 34 to 45 g $kg^{-1}$. Similarly, in the second experiment, diets formulated with 10 g $kg^{-1}$ cholesterol actually contained 9 g $kg^{-1}$ (Diet 11 and 12) while the diets with 120 g $kg^{-1}$ PL actually contained 127 g $kg^{-1}$ and 126 g $kg^{-1}$ PL (Diet 12 and 14), respectively.

The results for fatty acids analysis of the diets are shown in Table 5.2. As expected the increase in dietary supplemental PL from 0 to 90 g $kg^{-1}$ in diets used in the first experiment resulted in corresponding increases in the LOA (18:2n-6) and LNA (18:3n-3) contents. In experiment 1, the diet without PL supplementation (Diet 1) contained the lowest LOA level of 5.94 g $kg^{-1}$ while the highest level of LOA (23.68 g $kg^{-1)}$ was found in Diet 7 with 90 g $kg^{-1}$ PL supplementation. Similarly, the lowest level of LNA (0.33 g $kg^{-1}$) was detected in Diet 1 while the highest LNA level (2.29 g $kg^{-1}$) was also found in the Diet 7. In the second experiment, with the PL supplementation increasing further to 90 g $kg^{-1}$, the highest LOA and LNA were found in Diet 12 at 32.31 and 3.23 g $kg^{-1}$, respectively.

**Table 5.2** Fatty acid compositions (g $kg^{-1}$ dry weight) of the experimental diets used in the two experiments evaluating effects of dietary cholesterol and phospholipid on the performance of *P. pelagicus* early juveniles.

| Fatty acids composition | Experiment 1 | | | | | | | | Experiment 2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 14:0 | 1.40 | 1.95 | 1.75 | 1.68 | 1.54 | 1.89 | 1.72 | 1.53 | 1.99 | 1.72 | 2.18 | 1.87 | 1.76 | 1.76 |
| 15:0 | 0.13 | 0.18 | 0.19 | 0.18 | 0.16 | 0.18 | 0.18 | 0.15 | 0.20 | 0.17 | 0.23 | 0.24 | 0.18 | 0.19 |
| 16:0 | 4.14 | 7.48 | 8.21 | 9.98 | 6.39 | 8.84 | 10.15 | 5.98 | 9.30 | 9.79 | 12.56 | 12.98 | 9.75 | 11.93 |
| 18:0 | 0.75 | 1.42 | 1.67 | 2.03 | 1.24 | 1.72 | 2.07 | 1.18 | 1.87 | 2.01 | 2.56 | 2.89 | 1.93 | 2.45 |
| 16:1n7 | 2.01 | 2.78 | 2.50 | 2.44 | 2.20 | 2.73 | 2.51 | 2.14 | 2.78 | 2.49 | 2.95 | 2.87 | 2.44 | 2.52 |
| 18:1n9 | 3.97 | 6.59 | 6.84 | 7.87 | 5.37 | 7.02 | 8.04 | 5.04 | 7.26 | 7.60 | 8.85 | 10.09 | 7.33 | 8.87 |
| 18:2n6 | 5.94 | 13.92 | 16.97 | 23.40 | 12.35 | 18.71 | 23.68 | 11.04 | 19.27 | 22.43 | 25.25 | 32.21 | 22.01 | 28.84 |
| 18:3n3 | 0.33 | 1.16 | 1.62 | 2.33 | 1.10 | 1.78 | 2.29 | 0.92 | 1.79 | 2.25 | 2.40 | 3.23 | 2.17 | 2.90 |
| 20:3n-3 | 0.05 | 0.06 | 0.06 | 0.06 | 0.04 | 0.07 | 0.07 | 0.04 | 0.07 | 0.04 | 0.08 | 0.15 | 0.06 | 0.16 |
| 20:4n6 | 0.17 | 0.22 | 0.21 | 0.20 | 0.19 | 0.22 | 0.23 | 0.18 | 0.28 | 0.23 | 0.38 | 0.47 | 0.21 | 0.25 |
| 20:5n3 | 1.99 | 2.71 | 2.44 | 2.28 | 2.18 | 2.57 | 2.51 | 2.09 | 2.71 | 2.39 | 2.66 | 2.94 | 2.32 | 2.42 |
| 22:5n3 | 0.37 | 0.59 | 0.44 | 0.40 | 0.39 | 0.53 | 0.43 | 0.41 | 0.64 | 0.44 | 0.48 | 0.55 | 0.40 | 0.39 |
| 22:6n3 | 2.00 | 2.68 | 2.46 | 2.28 | 2.20 | 2.59 | 2.47 | 2.10 | 2.73 | 2.41 | 2.74 | 3.13 | 2.33 | 2.48 |
| ∑ SFA | 6.62 | 11.31 | 12.10 | 14.15 | 9.55 | 12.94 | 14.38 | 9.10 | 13.63 | 14.00 | 17.88 | 18.40 | 13.90 | 16.67 |
| ∑ MUFA | 7.50 | 22.52 | 11.39 | 12.48 | 9.27 | 11.88 | 12.71 | 8.86 | 12.38 | 12.30 | 14.46 | 16.27 | 11.78 | 13.70 |
| ∑ PUFA | 11.88 | 22.75 | 25.48 | 32.17 | 19.57 | 27.77 | 32.89 | 17.82 | 29.08 | 31.43 | 35.87 | 44.48 | 30.68 | 38.81 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (≥18:2n) | | | | | | | | | | | | | | |
| ∑ HUFA (≥20:3n) | 4.87 | 6.68 | 5.98 | 5.61 | 5.36 | 6.38 | 6.10 | 5.13 | 6.90 | 5.89 | 6.84 | 7.81 | 5.71 | 6.22 |
| ∑ n-3 | 5.55 | 8.32 | 8.02 | 8.29 | 6.81 | 8.59 | 8.72 | 6.41 | 9.02 | 8.51 | 9.44 | 11.10 | 8.22 | 9.28 |
| ∑ n-6 | 6.23 | 14.30 | 17.34 | 23.77 | 12.67 | 19.07 | 24.09 | 11.31 | 19.85 | 22.79 | 26.90 | 33.14 | 22.40 | 29.45 |
| n-3/n-6 | 0.89 | 0.58 | 0.46 | 0.65 | 0.54 | 0.45 | 0.36 | 0.57 | 0.45 | 0.37 | 0.36 | 0.33 | 0.37 | 0.32 |
| DHA/EPA | 1.01 | 0.99 | 1.01 | 1.00 | 1.01 | 1.00 | 0.98 | 1.01 | 1.01 | 1.01 | 1.03 | 1.06 | 1.00 | 1.03 |
| TOTAL | 30.87 | 63.25 | 54.96 | 64.41 | 43.76 | 58.97 | 66.09 | 40.91 | 61.99 | 63.61 | 75.05 | 86.96 | 62.06 | 75.41 |

*(∑) include minor fatty acids that are not shown in the Table

*5.3.2 Survival*

In the first experiment, none of the C1 crabs survived to the C4 stage when fed a diet with no supplemental cholesterol and PL (Diet 1) or with no supplemental cholesterol but a low level of PL at 30 g $kg^{-1}$ (Diet 2) (Fig 5.1 A). Potential compensatory effects between PL and cholesterol were indicated when comparing survival of crabs fed 3 diet treatments with no cholesterol supplementation (Diet 2, 3 and 4). Survival of crabs fed the diet supplemented with the highest level of PL at 90 g $kg^{-1}$ (Diet 4) jumped more than 60% compared to that of crabs fed the diets supplemented with lower levels of PL levels at 30 and 60 g $kg^{-1}$ (Diet 2 and 3). Conversely, supplementation of cholesterol at 5 and 10 g $kg^{-1}$ resulted in very similar survival rates (63.3-70.0%) among 5 diet treatments (Diet 5, 6, 7, 8 and 9) despite substantial differences in supplemental PL level (from 30 to 90 g $kg^{-1}$) in these diets (Fig 5.1 A). As the highest survival to the C4 stage in the first experiment (83.3%) was recorded for crabs fed Diet 10 with the highest supplemental level of both cholesterol (10 g $kg^{-1}$) and PL (90 g $kg^{-1}$), this prompted the second experiment to determine whether a further increase in dietary levels of these nutrients may lead to higher survival.

The results of the second experiment showed that further increase in cholesterol and PL supplementation to 12 and 120 g $kg^{-1}$, respectively, did not enhanced survival of the crabs (Fig 5.2). Survival of crabs fed three diets supplemented with higher cholesterol or PL levels (i.e. Diets 12, 13 and 14) was lower than that of crabs fed the diet supplemented with 10 g $kg^{-1}$ cholesterol and 90 g $kg^{-1}$ PL (Diet 11) (Fig 5.1 B).

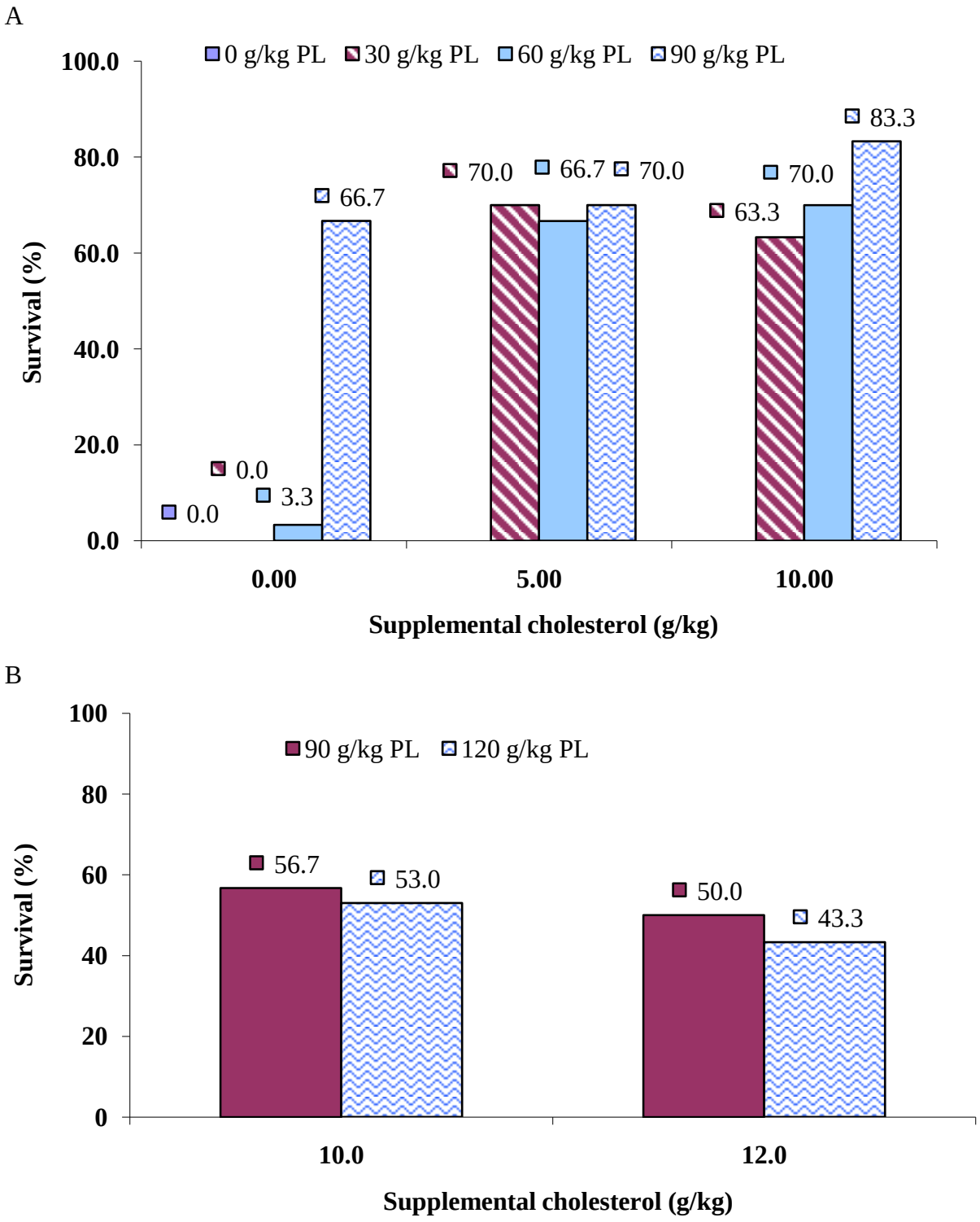



**Figure 5.1** Mean survival (%) to the fourth crab stage (C4) of the newly settled first stage *P. pelagicus* fed semi-purified diets formulated with various combinations of supplemental cholesterol and phospholipid levels in A. Experiment 1 B. Experiment 2

*5.3.3 Development time*

Among crabs fed diets with no supplemental cholesterol (Diet 1 to 4), a clear trend of reduced intermolt period of C1 to C2 *P. pelagicus* with increasing dietary PL levels was shown. The C1 intermolt duration reduced from 6.1 ± 0.5 days for the non-supplemented PL treatment (Diet 1) to 4.5 ± 0.1 days for the PL 90 g $kg^{-1}$ treatment (Diet 4) and the differences between treatments was often significant ($p<0.05$) (Table 5.3). Meanwhile, compared to diet treatments with no supplemental cholesterol, cholesterol supplementation at both 5 and 10 g $kg^{-1}$ levels combined with 30 to 90 g $kg^{-1}$ supplemental PL (Diet 5 to 10) supported significantly shortened intermolt period for C1 crabs. The only exception was with Diet 4, which had no supplemental cholesterol but a high level of PL (90 g $kg^{-1}$) supplementation. The C1 intermolt period of crabs fed this diet was virtually the same as those fed other diets with cholesterol supplementation (Diet 5 to 10) (Table 5.3).

Due to total mortality at the C2 stage, no intermolt period data is available for the crabs fed cholesterol and PL deficient diets (Diet 1 and Diet 2). Similarly, only one crab fed Diet 3 molted to the C4 stage, so the C3-C4 intermolt period data was excluded for this treatment. From C2 to C3 stages, Diet 3 had significantly longer molting interval, however no significant differences for intermolt periods of both C2 to C3 and C3 to C4 stages was detected between other treatments (Table 5.3). The cumulative mean development time from C1 to C4 was significantly affected by both dietary cholesterol and PL level (Table 5.3). The shortest development time was found for crabs fed the diets with the highest supplementation level of both cholesterol (10 g $kg^{-1}$) and PL (90 g $kg^{-1}$) (Diet 10). The second fastest development time was found in crabs fed the diet with high supplementation of PL (90 g $kg^{-}$

$^{1}$) but lower cholesterol level (5 g $kg^{-1}$) (Diet 7). Both diets supported significantly shorter development times than those for crabs fed either the diet with higher supplemented PL (90 g $kg^{-1}$) but deficient in cholesterol (Diet 4) or the diet with a high level of cholesterol (10 g $kg^{-1}$) but lower level of PL (30 g $kg^{-1}$) (Diet 8) ($p<0.05$). No significant difference was detected among all other treatments (Table 5.3).

**Table 5.3** Mean (± SE) intermolt period at each crabs stages and cumulative development time to C4 stage (in days) of *P. pelagicus* early juveniles fed semi-purified diets formulated with various combinations of cholesterol and phospholipid levels in the first experiment.

| Supplemented level | Intermolt Period | | | Cumulative development time |
|---|---|---|---|---|
| | C1-C2 | C2-C3 | C3-C4 | |
| Diet 1: 0 g $kg^{-1}$ cholesterol, 0 g $kg^{-1}$ PL | 6.1 ± 0.5[a] | - | - | - |
| Diet 2: 0 g $kg^{-1}$ cholesterol, 30 g $kg^{-1}$ PL | 5.3 ± 0.2[a,b] | - | - | - |
| Diet 3: 0 g $kg^{-1}$ cholesterol, 60 g $kg^{-1}$ PL | 4.9 ± 0.2[b,c] | 10.8 ± 2.9[a] | - | - |
| Diet 4: 0 g $kg^{-1}$ cholesterol, 90 g $kg^{-1}$ PL | 4.5 ± 0.1[c] | 6.5 ± 0.2[b] | 6.6 ± 0.2 | 17.3 ± 0.3[a] |
| Diet 5: 5 g $kg^{-1}$ cholesterol, 30 g $kg^{-1}$ PL | 4.5 ± 0.1[c] | 6.3 ± 0.2[b] | 6.4 ± 0.1 | 16.9 ± 0.2[a, b] |
| Diet 6: 5 g $kg^{-1}$ cholesterol, 60 g $kg^{-1}$ PL | 4.5 ± 0.1[c] | 5.9 ± 0.1[b] | 6.6 ± 0.3 | 16.9 ± 0.4[a, b] |
| Diet 7: 5 g $kg^{-1}$ cholesterol, 90 g $kg^{-1}$ PL | 4.4 ± 0.1[c] | 5.5 ± 0.1[b] | 5.9 ± 0.1 | 16.0 ± 0.2[b] |
| Diet 8: 10 g $kg^{-1}$ cholesterol, 30 g $kg^{-1}$ PL | 4.5 ± 0.1[c] | 6.3 ± 0.1[b] | 6.7 ± 0.3 | 17.4 ± 0.3[a] |
| Diet 9: 10 g $kg^{-1}$ cholesterol, 60 g $kg^{-1}$ PL | 4.5 ± 0.1[c] | 6.0 ± 0.3[b] | 6.5 ± 0.2 | 16.6 ± 0.4[a, b] |
| Diet 10: 10 g $kg^{-1}$ cholesterol, 90 g $kg^{-1}$ PL | 4.4 ± 0.1[c] | 5.7 ± 0.1[b] | 5.8 ± 0.1 | 15.8 ± 0.2[b] |

| | | | | |
|---|---|---|---|---|
| *Two-way ANOVA* | | | | |
| Cholesterol | 0.0001 | 0.052 | 0.077 | 0.038 |
| PL | 0.0001 | 0.261 | 0.005 | 0.0001 |
| Cholesterol x PL | 0.086 | 0.579 | 0.627 | 0.532 |

[abc] Different superscripts within a column indicate significant differences ($p<0.05$)

**Table 5.4** Mean (± SE) intermolt period at each crabs stages and cumulative development time to C4 stage (in days) of *P. pelagicus* early juveniles fed semi-purified diets formulated with various combinations of cholesterol and phospholipid levels in the second experiment. No significant differences were detected in all parameters among any diet treatments ($p>0.05$).

| Supplemented levels | Intermolt Period | | | Cumulative development time |
|---|---|---|---|---|
| | C1-C2 | C2-C3 | C3-C4 | |
| Diet 11: 10 g $kg^{-1}$ cholesterol, 90 g $kg^{-1}$ PL | 4.5 ± 0.1 | 5.9 ± 0.2 | 6.6 ± 0.5 | 16.7 ± 0.5 |
| Diet 12: 10 g $kg^{-1}$ cholesterol, 120 g $kg^{-1}$ PL | 4.5 ± 0.1 | 5.9 ± 0.2 | 5.9 ± 0.1 | 16.1 ± 0.4 |
| Diet 13: 12 g $kg^{-1}$ cholesterol, 90 g $kg^{-1}$ PL | 4.7 ± 0.1 | 6.04 ± 0.2 | 6.8 ± 0.4 | 16.9 ± 0.6 |
| Diet 14: 12 g $kg^{-1}$ cholesterol, 120 g $kg^{-1}$ PL | 4.4 ± 0.1 | 5.78 ± 0.2 | 6.2 ± 0.3 | 16.5 ± 0.6 |
| *Two-way ANOVA* | | | | |
| Cholesterol | 0.650 | 0.927 | 0.514 | 0.554 |
| PL | 0.440 | 0.481 | 0.074 | 0.294 |
| Cholesterol x PL | 0.216 | 0.467 | 0.929 | 0.871 |

In the second experiment, the further increase in supplemental cholesterol level above 10 g $kg^{-1}$ and PL level above 90 g $kg^{-1}$ did not appeared to affect the development time of early *P. pelagicus* as no significant differences were found between any treatments (Table 5.4). For both diet experiments, two-way ANOVA did not detect significant interactive effects ($p>0.05$) between dietary cholesterol and PL levels for either development time from C1 to C4 or intermolt periods of any single crab stage (Tables 5.3 and 5.4).

*5.3.4 Dry weight and carapace size of newly molted C4 stage*

In the first experiment, the highest mean dry weight (10.20 ± 0.38 mg) and carapace length (5.11 ± 0.03 mm) of newly molted C4 were again recorded for crabs fed the diet with the highest level of cholesterol (10 g $kg^{-1}$) and PL (90 g $kg^{-1}$) (Diet 10). However, no significant differences in either dry weight, or carapace size of C4 crabs were detected among any treatments (Table 5.5). Similar results were obtained for the second experiment (Table 5.6). Two-way ANOVA showed that for the both experiments, neither supplemental cholesterol nor PL had any significant effects on dry weight and carapace size of C4 crabs. It also did not detect any significant interactive effects ($p>0.05$) between the cholesterol and PL (Tables 5.5 and 5.6).

**Table 5.5** Mean (± SE) dry weight, carapace length and carapace width of newly molted 4$^{th}$ stage crabs of *P. pelagicus* fed semi-purified diets formulated with various combinations of cholesterol and phospholipid levels in the first experiment. No significant differences were detected for any of the parameters among diet treatments ($p>0.05$).

| Supplemented levels | Dry Weight (mg) | Carapace length (mm) | Carapace width (mm) |
|---|---|---|---|
| Diet 4: 0 g kg$^{-1}$ cholesterol, 90 g kg$^{-1}$ PL | 9.84 ± 0.56 | 4.95 ± 0.08 | 8.87 ± 0.21 |
| Diet 5: 5 g kg$^{-1}$ cholesterol, 30 g kg$^{-1}$ PL | 9.47 ± 0.55 | 5.06 ± 0.14 | 8.91 ± 0.21 |
| Diet 6: 5 g kg$^{-1}$ cholesterol, 60 g kg$^{-1}$ PL | 9.96 ± 0.58 | 5.10 ± 0.08 | 9.14 ± 0.15 |
| Diet 7: 5 g kg$^{-1}$ cholesterol, 90 g kg$^{-1}$ PL | 9.88 ± 0.51 | 5.08 ± 0.05 | 8.95 ± 0.10 |
| Diet 8: 10 g kg$^{-1}$ cholesterol, 30 g kg$^{-1}$ PL | 9.2 ± 0.38 | 5.02 ± 0.05 | 8.93 ± 0.08 |
| Diet 9: 10 g kg$^{-1}$ cholesterol, 60 g kg$^{-1}$ PL | 10.13 ± 0.32 | 5.00 ± 0.05 | 8.88 ± 0.07 |
| Diet 10: 10 g kg$^{-1}$ cholesterol, 90 g kg$^{-1}$ PL | 10.20 ± 0.38 | 5.11 ± 0.03 | 9.03 ± 0.09 |
| *Two-way ANOVA* | | | |
| Cholesterol | 0.943 | 0.267 | 0.731 |
| PL | 0.250 | 0.748 | 0.810 |
| Cholesterol x PL | 0.832 | 0.727 | 0.454 |

**Table 5.6** Mean dry weight, carapace length and carapace width (mean ± SE) of newly molted 4$^{th}$ stage crabs of *P. pelagicus* fed semi-purified diets formulated with various combinations of cholesterol and phospholipid levels in the second experiment. No significant differences were detected for any of the parameters among any diet treatments ($p>0.05$).

| Supplemented levels | Dry weight (mg) | Carapace length (mm) | Carapace width (mm) |
|---|---|---|---|
| Diet 11: 10 g kg$^{-1}$ cholesterol, 90 g kg$^{-1}$ PL | 8.84 ± 0.38 | 5.04 ± 0.04 | 8.96 ± 0.12 |
| Diet12: 10 g kg$^{-1}$ cholesterol, 120 g kg$^{-1}$ PL | 8.55 ± 0.45 | 5.04 ± 0.08 | 8.76 ± 0.11 |
| Diet 13: 12 g kg$^{-1}$ cholesterol, 90 g kg$^{-1}$ PL | 8.14 ± 0.70 | 4.87 ± 0.06 | 8.59 ± 0.13 |
| Diet 14: 12 g kg$^{-1}$ cholesterol, 120 g kg$^{-1}$ PL | 7.79 ± 0.34 | 4.89 ± 0.06 | 8.62 ± 0.13 |
| *Two-way ANOVA* | | | |
| Cholesterol | 0.142 | 0.011 | 0.043 |
| PL | 0.514 | 0.968 | 0.492 |
| Cholesterol x PL | 0.951 | 0.930 | 0.337 |

*5.3.5 Specific growth rate*

The growth of early *P. pelagicus* in first the experiment when measured as specific growth rate (SGR), was effectively improved by addition of cholesterol and phospholipid to the diet (Table 5.7). The highest SGR (% $day^{-1}$) for dry weight and carapace length came from the diet treatments in which crabs were fed diets supplemented with 90 g $kg^{-1}$ PL and cholesterol in the diet at any level. Often, SGR for carapace length and carapace width was significantly higher than for crabs fed diets that were supplemented with no cholesterol or PL at 30 g $kg^{-1}$ when they were fed diets supplemented with 90 g $kg^{-1}$ PL (Table 5.7).

**Table 5.7** Mean (± SE) specific growth rate (SGR) of dry weight, carapace width and carapace length of newly molted 4$^{th}$ stage crabs of *P. pelagicus* fed semi-purified diets formulated with various combinations of cholesterol and phospholipid levels in the first experiment.

| Supplemented levels | Specific growth rate (SGR) | | |
|---|---|---|---|
| | Dry Weight (% day$^{-1}$) | Carapace length (% day$^{-1}$) | Carapace width (% day$^{-1}$) |
| Diet 4: 0 g kg$^{-1}$ cholesterol, 90 g kg$^{-1}$ PL | 12.00 ± 0.37$^{ab}$ | 3.69 ± 0.09$^{ab}$ | 5.63 ± 0.14$^{a}$ |
| Diet 5: 5 g kg$^{-1}$ cholesterol, 30 g kg$^{-1}$ PL | 12.02 ± 0.37$^{ab}$ | 3.88 ± 0.16$^{abc}$ | 5.77 ± 0.14$^{ab}$ |
| Diet 6: 5 g kg$^{-1}$ cholesterol, 60 g kg$^{-1}$ PL | 11.69 ± 0.58$^{a}$ | 3.95 ± 0.09$^{abc}$ | 6.20 ± 0.19$^{b}$ |
| Diet 7: 5 g kg$^{-1}$ cholesterol, 90 g kg$^{-1}$ PL | 13.03 ± 0.34$^{ab}$ | 4.16 ± 0.07$^{c}$ | 6.15 ± 0.07$^{b}$ |
| Diet 8: 10 g kg$^{-1}$ cholesterol, 30 g kg$^{-1}$ PL | 11.56 ± 0.23$^{a}$ | 3.74 ± 0.06$^{ab}$ | 5.62 ± 0.05$^{a}$ |
| Diet 9: 10 g kg$^{-1}$ cholesterol, 60 g kg$^{-1}$ PL | 12.71 ± 0.19$^{ab}$ | 3.90 ± 0.06$^{abc}$ | 5.86 ± 0.05$^{ab}$ |
| Diet 10: 10 g kg$^{-1}$ cholesterol, 90 g kg$^{-1}$ PL | 13.31 ± 0.24$^{b}$ | 4.21 ± 0.04$^{c}$ | 6.23 ± 0.06$^{b}$ |
| *Two-way ANOVA* | | | |
| Cholesterol | 0.114 | 0.001 | 0.001 |
| PL | 0.207 | 0.001 | 0.001 |
| Cholesterol x PL | 0.065 | 0.542 | 0.205 |

$^{ab}$ Different superscripts within a column indicate significant differences ($p<0.05$)

**Table 5.8** Mean (± SE) specific growth rate (SGR) of dry weight, carapace width and carapace length of newly molted 4$^{th}$ stage crabs of *P. pelagicus* fed semi-purified diets formulated with various combinations of cholesterol and phospholipid levels in the second experiment.

| Supplemented levels | Specific growth rate | | |
|---|---|---|---|
| | Dry weight (% day$^{-1}$) | Carapace length (% day$^{-1}$) | Carapace width (% day$^{-1}$) |
| Diet 11: 10 g kg$^{-1}$ cholesterol, 90 g kg$^{-1}$ PL | 11.28 ± 0.26 | 3.93 ± 0.05$^{ab}$ | 5.69 ± 0.08$^{ab}$ |
| Diet 12: 10 g kg$^{-1}$ cholesterol, 120 g kg$^{-1}$ PL | 11.47 ± 0.34 | 4.05 ± 0.09$^{a}$ | 5.76 ± 0.08$^{a}$ |
| Diet 13: 12 g kg$^{-1}$ cholesterol, 90 g kg$^{-1}$ PL | 10.51 ± 0.51 | 3.67 ± 0.07$^{b}$ | 5.37 ± 0.09$^{b}$ |
| Diet 14: 12 g kg$^{-1}$ cholesterol, 120 g kg$^{-1}$ PL | 10.42 ± 0.25 | 3.69 ± 0.08$^{b}$ | 5.41 ± 0.10$^{b}$ |
| *Two-way ANOVA* | | | |
| Cholesterol | 0.015 | 0.001 | 0.001 |
| PL | 0.018 | 0.309 | 0.529 |
| Cholesterol x PL | 0.149 | 0.438 | 0.446 |

$^{abc}$ Different superscripts within a column indicate significant differences ($p<0.05$)

Meanwhile, significance differences of SGR in experiment 2 was only detected on carapace length and carapace width and it was the highest when crabs were fed diets that were supplemented with cholesterol and PL at 10 g $kg^{-1}$ and 120 g $kg^{-1}$, respectively. However the significant differences was not detected between this treatment and crabs that were fed with diets that were supplemented with same level of cholesterol but lower level of PL at 90 g $kg^{-1}$.

**5.4 Discussion**

The effects of varying dietary cholesterol and phospholipid levels on the performance of various commercially important crustaceans have been the subjects of intensive study since the 1970's. The results of these studies have suggested that dietary cholesterol and phospholipid requirements of different crustaceans are largely species-specific and they may vary substantially depending on developmental stage, dietary status and experimental conditions (Coutteau et al., 1997; Gong et al., 2000b); however, it is generally acknowledged that, to support normal survival, development and growth, crustaceans need to acquire both PL and cholesterol exogenously.

In the first experiment, diets with PL supplementation lower than 60 g $kg^{-1}$ and with cholesterol in the diets (5 and 10 g $kg^{-1}$) supported improved survival of *P. pelagicus* compared to when they were fed a similar level of PL in diets deficient in cholesterol. Inadequate dietary cholesterol has been reported to cause inferior survival in other crustaceans (D'Abramo et al., 1984; Chen and Jenn, 1991; Sheen et al., 1994b; Sheen, 2000) and this has been attributed to a lack of an ability in crustaceans to synthesize cholesterol

from precursors such as acetate and mevalonate (Teshima, 1997). A possible explanation for this is that unlike mammals, crustaceans may not posses one or most of the enzymes, such as squalene synthase/monooxygenase, HMG-CoA synthase/reductase and lanosterol synthase/dimethylase, that are required for precursors such as mevalonate to be converted to cholesterol (Brown and Goldstein, 1978).

Interestingly, despite deficient dietary cholesterol, survival of crabs fed the diet supplemented with a high level of PL (Diet 4, PL formulation at 90 g $kg^{-1}$; actual analyzed value; 103 g $kg^{-1}$) was similar to that of crabs fed diets with cholesterol supplementation. This indicates that juvenile *P. pelagicus* crabs may have the ability to compensate for the lack of cholesterol in their diets when PL is sufficiently high. Similar results have also been reported for Pacific white shrimp, *L. vannamei*, juveniles (Gong et al., 2000b). High PL supplementation elevated the levels of polyunsaturated fatty acids, particularly LOA and LNA, in the diets and this may improve survival in *P. pelagicus* juveniles observed in this study. Kanazawa et al. (1985) suggested that the effects of dietary phospholipid on survival and growth of crustaceans depends on the fatty acids components of the PL to some extent.

Similarly, deficiency in dietary cholesterol in diets also resulted in significantly prolonged intermolt period of C1 *P. pelagicus* when PL supplementation was lower than 60 g $kg^{-1}$. Sheen (2000) also reported that when juvenile mud crabs, *S. serrata,* were fed diets deficient in cholesterol, low molting frequency resulted. This may be due to restriction in the conversion of cholesterol to steroid and molting hormones thus leading in delayed development (Teshima and Kanazawa, 1983). In the first experiment, among diets deficient

in cholesterol, increasing dietary PL from 0 to 90 g $kg^{-1}$ led to a clear trend of enhanced development with shorter intermolt period in C1 crabs. Inclusion of the highest level of PL (90 g $kg^{-1}$) combined with cholesterol levels at 5 and 10 g $kg^{-1}$ also resulted in the two shortest cumulative development times to C4 (16.0 ± 0.2 and 15.8 ± 0.2 days, respectively) among all diet treatments. Using radioactively labeled cholesterol, it has been demonstrated with the Kuruma prawn, *M. japonicus*, that when diets are deficient in PL, cholesterol was retained longer in the midgut of the prawn and was incorporated into hemolymph. The addition of 3% soybean lecithin (PL) in the diet, however, accelerated the occurrence of cholesterol and cholesterol esters in the hemolymph (Teshima and Kanazawa, 1986). Increasing levels of PL in the diet is thought to enhance absorption and speed up the transport of cholesterol. This facilitates higher efficiency in the utilization of cholesterol as the precursor of molting hormones, which positively influences the molting cycle.

The capacity of PL to increase absorption of dietary cholesterol is likely to depend on the degree of saturation of the acyl moiety as has been shown in Sprague-Dawley rats. When the rats were fed soybean phosphatidylcholine (PC) containing 61% of 18:2 fatty acids, cholesterol absorption in lymph canula was significantly improved compared to those fed egg PC or hydrogenated PC, which containing 18% and 0% 18:2 fatty acids, respectively (Jiang et al., 2001). Although no interaction effect was observed on the addition of PL and cholesterol simultaneously, the shorter intermolt periods observed for crabs fed diets containing high levels of PL in the present study are probably linked with high levels of LOA (18:2n-6) and LNA (18:3n-3) associated with PL level in the diets. In penaeid prawns, such as *Penaeus penicillatus* (Chen and Jenn, 1991) and *P. monodon*, the addition of dietary

phospholipid significantly improved growth but was not affected by dietary cholesterol level. In the present study, however, except those cholesterol deficient diet treatments in which total mortality occurred prior to C4, both dietary cholesterol and PL level were found not to significantly affected growth of *P. pelagicus* early juveniles although a trend of increasing dry weight with increasing dietary PL level at both 5 and 10 g $kg^{-1}$ cholesterol levels appeared to existed.

Similarly, in the second experiment, both dietary cholesterol and PL were found not to significantly affect the growth of *P. pelagicus* early juveniles; a trend of decreasing dry weight with increasing dietary PL and cholesterol was indicated. In addition, crabs fed diets formulated with cholesterol and PL higher than 10 g $kg^{-1}$ and 90 g $kg^{-1}$, respectively, also had lower survival. This negative growth response may be explained by the nutrient-response curve theory where when animals fed nutrients at their optimal level shows no further beneficial effects when fed higher levels of the nutrient (Mercer, 1982). It has also been demonstrated that provision of excessive levels of lipids in the diets of crustaceans (*M. rosenbergii* and *P. monodon*) adversely affects lipids utilization and retards growth and lipid storage capacity (Sheen and D'Abramo, 1991; Glencross et al., 2002c).

In summary, the study demonstrated that dietary inclusion of cholesterol and PL are is necessary to maximize growth, survival and development of early juvenile *P. pelagicus.* Cholesterol deficiency often led to total mortality of early *P. pelagicus*. However, it appears that supplementation of PL at high levels could largely compensate for cholesterol deficient. Dietary PL requirements from the soybean source was suggested to be between 12.5 g $kg^{-1}$ to

65 g $kg^{-1}$ for crustaceans (Coutteau *et al.*, 1997), but the results of this study suggested that higher PL levels (around 90 g $kg^{-1}$) are required for the best performance of early *P. pelagicus*. The results are an important step towards formulating practical diets for *P. pelagicus.*

# CHAPTER 6

## Assessment of survival, development and growth of *Portunus pelagicus* early juveniles, fed semi-purified diets supplemented with different levels of triglyceride

---

### 6.1 Introduction

Among the major nutrients, dietary lipids is one of the most important for crustaceans, providing them with concentrated energy, cell membrane components and also the precursors for important steroids including molting and reproductive hormones (Lim et al., 1997; Oliva-Teres, 2000). Animal lipids can be divided into two major groups, polar lipids and neutral lipids, based on their degree of affiliation with water being either hydrophilic (polar lipid) or hydrophobic neutral lipid (Gurr and Harwood, 1991). In many nutritional studies, dietary lipids refer to the addition of neutral lipid which could be in the form of triglyceride, ethyl esters, methyl esters and free fatty acids (Castell and Covey, 1976; Davis and Robinson, 1986; Sheen and D'Abramo, 1991; Sheen et al., 1994a; Glencross and Smith, 1997; Sheen, 1997; Sheen and Wu, 1999).

Optimal dietary lipids level has been shown to be varied among crustaceans. For example, when supplemented as a mixture of cod liver and corn oil at 2:1, the optimal level was found to be between 2% to 10% for giant freshwater prawn, *Macrobrachium rosenbergii* juveniles (Sheen and D'Abramo, 1991) and 3.6 to 9.6% for Chinese prawn, *Penaeus chinensis,* juveniles (Sheen, 1997). However, when supplemented as cod liver oil only, the optimal level was 5% for adult American lobster, *Homarus americanus* (Castell and Covey,

1976) and when only menhaden fish oil was used, it was 0 to 6% as for white crayfish, *Procambarus acutus* juveniles (Davis and Robinson, 1986). Separate studies on black tiger prawn, *Penaeus monodon* juveniles, showed similar dietary lipid requirements ranging from 4.5 to 10.5% when a mixture of marine oil, plant oil and purified fatty acids was used to supplemented the diets (Glencross et al., 2002c) and 4% to 11.3% when a mixture of cod liver oil and corn oil in a ratio of 2:1 was used (Sheen et al., 1994a). In most of these studies, supplementation of lipid exceeding optimal dietary levels was shown to impair growth rate; excess dietary lipid levels have been associated with nutrient imbalances and toxic products from lipid oxidation (Akiyama et al., 1992). Mud crab, *Scylla serrata*, juveniles, however, appear to have greater ability than other crustaceans to utilize high dietary lipids levels, as no adverse effects on growth or development was demonstrated when crabs were fed diets containing up to 13.8% lipid (fish oil:corn oil; 2:1) (Sheen and Wu, 1999).

On the other hand, diets containing low or sub-optimal levels of lipid also resulted in reduced growth in adult, *H. americanus* (Castell and Covey, 1976) and *M. rosenbergii* juveniles (Sheen and D'Abramo, 1991) which is likely to be linked to inadequate supply of essential fatty acids in the diets. Essential fatty acids, such as linoleic acid (LOA; 18:2n-6), linolenic acid (LNA; 18:3n-3), ecosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (22:6n-3) and arachidonic acid (ARA; 20:4n-6), are needed in promoting growth and survival of crustaceans and have to be provided exogenously because crustaceans generally lack the ability to synthesize them *de novo* (Baum et al., 1990; Merican and Shim, 1997; Glencross and Smith, 1999; Glencross et al., 2002d; L.Gonzalez-Felix et al., 2002).

Past studies have clearly demonstrated the highly species-specific nature of dietary lipid requirements of crustaceans. It is therefore necessary to conduct relevant research for any aquaculture candidate species, such as *P. pelagicus*, to determine their dietary lipid requirements. This experiment used semi-purified diets supplemented with different levels of triglyceride to assess the effects of dietary triglyceride levels on survival, development and growth of *P. pelagicus* early juveniles.

## 6.2 Materials and Method

### *6.2.1 Source of crabs*

Broodstock capture and husbandry and larval culture protocol as were described in Chapter 2 (Section 2.2.1).

### *6.2.2 Diet preparation*

Based on previous studies (Chapter 5) six iso-lipidic diets were formulated to contain phospholipid (PL) at optimal level of 90 g $kg^{-1}$ of diet dry weight and cholesterol at 10 g $kg^{-1}$ of diet weight (Table 6.1). The triglyceride was supplied as a mixture of fish oil and corn oil at a ratio of 2:1 but at various levels of 0, 20, 40, 60, 80, 100 g $kg^{-1}$ of diet weight, respectively (Table 6.1). The diets were made iso-energetic by manipulating the level of carbohydrate (corn starch) with α-cellulose used as filler. Information provided by the manufacturer showed that the phospholipid used in this study was derived from soybean with purity >90% and contained 55% purified phosphatidylcholine (PC) and 20% phosphatidylethanolamine (PE). The phospholipid also contained 65% >18:2 polyunsaturated fatty acids (PUFA) (of total fatty acids).

To prepare the diets, fish meal was pulverized and sieved through 100 µm mesh before being added to a chloroform/methanol solution (2:1, v:v) (Folch et al., 1957). The solution was then filtered with the aid of a vacuum pump and this defatting process was repeated three times to ensure maximum removal of lipid from the fish meal. All dry and wet ingredients of the diets were then mixed well in separate mixing bowls before being combined and throughout blended using an electric mixer. Subsequently, agar the binder used in this study was dissolved in 100 mL distilled water heated to $\geq 80^{o}$C. As the agar solution turned clear, it was allowed to cool to $40^{o}$C when the diet mixture was added to the agar solution. The diet mixture quickly became gelatinized when temperature reduced to $37^{o}$C and the gelatinized diets were then cut into small pieces of approximately 2 $mm^3$ and stored in a freezer at $-20^{o}$C until used.

**Table 6.1** Formulation of the experimental diets (g $kg^{-1}$) supplemented with different levels of triglyceride. The analyzed contents of total lipid in the diets are also shown at the lower part of the Table. Diet formulation is based on Holme (2007a), Sheen and Wu (1999) and Genodepa (2004).

| Ingredient | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|---|---|
| Defatted fish meal[1] | 460 | 460 | 460 | 460 | 460 | 460 |
| Triglyceride | | | | | | |
| (fish oil[2a]:corn oil[2b]; 2:1) | 0 | 20 | 40 | 60 | 80 | 100 |
| Cholesterol[2c] | 10 | 10 | 10 | 10 | 10 | 10 |
| Phospholipid[2d] | 90 | 90 | 90 | 90 | 90 | 90 |
| Vitamin mixture[3a] | 40 | 40 | 40 | 40 | 40 | 40 |
| Mineral Mixture[3b] | 40 | 40 | 40 | 40 | 40 | 40 |
| Choline chloride[2e] | 10 | 10 | 10 | 10 | 10 | 10 |
| DCP[2f] | 6 | 6 | 6 | 6 | 6 | 6 |
| Agar[2g] | 120 | 120 | 120 | 120 | 120 | 120 |
| Starch[2h] | 222 | 177 | 133 | 88 | 44 | 0 |
| Cellulose[2i] | 2 | 26 | 51 | 75 | 99 | 124 |
| *Analyzed* | | | | | | |
| Total Lipid | 119 | 126 | 155 | 176 | 210 | 228 |

[1] Skretting Tasmania

[2] Sigma-Aldrich Pty Ltd [a]from menhaden F8020 [b]C8267 [c]C8667 [d]P3644 [e]98% powder C7527 [f]dibasic calcium phosphate C4131 [g]A7002 [h]S4126 (corn) [i]C8002 alpha

[3] Rabar Pty Ltd [a]ZZ600 DPI, each 1kg contains: vitamin A 2miu, vitamin D3 0.8miu, vitamin E 40g, vitamin K 2.02g, inositol 50g, vitamin B3 30.40g, vitamin B5 9.18g, vitamin B9 2.56g, vitamin B2 4.48g, vitamin B12 0.004g, biotin 0.1g, vitamin B6 4g, vitamin B1 3.4g, vitamin C 44.4g, para amino benzoic acid 20g, tixosil 5g, antioxidant 30g [b]ZZ603 DO 067 DPI, each 1kg contains: copper 1g, cobalt 100mg, magnesium 59.4mg, manganese 5g. iodine 800mg, selenium 20mg, iron 8mg, zinc 20g, aluminium 100mg, chromium 100mg

*6.2.3 Experimental design and set-up*

Newly metamorphosed C1 crabs were obtained as described in Chapter 2 (Section 2.2.2). They were individually placed into 750 mL circular culture vessels to start the experiment. Three replicates were set up for each dietary treatment with 15 individually cultured C1 crabs as a replicate, bringing the total culture units to 270. The individual housing of the experimental crabs was necessary to eliminate any compounding effects of cannibalism and to allow precise recording of consecutive intermolt periods for each crab. The mean (± SE) initial dry weight was 1.28 ± 0.04 mg while the mean carapace width and length was 3.27 ± 0.03 mm and 2.46 ± 0.21 mm, respectively.

Throughout the experiment, a 100% water exchange was carried out daily in the morning for all culture units by transferring crabs individually into new culture vessels and any molts and mortality were recorded. After the water exchange, all surviving crabs were fed to satiation with their designated diets and the used culture vessels were sanitized in hypochlorite solution and dried overnight for use in the following morning. Throughout the experiment, water temperature and salinity were monitored daily and maintained at 28 ± 1$^{o}$C and 30 ± 2‰, respectively, while photoperiod was set at 14:10 (light: dark).

Towards the end of the experiment, any crabs that had molted to the 4$^{th}$ stage crabs (C4) at the morning inspection were removed from their culture unit in the afternoon for the measurement of carapace size and dry weight. Carapace measurements and dry weight determination were carried out as described in Chapter 3 (Section 3.2.3). The experiment was terminated when all crabs were either molted to the C4 stage or had died.

*6.2.4 Diet lipids and fatty acids content analysis*

To determine the total lipid content of the experimental diets, they were first extracted using methanol and chloroform (2:1, v/v) based on the method of Folch (1957). The lipid aliquot was separated and quantified gravimetrically. Fatty acid compositions of the triglyceride used in diet ingredients and experimental diets were analyzed following transesterification with 0.4 M KOH-methanol to produce fatty acid methyl esters (FAME). FAME were injected into an Agilent 6890 gas chromatograph fitted with an HP-5.5% Phenyl Methyl Siloam capillary column (30.0 mm x 25 mm, Agilent 19091J-413, USA) and substantiated by flame ionization detection (FID). The injector, detector and column temperature were set as described by Wu et al. (2007). Peaks were identified by comparing retention times with fatty acid standards (Sigma Chemical Co, St Louis, MO, USA) and individual fatty acids were quantified by reference to the internal standard (19:0).

*6.2.5 Data and statistical analysis*

Specific growth rate (SGR) based on dry weight, carapace width and carapace length of the newly molted C4 crabs for each diet treatment were calculated using the equation described in Chapter 3 (Section 3.2.5). Survival (%), development time (days) and SGR data of dry weight, carapace width and length (% $day^{-1}$) were analyzed using one-way ANOVA after confirmation of normality and homogeneity of variances. Survival data were arcsine transformed before analysis. Significant differences between treatments were determined using Tukey's test and the level of significance difference was set at $p<0.05$. All statistics were performed using the SPSS statistic software, version 17.

## 6.3 Results

### *6.3.1 Dietary lipid and fatty acid contents*

Table 6.2 shows the fatty acid profiles of the fish oil and corn oil used in this study and the fatty acid compositions of the experimental diets are shown in Table 6.3. The results show that fish oil contained a more diverse and balanced fatty acid profile compared to corn oil which contained predominantly polyunsaturated fatty acids (PUFA) at 57.89 g 100 $g^{-1}$ and monounsaturated fatty acids (MUFA) at 28.39 g 100 $g^{-1}$. Total lipid contents of the experimental diets increased concomitantly with increased level of triglyceride addition (fish oil:corn oil; 2:1) ranging from the lowest level of 119 g $kg^{-1}$ in the basal diet, to 126, 155, 176, 209 and 228 g $kg^{-1}$, for diets supplemented with triglyceride at 20, 40, 60, 80 and 100 g $kg^{-1}$, respectively (Table 6.1).

While fatty acid compositions of the experimental diets generally reflected the composition of the triglyceride included in them, they were overall at higher levels because some originated from the phospholipid components of the diet. Despite no addition of fish oil and corn oil, the basal diet contained the highest percentage of PUFA, mainly linoleic acid (LOA; 18:2n-6), likely due to phospholipid addition. In other experimental diets, the PUFA percentage decreased as a result of triglyceride addition (Table 6.3). As a result, diets with higher triglyceride inclusion contained higher percentages of HUFA, mainly ecosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). The n3 to n6 fatty acids ratio increased slightly at each incremental addition of triglyceride to the diets, however, the EPA and DHA ratio remain relatively stable among all diets (Table 6.3).

**Table 6.2** Fatty acid compositions (g 100 $g^{-1}$) of the fish oil and corn oil used in the formulation of experimental diets fed to *Portunus pelagicus* early juveniles.

| Major fatty acids | Fish oil | Corn oil |
|---|---|---|
| 14:0 | 7.76 | 0.06 |
| 16:0 | 16.56 | 10.99 |
| 16:1n-7 | 11.0 | - |
| 16: 1n-5 | 0.38 | - |
| 17:0 | 0.54 | 0.12 |
| 18:0 | 2.90 | 1.95 |
| 18:1n-9 | 8.43 | 27.73 |
| 18:1n-7 | 2.86 | - |
| 18:2n-6 | 5.15 | 57.00 |
| 18:3n-3 | 1.26 | 0.90 |
| 20:1n-9 | 1.72 | 0.36 |
| 20:4n-6 | 0.91 | - |
| 20:4n-3 | 1.59 | - |
| 20:5n-3 | 11.53 | - |
| 22:6n-3 | 11.84 | - |
| | | |
| ∑ SFA | 28.98 | 13.12 |
| ∑ MUFA | 26.54 | 28.39 |
| ∑ PUFA (≥ 18:2n) | 10.55 | 57.89 |
| ∑ HUFA (≥ 20:3n) | 28.49 | - |
| | | |
| ∑ n-3 | 31.85 | 0.89 |
| ∑ n-6 | 6.70 | 57.00 |
| n-3/n-6 | 4.77 | 0.01 |
| DHA/EPA | 1.03 | |

* ∑ include minor fatty acids percentage that is not shown in the Table

**Table 6.3** Fatty acid compositions (g 100 $g^{-1}$) of the experimental diets with different level of supplemented triglyceride (fish oil:corn oil ; 2:1)

| Major fatty acids | Diet 1<br>0 g $kg^{-1}$ | Diet 2<br>20 g $kg^{-1}$ | Diet 3<br>40 g $kg^{-1}$ | Diet 4<br>60 g $kg^{-1}$ | Diet 5<br>80 g $kg^{-1}$ | Diet 6<br>100 g $kg^{-1}$ |
|---|---|---|---|---|---|---|
| 14:0 | 0.80 | 1.65 | 2.22 | 2.61 | 3.25 | 3.37 |
| 16:0 | 18.07 | 17.32 | 16.46 | 16.24 | 16.55 | 16.28 |
| 16:1n-7 | 0.49 | 2.01 | 3.13 | 3.70 | 4.42 | 4.63 |
| 18:0 | 4.26 | 3.81 | 3.50 | 3.46 | 3.24 | 3.25 |
| 18:1n-9 | 10.46 | 11.84 | 12.04 | 12.52 | 12.66 | 13.11 |
| 18:1n-7 | 1.65 | 1.80 | 1.88 | 1.93 | 1.99 | 2.04 |
| 18:2n-6 | 54.08 | 48.51 | 42.67 | 36.69 | 37.00 | 35.74 |
| 18:3n-3 | 0.00 | 0.55 | 0.92 | 1.11 | 1.32 | 1.27 |
| 18:4n-3 | 0.53 | 0.36 | 0.25 | 0.08 | 0.27 | 0.52 |
| 20:1n-9 | 0.00 | 0.04 | 0.31 | 0.36 | 0.21 | 0.52 |
| 20:4n-6 | 0.49 | 0.25 | 0.57 | 0.34 | 0.35 | 0.38 |
| 20:5n-3 | 1.06 | 2.07 | 3.33 | 4.05 | 4.63 | 5.06 |
| 22:5n-3 | 0.00 | 0.34 | 0.62 | 0.79 | 0.83 | 0.94 |
| 22:6n-3 | 1.11 | 2.18 | 3.41 | 4.17 | 4.55 | 5.07 |
| | | | | | | |
| ∑ SFA | 23.56 | 23.14 | 23.26 | 23.10 | 23.93 | 23.69 |
| ∑ MUFA | 12.61 | 16.17 | 18.56 | 19.66 | 20.52 | 21.23 |
| ∑ PUFA (≥ 18:2n) | 63.85 | 60.06 | 57.02 | 55.18 | 53.76 | 53.00 |
| ∑ HUFA (≥ 20:3n) | 2.66 | 5.19 | 8.48 | 10.00 | 11.23 | 12.41 |
| | | | | | | |
| ∑ n-3 | 3.19 | 5.75 | 9.22 | 10.54 | 11.99 | 12.34 |
| ∑ n-6 | 54.57 | 48.76 | 43.35 | 40.33 | 37.84 | 38.95 |
| n-3/n-6 | 0.06 | 0.12 | 0.21 | 0.26 | 0.32 | 0.32 |
| DHA/EPA | 1.05 | 1.05 | 1.02 | 1.03 | 0.98 | 1.00 |

* ∑ include minor fatty acids percentage that is not shown in the Table

*6.3.2 Survival, development and growth of the crabs*

The overall survival of *P. pelagicus* from newly settled C1 juveniles to the C4 stage was relatively high for all diet treatments (≥ 88%) including those fed the basal diet without triglyceride supplementation. Highest survival of 95.6% was obtained for crabs fed the diet supplemented with the highest level of triglyceride (40 g $kg^{-1}$) where the total lipid level of the diet was 155 g $kg^{-1}$. No significant differences were detected for survival between diet treatments ($p>0.05$) (Fig 6.1).

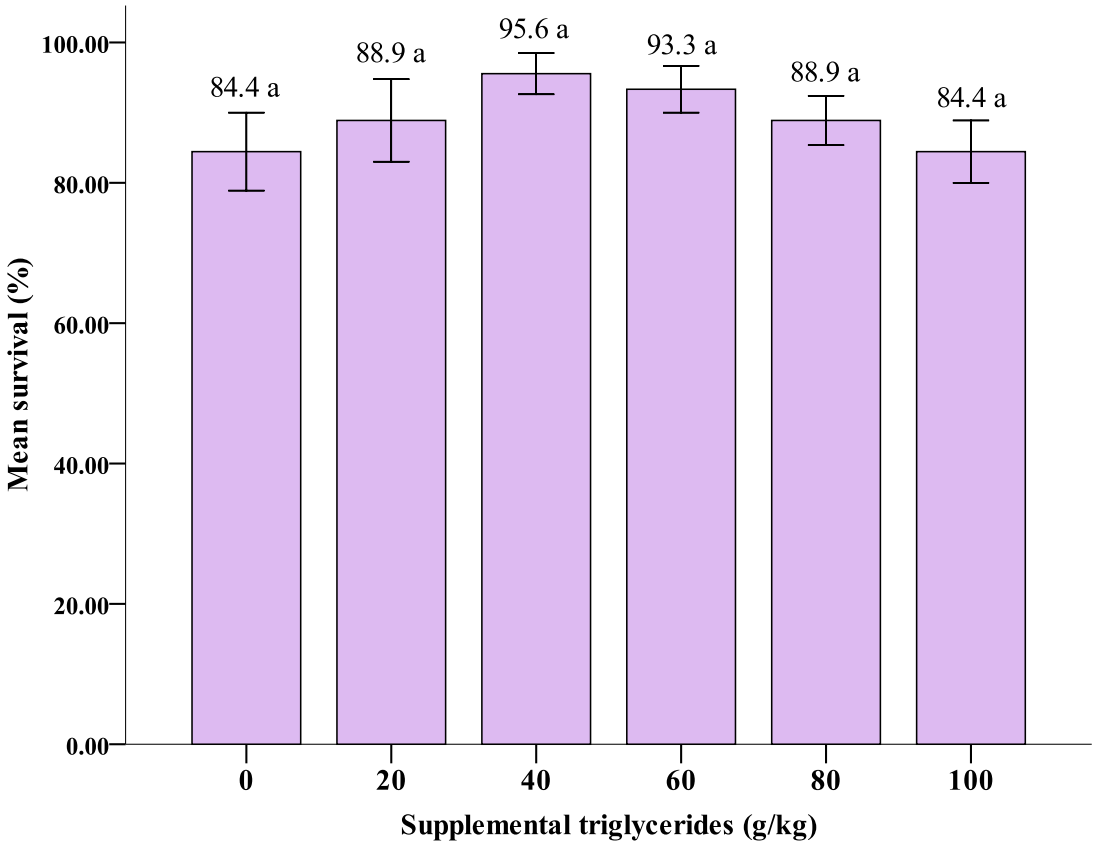



**Figure 6.1** Mean survival (%) of *Portunus pelagicus* fed diets containing different levels of triglyceride (fish oil:corn oil; 2:1) throughout the culture period. No significant differences were found between the treatments ($p>0.05$).

Significant differences in development time were detected for the intermolt period from C3 stage to C4 stage among diet treatments. Crabs fed the diet with 20 g $kg^{-1}$ triglyceride supplementation (analyzed total lipids at 126.0 g $kg^{-1}$ diet dry weight) developed significantly faster from C3 to C4 than those in other diet treatments ($p<0.05$). However, intermolt periods from C1 to C2, and from C2 to C3, did not show any significant differences among any diet treatments ($p<0.05$) (Table 6.4).

**Table 6.4** Mean (± SE) intermolt period for each molt stages and cumulative development time from C1 to C4 (in days) of *Portunus pelagicus* fed semi-purified diets containing different levels of triglyceride (fish oil : corn oil; 1:1).

| Supplemented triglyceride level (g $kg^{-1}$) | C1 to C2 | C2 to C3 | C3 to C4 | Cumulative development time (C1 to C4) |
|---|---|---|---|---|
| 0 | 3.4 ± 0.1 | 4.9 ± 0.1 | 5.5 ± 0.1[a] | 13.8 ± 0.3 |
| 20 | 3.5 ± 0.1 | 4.7 ± 0.1 | 4.9 ± 0.1[b] | 13.1 ± 0.2 |
| 40 | 3.3 ± 0.1 | 4.6 ± 0.1 | 5.2 ± 0.1[a] | 13.1 ± 0.2 |
| 60 | 3.5 ± 0.1 | 4.7 ± 0.1 | 5.2 ± 0.1[a] | 13.3 ± 0.2 |
| 80 | 3.5 ± 0.1 | 4.9 ± 0.1 | 5.5 ± 0.2[a] | 13.8 ± 0.3 |
| 100 | 3.4 ± 0.1 | 4.8 ± 0.1 | 5.4 ± 0.2[a] | 13.4 ± 0.2 |

[ab] Different superscripts within a column indicate significant differences ($p<0.05$)

**Table 6.5** Mean (± SE) final dry weight, carapace width and length and mean specific growth rate (SGR) (± SE) of newly molted 4$^{th}$ stage juvenile *Portunus pelagicus* fed diets containing different levels of triglyceride (fish oil:corn oil at 2:1).

| Supplemented triglyceride level (g kg$^{-1}$) | Newly molted C4 stage | | | Specific growth rate (SGR) | | |
|---|---|---|---|---|---|---|
| | Dry weight (mg) | Carapace width (mm) | Carapace length (mm) | Dry weight (% day$^{-1}$) | Carapace width (% day$^{-1}$) | Carapace length (% day$^{-1}$) |
| 0 | 5.93 ± 0.41$^{a}$ | 7.71 ± 0.16$^{ab}$ | 4.39 ± 0.08 | 10.86 ± 0.51$^{a}$ | 6.20 ± 0.15$^{a}$ | 4.16 ± 0.13$^{a}$ |
| 20 | 6.98 ± 0.36$^{ab}$ | 8.13 ± 0.12$^{ab}$ | 4.57 ± 0.08 | 12.88 ± 0.39$^{bc}$ | 6.99 ± 0.11$^{ab}$ | 4.73 ± 0.13$^{b}$ |
| 40 | 7.81 ± 0.40$^{b}$ | 8.31 ± 0.17$^{a}$ | 4.63 ± 0.08 | 13.70 ± 0.37$^{c}$ | 7.12 ± 0.16$^{b}$ | 4.83 ± 0.13$^{b}$ |
| 60 | 6.43 ± 0.35$^{ab}$ | 7.90 ± 0.16$^{ab}$ | 4.49 ± 0.06 | 11.93 ± 0.45$^{ab}$ | 6.61 ± 0.15$^{ab}$ | 4.49 ± 0.10$^{ab}$ |
| 80 | 6.58 ± 0.15$^{ab}$ | 8.13 ± 0.11$^{ab}$ | 4.53 ± 0.04 | 11.79 ± 0.17$^{ab}$ | 6.57 ± 0.09$^{ab}$ | 4.40 ± 0.07$^{ab}$ |
| 100 | 6.00 ± 0.36$^{a}$ | 7.59 ± 0.13$^{b}$ | 4.34 ± 0.08 | 11.32 ± 0.43$^{ab}$ | 6.26 ± 0.13$^{a}$ | 4.19 ± 0.14$^{a}$ |

$^{abc}$ Different superscript letters within a column indicate significant differences ($p<0.05$)

The growth of early *P. pelagicus* juveniles, determined as specific growth rate (SGR) was effectively improved by addition of an appropriate level of triglyceride in their diets (Table 6.5). The highest SGR (% $day^{-1}$) for dry weight, resulted from the diet treatment in which crabs were fed the diet supplemented with 40 g $kg^{-1}$ triglyceride and it was significantly higher than that in all other treatments ($p<0.05$) with the exception of the diet treatment supplemented with 20 g $kg^{-1}$ triglyceride ($p>0.05$). The SGR based on carapace width and carapace length was also highest for crabs fed the diet supplemented with 40 g $kg^{-1}$ triglyceride; however, it was not significantly different from crabs fed diets supplemented with 20, 60 and 80 g $kg^{-1}$ triglyceride ($p<0.05$). The lowest SGR for both dry weight and carapace measurement were recorded for crabs fed the diet without supplemented triglyceride, while the second lowest was shown by crabs fed the diet with 100 g $kg^{-1}$ triglyceride supplementation (Table 6.5).

## 6.4 Discussion

The importance of dietary lipid, phospholipid, essential fatty acids and sterol to the performance of aquaculture species has been the subject of considerable research over the last few decades. The present study has shown that supplementation of appropriate levels of triglyceride significantly improved the growth rate of *P. pelagicus.* Although during the first two stages of crab development (i.e. from C1 to C2 and C2 to C3), the differences in intermolt periods were not significant, supplemented dietary triglyceride supported significantly improved growth rates for C3 to C4; the final stage of crab development tested in the present study. It suggests that if the experiment was run for a longer period, the discrepancies between diet treatments may have become more obvious.

The dietary lipid requirement for crustacean is attributed to many factors; the most important being the fatty acids constituent of the lipid supplied. Crustaceans may need lower dietary lipid levels when their fatty acids requirements are already being met and this depending on the essential fatty acids content, digestibility, amount and also the form of fatty acids. In *P. monodon* juveniles, highest weight gain was achieved when they were fed DHA at 1.5% and similar weight gain was only able to be achieved when the prawns were fed 2.5% LNA (Merican and Shim, 1997). In addition, feeding a diet containing a mixture of LOA and LNA to *P. chinensis* juveniles supported a greater growth rate compared to those fed diets containing the fatty acids individually. Secondly, digestibility of neutral lipid has been shown to be significantly decreased when the dietary level of neutral lipid was increased to 13.5% (Glencross et al., 2002b). Digestibility of unsaturated fatty acids also tends to increase with increased chain length (Merican and Shim, 1994). The form of fatty acids supplied in the diet of crustaceans could also influence the level of dietary lipid required. Fatty acids in triacylglycerides and free fatty acids are better digest and utilized than fatty acids in methyl-ester or ethyl-ester form (Glencross and Smith, 1997). D'Abramo (1997) added that the lipid requirements of crustacean are contributed also influenced by the quality and quantity of dietary protein and the quantity, quality and availability of other energy sources.

Most prior studies on crustaceans have used oil mixtures as dietary lipid sources and their dietary total lipid requirements have been reported to generally range from 2% to 10% (Castell and Covey, 1976; Davis and Robinson, 1986; Sheen and D'Abramo, 1991; Sheen and Wu, 1999). The diets used in the present study contained substantially higher total lipid

levels (ranging between 119 g $kg^{-1}$ (0% triglyceride inclusion) to 228 g $kg^{-1}$ (10% triglyceride inclusion), than most other studies due to inclusion of a high level of PL, which were found optimal for *P. pelagicus* in Chapter 5. The triglyceride supplementation levels tested in this study did not significantly affect survival of *P. pelagicus* as reported for other crustacean species (Davis and Robinson, 1986; Sheen and D'Abramo, 1991; Gonzalez-Felix et al., 2002b; Hernández-Vergara et al., 2003).

Prolonged intermolt periods, low survival and slow swimming activity were shown by larvae of the mud crab, *S. serrata*, when fed *Artemia* which lacked essential fatty acids (Suprayudi et al., 2004). It was also demonstrated that when megalopae of *S. serrata* were fed diets lacking of EPA, DHA and ARA, there was higher occurrence of molt death syndrome (MDS) during metamorphosis to the first crab stage, resulting in lower survival (Holme et al., 2007b). EPA, DHA and ARA play important roles as precursors of biologically active hormones, such as eicosanoids, and provide higher energy value than shorter-chained fatty acids when catabolised (Bell et al., 1986; Merican and Shim, 1995). Eicosanoids regulate the molting process in crustaceans (Gonzalez-Felix et al., 2003b) and are generated *in situ* when needed (Gurr and Harwood, 1991). In the present study, *P. pelagicus* fed the basal diet achieved similar development time to those fed most other diet treatments and no MDS was observed. However, the basal diet used contained a reasonable level of essential fatty acids resulting from inclusion of dietary PL and this was probably adequate for *P. pelagicus* juveniles. It is also possible that no discrepancies found in development time of *P. pelagicus* fed the basal diet with most other diets were due to the optimal level of cholesterol (Chapter 3) included in the diets of the this study as ecdysteroids,

another molt hormone is synthesized by conversion of dietary cholesterol to 7-dehydrocholesterol and other oxidation products in steroidogenic glands (Skinner, 1985; Grieneisen et al., 1993).

Based on the results of this study, early juveniles of *P. pelagicus* require between 126.0 g $kg^{-1}$ to 155.0 g $kg^{-1}$ total lipids in their diet for optimal growth and development. Juveniles of the mud crab, *S. serrata*, achieved highest weight gain when they were fed a diet containing 11.6% (116 g $kg^{-1}$) of total lipids although no significant differences were found among crabs that were fed diets containing total lipid levels ranging from 5.3% to 13.8% (53 to 138 g $kg^{-1}$) (Sheen and Wu, 1999). The higher levels of total lipids in both studies exceed the levels commonly recommended for crustaceans. However, it is worth noting that for *S. serrata* juveniles, high weight gain was obtained when they were fed diets containing mostly neutral lipids. In comparison, the diets used for *P. pelagicus* in this study contained only 20 to 40 g $kg^{-1}$ supplemented neutral lipids in the form of triglyceride mixture. As food intake of crustaceans is influenced by diet energy content (D'Abramo, 1997), it is likely that the low requirements for neutral lipids in the present study are due to high level of PL supplementation (90 g $kg^{-1}$). This suggests that a combination of 20 to 40 g $kg^{-1}$ neutral lipid and 90 g $kg^{-1}$ PL satisfies the energy requirement of *P. pelagicus* juveniles.

Although no significant differences in survival, development and growth were found between crabs fed diets that contained 60, 80 and 100 g $kg^{-1}$ triglyceride, there was a tendency towards decreasing growth and survival with further increase in triglyceride above 40 g $kg^{-1}$. Excessively high levels of dietary lipid have been associated with high lipid

accumulation in the digestive gland of crustaceans, a reason suggested for slow growth of western rock lobster, *Panulirus cygnus* (Tsvetnenko et al., 2001). In larvae of marine fish, high dietary neutral lipids has been reported to affect larval growth because of increases in lipid droplet accumulation in enterocytes causing poor fatty acid absorption (Morais et al., 2007). High levels of neutral lipids in the diets of juvenile *P. monodon* has also been shown to reduce the apparent digestibility of neutral lipid due to a limited capacity within juvenile *P. monodon* to digest dietary neutral lipids (Glencross et al., 2002b).

This study used a 2:1 ratio of fish oil to corn oil based on previous dietary lipid studies with other crustaceans (Sheen and D'Abramo, 1991; Sheen, 1997; Sheen and Wu, 1999; Holme et al., 2006). As fish oil contains high levels of HUFA, particularly the n-3 HUFA, EPA (20:5n-3) and DHA (22:6n-3), increasing lipid level in the diets subsequently increased the level of n-3 HUFA. Excessive levels of n-3 HUFA have been suggested to reduce growth and survival of the larvae of Kuruma prawn, *M. japonicus* (Kanazawa et al. (1985). In summary, this study showed that with the optimal inclusion level of phospholipid and cholesterol, supplementation of triglyceride at 20 to 40 g $kg^{-1}$ supported better growth and development of early juvenile *P. pelagicus*. This finding has clear application in production of species-specific diets for *P. pelagicus*.

# CHAPTER 7

## The effects of dietary soybean oil to fish oil ratios on survival, development and growth of *Portunus pelagicus* early juveniles

---

### 7.1 Introduction

Various lipid constituents, including essential fatty acids, sterols, phospholipid and fat soluble vitamins play important roles in crustaceans physiology and as sources of concentrated energy (Sheen and Wu, 1999; Oliva-Teres, 2000). The dietary requirements of crustacean for various lipid components are largely species-specific and are also affected by other factors, such as their developmental stage and the environment under which they are cultured (Sheen and D'Abramo, 1991). Among different lipid constituents, fatty acids, particularly polyunsaturated fatty acids (PUFA) e. g. linoleic acid (LNA; 18:2n-6) and linolenic acid (LOA; 18:3n-3) and highly unsaturated fatty acids (HUFA) e.g. arachidonic acid (ARA; 20:4n-6), docosahexaenoic acid (DHA; 22:6n-3) and ecosapentaenoic acid (EPA; 20:5n-3), are most commonly studied and have been found to be essential in promoting survival and growth of crustaceans (Baum et al., 1990; Merican and Shim, 1997; Glencross and Smith, 1999; Glencross et al., 2002d; L.Gonzalez-Felix et al., 2002). Such studies can be traced back to the work of Kanazawa et al. (1979a) on the Kuruma prawn, *Marsupenaeus japonicus*, in which it was found that *M. japonicus* have no (or limited) ability to elongate palmitic acids to produce longer carbon chain fatty acids. Crustacean inability to synthesized HUFA was later suggested to be linked to inactive Δ6 and Δ5 enzymes in crustacean that are

responsible for desaturation of 18:3n-3 to 18:4n-3, before being elongated to 20:4n-3 and 20:5n-3 (Gonzalez-Baro and Pollero, 1998).

Among the essential fatty acids, some have been found to be more effective than others in promoting survival and/or growth of crustaceans (Read, 1981; Lim et al., 1997; Gonzalez-Felix et al., 2003b). For instance, the superiority of HUFA to PUFA in promoting survival and growth have been demonstrated for mud crabs, *Scylla serrata,* juveniles (Sheen and Wu, 2003) and black tiger prawn, *Penaeus monodon,* juveniles (Merican and Shim, 1997). Within PUFA groups, n-3 PUFA have been found to have greater nutritional value in promoting survival and growth for range of crustaceans compared to n-6 PUFA supplemented at the same level (Read, 1981; Xu et al., 1993; Xu et al., 1994; Gonzalez-Felix et al., 2003a). For example, Chinese prawn, *Penaeus chinensis,* juveniles, fed a diet with 1% LNA inclusion have been shown to have significantly superior growth rate, molting frequency and survival than those fed a similar diet with 1% LOA (Xu et al., 1993). In addition, ARA, a n-6 HUFA, has been found to be significantly inferior in promoting survival, molting frequency and weight gain for the same prawn species than DHA, a n-3 HUFA (Xu et al., 1994). Due to different nutritional values between essential fatty acids, it has been proposed that requirements of all HUFA should be considered simultaneously rather than as individuals (Sargent et al., 1999). However, in order to achieve an optimal dietary level of each essential fatty acid while considering the interaction effects among them, one need to use purified fatty acids which are costly, laborious and often impractical to use for large scale feed production. Feeds and feeding represent the largest operating cost in intensive and semi-intensive fish and crustacean farming operations (FAO, 2006).

Traditionally, fish oil has been used extensively as the source of lipids for formulated feeds in aquaculture. Fish oil in general is considered to have high nutritional value due to high content of essential fatty acids, particularly EPA and DHA (Merican and Shim, 1996; Zhou et al., 2007). It has been reported that in 2006, the aquafeed industry consumed 835 000 tonnes of fish oil, approximately 88.5% of annual global fish oil production (Tacon and Metian, 2008). This has also led to an increase in the prices of fish oil that substantially increased the cost of formulated feeds. To reduce dependence on this limited resource and to sustain growth of the aquaculture industry, enormous efforts have been devoted to find alternatives, such as various plant oils, for fish oil. Some plant oils such as soybean oil, peanut oil and rapeseed oil are known to have abundant unsaturated fatty acids such as LOA and LNA. They have higher energy contents compare to terrestrial animal oils and other plant oils that have higher saturated fatty acid contents (Wiseman, 1991). Soybean oil is among plant oils that are rich in LOA (Zhou et al., 2007), which is known to promote growth in crustaceans (Glencross and Smith, 1999). Fortunately, research to date has found that partial or full replacement of fish oil by plant oil in diets formulated for crustaceans and fish is plausible (Bell et al., 2001; Vasagam et al., 2005; Piedecausa et al., 2007) and sometimes, even yields better results than using fish oil alone (Deshimaru et al., 1979; Kamarudin and Roustaian, 2002; Holme et al., 2007b). The later cases are likely linked to the fact that mixing two or more oil sources leads to a more balanced fatty acid profile in diets of cultured aquatic animals.

The objective of this experiment was to evaluate the potential of partial or total replacement of dietary fish oil with soybean oil, a widely available plant oil source, in formulated diet for *P. pelagicus* juveniles. Performances of *P. pelagicus* were evaluated with standard aquaculture parameters such as survival, development time and growth. All experimental diets were analyzed to obtain their fatty acid profiles.

## 7.2 Materials and Method

### *7.2.1 Source of crabs*

Broodstock capture and husbandry and larval culture protocol were described in Chapter 2 (Section 2.2.1).

### *7.2.2 Diet preparation*

Seven iso-caloric semi-purified diets were formulated to contain a same level of 40 g $kg^{-1}$ triglyceride but consisted of different ratios of soybean oil (SO) and fish oil (FO). The SO:FO ratios in the seven experimental diets were 1:0, 3:1, 2:1, 1:1, 1:2, 1:3 and 0.1, respectively (Table 7.1). Fish oil (menhaden) used in this study were obtained from Sigma-Aldrich (Australia) and soybean oil were contributed by CSD Grains Pty Ltd Australia and their fatty acid compositions were given in Table 7.2. Prior to diet preparation, fish meal was pulverized and defatted by vigorously mixing fishmeal in a solvent mixture of chloroform and methanol (2:1, v/v) (Folch et al., 1957). The process was repeated three times and fishmeal was then aired in fume hood for 24 h before sieved to remove particles larger than 100 µm.

Dry and wet ingredients (Table 7.1) of the experimental diets were then individually weighed with Sartorius TE2145 electronic balance (0.0001 g) and were combined together in an electric mixer to form a homogenous blend. Subsequently, agar was prepared by dissolving it in a hot water at above 80$^{o}$C (Sheen, 2000). As the solution became clear, the solution was cooled to 40$^{o}$C. The prior mentioned diet ingredient mixture was added to the agar solution and all ingredients thoroughly mixed. The agar quickly became gelatinized as temperature dropped to 37$^{o}$C. The diets were subsequently cut into small pieces of approximately 2 mm$^{3}$ and stored in -20$^{o}$C until used.

**Table 7.1** Formulation of the experimental diets (g $kg^{-1}$ dry diet) supplemented with different ratios of soybean oil to fish oil. Diet formulation was modified based on Holme (2007a), Sheen and Wu (1999) and Genodepa (2004).

| Ingredients | Level (g $kg^{-1}$) |
|---|---|
| *Basal diet composition* | |
| Defatted fish meal[1] | 500 |
| Phospholipid[2a] | 90 |
| Cholesterol[2b] | 10 |
| Vitamin Mix[3a] | 40 |
| Mineral Mix[3b] | 40 |
| Choline Chloride[2c] | 10 |
| DCP[2d] | 6 |
| Starch[2e] | 60 |
| Cellulose[2f] | 64 |
| Agar (binder)[2g] | 120 |
| | |
| *Ratio Soybean oil*[4] *: Fish oil*[2h] | |
| Diet 1; 1 : 0 | 40 : 0 |
| Diet 2; 3 : 1 | 30 : 10 |
| Diet 3; 2 : 1 | 27 : 13 |
| Diet 4; 1 : 1 | 20 : 20 |
| Diet 5; 1 : 2 | 13 : 27 |
| Diet 6; 1 : 3 | 10 : 30 |
| Diet 7; 0 : 1 | 0 : 40 |

[1] Skretting Tasmania

[2] Sigma-Aldrich Pty Ltd [a]P3644, [b]C8667, [c]98% powder C7527, [d]dibasic calcium phosphate C4131, [e]S4126 (corn), [f]C8002 alpha, [g]A7002, [h]F8020 from menhaden

[3] Rabar Pty Ltd [a]ZZ600 DPI, each 1kg contains: vitamin A 2miu, vitamin D3 0.8miu, vitamin E 40g, vitamin K 2.02g, inositol 50g, vitamin B3 30.40g, vitamin B5 9.18g, vitamin B9 2.56g, vitamin B2 4.48g, vitamin B12 0.004g, biotin 0.1g, vitamin B6 4g, vitamin B1 3.4g, vitamin C 44.4g, para amino benzoic acid 20g, tixosil 5g, antioxidant 30g [b]ZZ603 DO 067 DPI, each 1kg contains: copper 1g, cobalt 100mg, magnesium 59.4mg, manganese 5g. iodine 800mg, selenium 20mg, iron 8mg, zinc 20g, aluminium 100mg, chromium 100mg

[4] CSD Grains Pty Ltd

*7.2.3 Experimental design and set up*

Newly metamorphosed C1 crabs were obtained as described in Chapter 2 (Section 2.2.2) and were placed individually into 750 mL circular culture vessels to start the feeding experiment. As *P. pelagicus* juveniles are highly cannibalistic, to keep them separately in individual culture units throughout the experiment is a must for nutritional studies as this eliminates potential nutritional contribution from cannibalism while also allow precise records of consecutive intermolt periods for each crab. All culture units were labeled for identification and kept in water bathes to maintain the temperature at 28 ± 2$^{o}$C.

A total of 280 culture units were set up with 40 crabs per treatment. At the starting point of the experiment, the average dry weight of the newly settled C1 crabs was 1.11 ± 0.27 mg and mean carapace width and length were 3.25 ± 0.22 mm and 2.38 ± 0.11 mm, respectively. During the experiment, a 100% water exchange was carried out daily in the morning by transferring crabs individually into new culture units while the previous units were sanitized in hypochlorite solution and dried overnight for the use in the next day. Any mortality and molting found during the daily water exchange were recorded and crabs were then fed to satiation with their designated diets. Throughout the experiment, salinity was maintained at 30 ± 2‰ and pH between 7.5 to 8.1 while photoperiod was set at light:dark = 14:10 h at all times.

Toward the end of the experiment, during the daily checking in the morning, any crabs that were found molted to C4 stage, the designed final stage for the experiment, were recorded but removed from the culture in the afternoon for the measurement of carapace

length, carapace width and dry weight determination. The measuring of crabs in the afternoon was to allow sufficient time for newly molted crabs to harden their new exoskeleton. Carapace measurements and dry weight determination were carried out as described in Chapter 3 (Section 3.2.3). The experiment was terminated when all crabs were either molted to the C4 stage or died.

*7.2.4 Fatty acids analysis*

Fish oil and soybean oil used in the experimental diets as well as all experimental diets were analyzed for their fatty acid compositions. To carry out the fatty acid analysis, lipid was first extracted from the samples using method by Folch et al. (1957). Fatty acid methyl esters (FAME) were then derived using 14% boron trifluoride-methanol (Wijngaarden, 1967) and were analyzed on an Agilent Technologies 6890 gas chromatograph using split injection with helium carrier gas and a flame ionization detector. DB23 fused silica capillary column of 30 mm x 0.25 mm, with a 0.25 µm coating (Agilent Technologies, USA), was set to held oven temperature at 140°C for 5 minutes. It was then elevated at 3°C minute$^{-1}$ to 210°C where it was held until all FAME of interest had been eluted. FAME were identified by comparing their retention times with standards (Sigma-Aldrich Co, USA), and were quantified by comparison with the response of an internal standard, heneicosanoic acid.

*7.2.5 Data and statistical analysis*

The specific growth rate (SGR) based on dry weight or carapace size increases of the experimental crabs from C1 to C4 were calculated using the equation described in Chapter 3 (Section 3.2.5). Survival, development time and SGR of the experimental crabs are presented

as mean ± standard error (SE) and subjected to one-way analysis of variances (ANOVA) to determine whether any significant differences exist among treatments. Before analysis, the data were first tested to confirm the normality and homogeneity of variance. Log or arcsine transformation of data was performed before further analysis when variances were found not homogenous. Survival data was arcsine transformation before analysis. If any significant differences were detected by ANOVA ($p<0.05$), the differences were identified using Tukey's test. All statistical tests were conducted using SPSS, version 17.

## 7.3 Results

### *7.3.1 Fatty acid compositions*

Table 7.2 shows fatty acid profiles of soybean oil and fish oil used for the formulation of experimental diets and the fatty acid compositions of all seven semi-purified experimental diets with various soybean oil to fish oil ratios are shown in Table 7.3. The results show that the soybean oil contained predominantly C18 fatty acids with linoleic acid (LOA; 18:2n-6), a n-6 PUFA, as the most dominant fatty acid making up more than half of total fatty acids (54.45 g 100 $g^{-1}$). It was followed by oleic acid (18:1n-9) at 21.45 g 100 $g^{-1}$ (Table 7.2). The soybean oil only contained one n-3 group fatty acids, i.e. linolenic acid (LNA; 18:3n-3) at 8.29 g 100 $g^{-1}$, and was void of any highly unsaturated fatty acids (HUFA; ≥ 20:3n). The fish oil had a more diverse fatty acid profile, made up of 28.98 g 100 $g^{-1}$ saturated fatty acids (SFA), 26.54 g 100 $g^{-1}$ monounsaturated fatty acids (MUFA), 10.55 g 100 $g^{-1}$ polyunsaturated fatty acids (PUFA) and 28.49 g 100 $g^{-1}$ HUFA. Compared to soybean oil, fish oil contained substantially less LNA (1.26 g 100 $g^{-1}$) but high levels of ecosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), at 11.53 g 100 $g^{-1}$ and 11.84

g 100 $g^{-1}$, respectively. The fish oil also contained n-6 group HUFA, i.e. (ARA; 20:4n-6) at 0.90 g 100 $g^{-1}$ and LOA in the fish oil was notably lower (5.15 g 100 $g^{-1}$) than the soybean oil (Table 7.2).

Fatty acids composition (presented as g $kg^{-1}$ of diet dry weight) reflected the ratio of fish oil and soybean oil in the diets (Table 7.3). The diet containing only soybean oil (SO:FO ratio 1:0), had the highest level of PUFA among all diets, mainly due to a high level of LOA (56.93 g $kg^{-1}$) in the diet. LOA level in the diets decreased steadily with increasing ratio of fish oil and dropped almost by half (32.17 g $kg^{-1}$) in the diet contained fish oil only (SO:FO ratio 0:1). Similarly, the increased ratio of fish oil in the diets reduced the LNA level substantially. In contrast, HUFA level increased concomitantly with the increased ratio of fish oil as it went up from less than 0.21 g $kg^{-1}$ in the diet supplemented with the soybean oil only, to 11.71 g $kg^{-1}$ in the diet supplemented solely with fish oil (Table 7.3). In particular, among major HUFAs, EPA level increased from less than 0.05 g $kg^{-1}$ to 4.75 g $kg^{-1}$ while DHA increased from 0.21 g $kg^{-1}$ to 5.31 g $kg^{-1}$ as soybean oil to fish oil ratio in the diets increased from 1:0 to 0:1. Meanwhile, ARA level in the diets was however more similar in all diets that contained fish oil although the fish oil was added at different amount. The major saturated fatty acids (SFA) in all diets were palmitic acid (16:0) and stearic acid (18:0) and the monounsaturated fatty acids (MUFA) were dominated by oleic acid. Total SFA and MUFA remained relatively stable in all diets regardless their soybean oil to fish oil ratio (Table 7.3).

**Table 7.2** Fatty acid compositions (g 100 $g^{-1}$) of the soybean oil and fish oil used in the formulation of experimental diets fed to *Portunus pelagicus* early juveniles.

| Major fatty acid | Soybean oil | Fish oil |
|---|---|---|
| 14:0 | - | 7.75 |
| 15:0 | - | 0.70 |
| 16:0 | 10.04 | 16.56 |
| 18:0 | 3.84 | 2.86 |
| 22:0 | 0.37 | - |
| 16:1n-7 | - | 11.01 |
| 18:1n-9 | 21.45 | 8.40 |
| 18:1n-7 | 1.38 | 2.9 |
| 18:2n-6 | 54.45 | 5.15 |
| 18:3n-3 | 8.29 | 1.26 |
| 20:4n-6 | - | 0.90 |
| 20:5n-3 | - | 11.53 |
| 22:6n-3 | - | 11.84 |
| | | |
| ∑ SFA | 14.25 | 28.98 |
| ∑ MUFA | 23.00 | 26.54 |
| ∑ PUFA (≥18:2n) | 62.74 | 10.55 |
| ∑ HUFA (≥20:3n) | - | 28.49 |
| | | |
| ∑ n-3 | 8.30 | 31.85 |
| ∑ n-6 | 54.50 | 6.70 |
| n-3/n-6 | 0.15 | 4.77 |
| DHA/EPA | - | 1.03 |

*(∑) include minor fatty acids percentage that is not shown in Table

**Table 7.3** Fatty acids composition (g $kg^{-1}$ dry weight) of experimental diets supplemented with different ratios of soybean oil to fish oil.

| Major fatty acids | Dietary soybean oil : fish oil (SO:FO) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:0 | 3:1 | 2:1 | 1:1 | 1:2 | 1:3 | 0:1 |
| 14:0 | 0.16 | 0.87 | 0.99 | 1.35 | 1.78 | 1.86 | 2.63 |
| 15:0 | - | 0.12 | 0.14 | 0.17 | 0.22 | 0.23 | 0.30 |
| 16:0 | 14.71 | 14.48 | 14.69 | 14.58 | 15.11 | 14.91 | 15.87 |
| 16:1n-7 | 0.16 | 1.23 | 1.44 | 2.05 | 2.62 | 2.79 | 3.82 |
| 17:0 | 0.14 | 0.18 | 0.20 | 0.22 | 0.26 | 0.27 | 0.33 |
| 18:0 | 4.29 | 3.82 | 3.84 | 3.65 | 3.57 | 3.48 | 3.42 |
| 18:1n-9 | 15.68 | 13.00 | 12.75 | 11.43 | 10.39 | 9.76 | 8.44 |
| 18:1n-7 | 1.48 | 1.58 | 1.65 | 1.72 | 1.81 | 1.81 | 2.03 |
| 18:2n-6 | 56.93 | 47.42 | 47.00 | 42.13 | 38.53 | 36.45 | 32.17 |
| 18:3n-3 | 7.31 | 6.09 | 6.08 | 5.42 | 4.95 | 4.68 | 4.14 |
| 18:4n-3 | - | 0.36 | 0.45 | 0.63 | 0.81 | 0.87 | 1.20 |
| 20:0 | 0.24 | 0.20 | 0.20 | 0.18 | 0.17 | 0.16 | 0.15 |
| 20:1n-9 | 0.14 | 0.29 | 0.32 | 0.40 | 0.49 | 0.51 | 0.67 |
| 20:2n-6 | - | - | - | - | 0.11 | 0.12 | 0.14 |
| 20:4n-6 | - | 0.14 | 0.17 | 0.20 | 0.26 | 0.29 | 0.38 |
| 20:5n-3 | - | 1.45 | 1.80 | 2.52 | 3.21 | 3.45 | 4.75 |
| 22:0 | 0.33 | 0.27 | 0.28 | 0.24 | 0.23 | 0.21 | 0.19 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 22:5n-6 | - | - | - | - | 0.12 | - | 0.17 |
| 22:5n-3 | - | 0.44 | 0.52 | 0.63 | 0.77 | 0.81 | 1.10 |
| 22:6n-3 | 0.21 | 1.70 | 2.05 | 2.89 | 3.60 | 3.87 | 5.31 |
| 24:1n-9 | - | - | - | - | 0.11 | 0.12 | 0.17 |
| | | | | | | | |
| ∑ SFA | 19.89 | 20.0 | 20.33 | 20.40 | 21.34 | 21.12 | 22.90 |
| ∑ MUFA | 17.47 | 16.1 | 16.16 | 15.60 | 15.42 | 15.01 | 15.37 |
| ∑ PUFA (≥18:2n) | 64.24 | 53.9 | 53.53 | 48.18 | 44.39 | 42.12 | 37.65 |
| ∑ HUFA (≥20:3n) | 0.21 | 3.7 | 4.53 | 6.24 | 7.96 | 8.42 | 11.71 |
| | | | | | | | |
| ∑ n-6 | 56.93 | 47.56 | 47.17 | 42.33 | 39.01 | 36.86 | 32.85 |
| ∑ n-3 | 7.51 | 10.05 | 10.88 | 12.09 | 13.34 | 13.69 | 16.50 |
| n-6/n-3 | 7.58 | 4.73 | 4.33 | 3.50 | 2.93 | 2.69 | 1.99 |
| DHA/EPA | - | 1.13 | 1.11 | 1.16 | 1.13 | 1.11 | 1.10 |
| TOTAL | 101.94 | 87.63 | 90.41 | 94.54 | 89.11 | 93.67 | 86.67 |

*(∑) include minor fatty acids that are not shown in the Table

* - indicates less than 0.05 g $kg^{-1}$

*7.3.2 Survival, development and growth of the crabs*

Highest survival of 75.0 ± 6.3% was recorded for crabs fed the diet containing SO:FO at 1:1 ratio (Fig 7.1). This is followed by around 60.0% survival for crabs in 4 diet treatments; those that were either fed the diet solely supplemented with fish oil (SO:FO ratio 0:1) or diets with SO:FO ratios at 2:1, 3:1 and 1:3. A lower survival of 55.0 ± 9.1% was recorded for crabs fed the diet with a SO:FO ratio of 1:2 and the lowest survival of 37.5 ± 10.9% was found for crabs fed the diet containing soybean oil only.

No significant differences were detected for intermolt period during the first molt of the crabs (C1 to C2 stage). However from C2 onwards, the differences in development rate of the crabs from different diet treatments became significant ($p<0.05$). For example, significantly longer C2 intermolt duration was recorded for crabs fed the diet supplemented with soybean oil only when compared to the rest of the treatments ($p<0.05$) except for those fed with SO:FO ratio at 2:1 ($p>0.05$). The shortest overall development time from C1 to C4 (14.9 ± 0.2 days) was again obtained for crabs fed the diet with an SO:FO ratio of 1:1, which was significantly ($p<0.01$) and more than 4 days shorter than the longest development time (19.1 ± 1.8 days) recorded for crabs fed the diet supplemented with soybean oil only (Table 7.4).

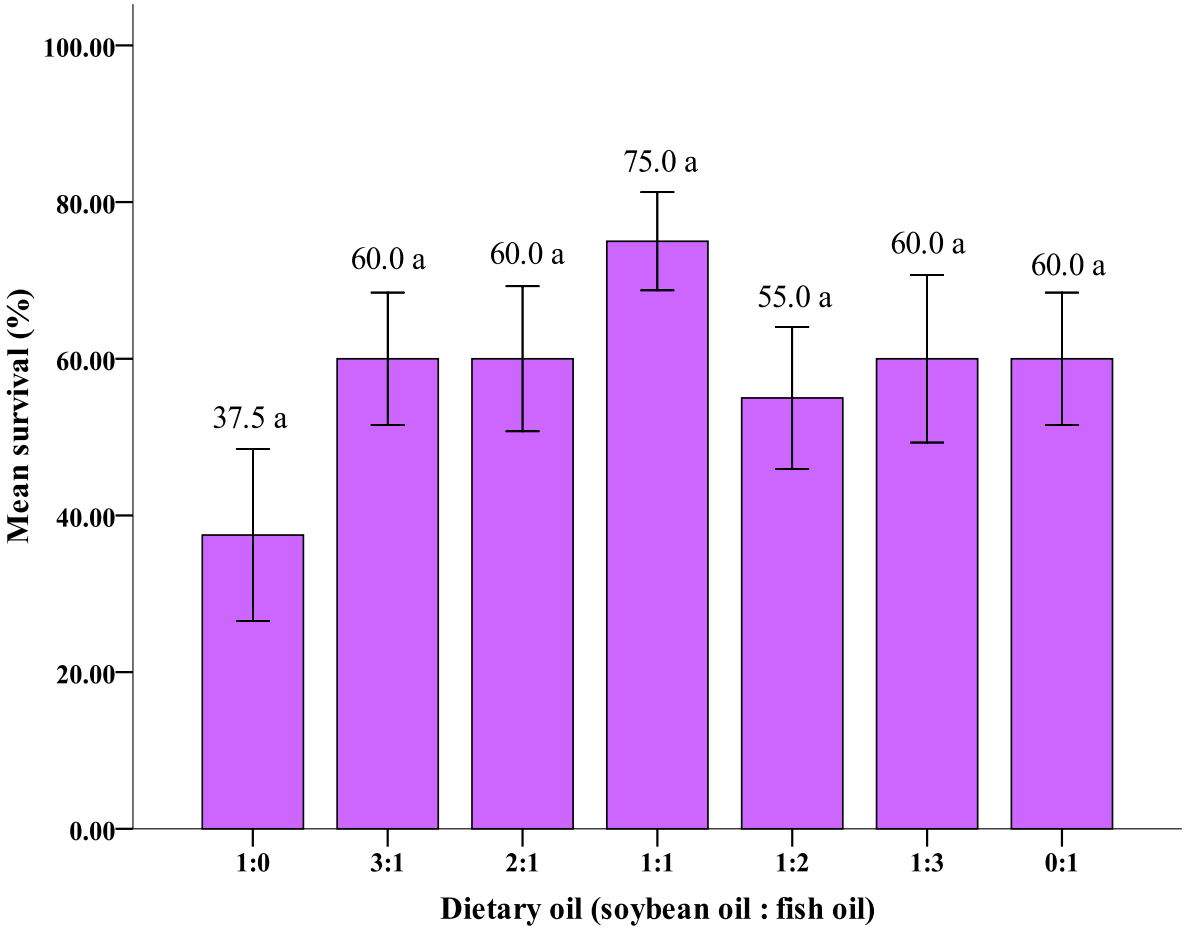



**Figure 7.1** Mean survival (%) of *Portunus pelagicus* fed diets supplemented with different ratios of soybean oil to fish oil (SO:FO) throughout the culture period. No significant differences were found between the treatments ($p>0.05$).

**Table 7.4** Mean (± SE) intermolt period at each crabs stages and cumulative development time to C4 stage (in days) of *Portunus pelagicus* fed semi-purified diets formulated to contain different soybean oil to fish oil ratios.

| Dietary oil (soybean oil : fish oil ratio) | C1-C2 | C2-C3 | C3-C4 | Culture period |
|---|---|---|---|---|
| 1:0 | 4.6 ± 0.2 | 7.0 ± 0.4$^{a}$ | 6.9 ± 0.4$^{a}$ | 19.1 ± 1.74$^{a}$ |
| 3:1 | 4.2 ± 0.1 | 5.9 ± 0.2$^{b}$ | 6.0 ± 0.3$^{ab}$ | 15.6 ± 0.4$^{b}$ |
| 2:1 | 4.4 ± 0.2 | 6.1 ± 0.2$^{ab}$ | 6.3 ± 0.2$^{ab}$ | 16.3 ± 0.3$^{b}$ |
| 1:1 | 4.2 ± 0.2 | 5.4 ± 0.2$^{b}$ | 5.7 ± 0.1$^{b}$ | 14.9 ± 0.2$^{b}$ |
| 1:2 | 4.5 ± 0.2 | 5.7 ± 0.2$^{b}$ | 6.2 ± 0.2$^{ab}$ | 15.8 ± 0.3$^{b}$ |
| 1:3 | 4.0 ± 0.2 | 5.2 ± 0.1$^{b}$ | 6.8 ± 0.3$^{ab}$ | 15.3 ± 0.4$^{b}$ |
| 0:1 | 4.6 ± 0.2 | 5.8 ± 0.3$^{b}$ | 5.8 ± 0.3$^{ab}$ | 15.8 ± 0.5$^{b}$ |

$^{abc}$ Different superscripts within a column indicate significant differences ($p<0.05$)

No significant differences were detected for dry weight or carapace measurement of crabs fed different dietary soybean oil and fish oil ratios, however, when the data were analyzed for specific growth rates (SGR), differences became apparent. The specific growth rates (SGR) based on dry weight of the newly molted C4 crabs was the highest for crabs fed the diet with a SO:FO ratio of 1:1 and the lowest for crabs fed the diet with soybean oil only (Table 7.5). In general, diets that contained no or low ratios of fish oil (SO:FO ratio at 1:0, 3:1 and 2:1, respectively) had SGR (dry weight) lower than 10.0% day$^{-1}$ and significant differences were detected between SGR (dry weight) of crabs fed the diets with SO:FO ratios of 1:0 and 2:1, and those fed diets containing SO:FO ratios of 0:1, 1:1 and 1:3 ($p<0.05$) (Table 7.5). The greatest SGR value for carapace width was again obtained for crabs fed the

diet with a SO:FO ratio of 1:1 which was significantly higher than the crabs fed the diets with SO:FO ratios of 1:0 and 2:1 ($p<0.05$). The SGR (carapace width) of treatment with SO:FO ratio of 1:0 was also significantly lower than in all other treatments ($p<0.05$) except the treatment with a dietary SO:FO ratio of 2:1. In contrast to SGR for dry weight and carapace width, no significant differences were detected for SGR for carapace length among all diet treatments ($p>0.05$) (Table 7.5).

**Table 7.5** Mean (± SE) dry weight, carapace width, carapace length and mean (± SE) specific growth rate (SGR) for dry weight, carapace width and carapace length of newly molted fourth crab stage *Portunus pelagicus* that had been fed semi-purified diets formulated to contain different soybean oil to fish oil ratios.

| Dietary oil (soybean oil : fish oil ratio) | Newly molted C4 stage | | | Specific growth rate (SGR) | | |
|---|---|---|---|---|---|---|
| | Dry weight (mg) | Carapace width (mm) | Carapace length (mm) | Dry weight (% day$^{-1}$) | Carapace width (% day$^{-1}$) | Carapace length (% day$^{-1}$) |
| 1:0 | 5.23 ± 0.20 | 6.70 ± 0.40 | 4.47 ± 0.40 | 8.87 ± 0.21$^{a}$ | 4.06 ± 0.42$^{a}$ | 3.50 ± 0.42 |
| 3:1 | 5.41 ± 0.20 | 7.12 ± 0.09 | 4.01 ± 0.07 | 9.92 ± 0.85$^{ab}$ | 4.93 ± 0.42$^{bc}$ | 3.28 ± 0.10 |
| 2:1 | 5.01 ± 0.27 | 7.01 ± 0.14 | 4.01 ± 0.10 | 9.14 ± 0.32$^{a}$ | 4.72 ± 0.13$^{ab}$ | 3.20 ± 0.15 |
| 1:1 | 5.72 ± 0.25 | 7.42 ± 0.12 | 4.12 ± 0.05 | 10.89 ± 0.31$^{b}$ | 5.52 ± 0.11$^{c}$ | 3.68 ± 0.08 |
| 1:2 | 5.53 ± 0.30 | 7.10 ± 0.14 | 3.99 ± 0.06 | 10.04 ± 0.33$^{ab}$ | 4.92 ± 0.13$^{bc}$ | 3.26 ± 0.09 |
| 1:3 | 5.81 ± 0.26 | 7.27 ± 0.12 | 4.09 ± 0.06 | 10.77 ± 0.30$^{b}$ | 5.27 ± 0.11$^{bc}$ | 3.54 ± 0.11 |
| 0:1 | 5.97 ± 0.33 | 7.44 ± 0.15 | 4.15 ± 0.07 | 10.55 ± 0.32$^{b}$ | 5.24 ± 0.12$^{bc}$ | 3.52 ± 0.10 |

$^{abc}$ Different superscripts within a column indicate significant differences ($p<0.05$)

## 7.4 Discussion

The results of this study demonstrate that replacing half the dietary fish oil with soybean oil in the formulation of diets for early juvenile *P. pelagicus* did not negatively affected survival, development and growth of the crabs. Previous studies have shown other examples of successful partial replacement of dietary fish oil with vegetable oils with other crustacean species. For instance, when megalopal larvae of the mud crab, *S. serrata,* were fed dry microbound diets with different fish oil to corn oil ratios, the diet containing fish oil to corn oil ratio at 1:1 gave the best survival to the first crab stage which had very similar dry weight and carapace width to megalopae fed the diet formulated with fish oil only (Holme et al., 2006). For juvenile Kuruma prawn, *M. japonicus*, at a same lipid supplementation level of 6%, a mixture of Pollack liver oil and soybean oil at ratios between 3:1 to 1:1 produced remarkably high growth and feed efficiency (Deshimaru et al., 1979). However, while no significant differences in dry weight was found when larvae of the giant freshwater prawn, *Macrobrachium rosenbergii*, were fed diets formulated with fish oil or corn oil or a mixture of them, survival of the larvae was significantly lower when they were fed the diet formulated with corn oil only (Kamarudin and Roustaian, 2002).

The best overall performance when fish oil and soybean oil were supplemented at equal quantity (1:1) suggests that *P. pelagicus* might prefer a more balanced dietary n-3 HUFA and n-6 PUFA profile, which is provided by the fish oil and soybean oil, respectively. However, it was also clear that the diet with soybean oil as sole lipid source suppressed survival, development and growth of *P. pelagicus*. It has been proposed that variations in essential fatty acid requirements of different crustaceans reflects their biosynthetic and

bioconversion ability to elongate and desaturate shorter chain fatty acids (Kanazawa et al., 1979a; Zhou et al., 2007). For example, it has been suggested that juvenile Pacific white shrimp, *Litopenaeus vannamei,* have limited capacity to synthesize HUFA from PUFA, therefore the inclusion of n-3 HUFA in the diet significantly improved their growth (Gonzalez-Felix et al., 2003a). In the present study, the generally lower growth rates of *P. pelagicus* fed diets with higher soybean oil inclusions may also related to their insufficient ability to synthesize HUFA.

Differences in fatty acid requirements among aquatic species have also been suggested to be linked with the metabolic pattern associated with their feeding habits (Ackman, 1967). For instance, marine fish are often carnivorous /piscivorous and their natural diets are rich in n-3 and n-6 HUFAs, which may results in an evolutionary down-regulation of their desaturase and/or elongase activities. On the other hand, freshwater fish with natural diets rich in PUFA may lead to enhanced capacity to synthesize PUFA to HUFA (Sargent et al., 2002). Therefore, dietary substitution of fish meal and fish oil with alternative materials has been suggested to be considerably more successful for herbivorous/omnivorous species than carnivorous species (Tacon and Metian, 2008). For *P. pelagicus*, previous studies have revealed that in nature, they consume a wide range of benthic invertebrates and to a lesser extent, teleost and plant materials (Williams, 1982; Wassenberg and Hill, 1987; Lestang et al., 2003).

In the present study, the best growth was achieved when crabs were fed the diet with n-6/n-3 fatty acid ratio at around 3.5. This ratio markedly increased with increasing vegetable

oil inclusion in the diets. In juvenile black seabream, *Acanthoparus schlegeli*, high n-6/n-3 fatty acids ratios lead to lipid being deposited in the liver and this has been suggested as a possible reason that limits their ability to utilize more vegetable oil in their diets (Peng et al., 2008). High dietary n-6/n-3 fatty acid ratios have also been suggested to have inhibitory effects on crustacean growth (New, 1976). A more recent study on juvenile black tiger prawn, *P. monodon*, has shown that growth tended to increase with n-3 fatty acids content in the diet and peaked at the level of 35 g 100 $g^{-1}$ with further increases leading to reduced growth (Glencross et al., 2002d). It was also shown that increase in dietary n-6 fatty acids only slightly improved growth as high levels of n-6, or similar to level of n-3 fatty acids negatively affected them (Glencross et al., 2002d). In addition, n-6 PUFA, LOA was found to be less digestible than n-3 group PUFA, LNA (18:3n-3) in juvenile and adult of *P. monodon* (Merican and Shim, 1995; Glencross and Smith, 1999).

It has been demonstrated that when juvenile Pacific white shrimp, *L. vannamei*, were fed a diet containing soybean oil only, their muscle and hepatopancreas contained lower level of cholesterol compared to those fed the diet containing fish oil (Cheng and Hardy, 2004). Similar results have been reported for the muscle cholesterol content of *P. monodon* (Vasagam et al., 2005) and juvenile black seabream, *A. schlegeli* (Peng et al., 2008). Peng et al. (2008) further suggested that the results for *A. schlegeli* was due to soybean oil being rich in oleic acid, linoleic acid and linolenic acid, all of which are capable of reducing cholesterol absorption. Plant oils are known to contain phytosterols (Phillips et al., 2002), which can decrease cholesterol and low density lipoprotein-cholesterol levels in teleost fish by reducing intestinal absorption efficiency (Vanstone et al., 2002; Gilman et al., 2003). Whether similar

mechanisms exist in crustaceans requires further research as crustacean are known to use cholesterol more extensively but unlike most fishes, crustaceans cannot synthesize cholesterol *de novo* (Sheen, 2000). The present study showed that *P. pelagicus* fed a diet containing soybean oil only had significantly prolonged intermolt periods. Cholesterol has long been known as a precursor of molting hormones, such as ecdysteroids, that are needed for ecdysis in crustaceans (Grieneisen et al., 1993).

The retarded development found in *P. pelagicus* fed the diet containing soybean oil only may also be linked to the low n-3 HUFA content of the diet. It has been shown that n-3 HUFA is important for survival, molting and growth of crustacean (Xu et al., 1993; D'Abramo, 1997; Boonyaratpalin, 1998; Coutteau et al., 2000). For example, feeding larvae of the mud crab, *S. serrata*, with *Artemia* lacking HUFA has been shown to have detrimental effects on survival and leads to prolonged intermolt periods (Suprayudi et al., 2004). A similar result was reported for larvae of the swimming crab, *Portunus trituberculatus*, fed rotifers containing low level of n-3 HUFA (Hamasaki et al., 1998; Takeuchi, 2000). Megalopae of *S. serrata* fed a formulated diet containing no n-3 HUFA, showed mortality due to molt death syndrome (crabs having problems in extracting themselves from old exoskeleton during metamorphosis) (Holme et al., 2007b). This may be related to the function of HUFA as a precursor of eicosanoids, hormones generated in situ through cyclo-oxygenases and lipoxygenases pathways for molting and cell membrane receptors (Sargent et al., 1999; Gonzalez-Felix et al., 2003b).

The growth enhancing effects of n-3 HUFA for aquatic animals seems undisputed and this is reflected in the present study when *P. pelagicus* fed the diets containing high soybean oil ratios generally had lower specific growth rate (SGR) which was often significantly lower than that of crabs fed the diet containing high level of fish oil. However, it is worth noting that while the SGR (dry weight) of crabs fed the diet containing soybean oil only was the lowest, it was not significant different from crabs fed the diets containing lower ratios of fish oil, such as the diets with SO:FO ratios of 2:1 and 3:1. Such a result suggests that a threshold level of dietary n-3 HUFA may exist for good growth performance of *P. pelagicus.*

In summary, this study demonstrated that soybean oil can be used to substitute at least half of fish oil for the formulation of diets for *P. pelagicus* juveniles and obtained overall best performance. The results also suggest a threshold level of dietary n-3 fatty acids for good growth performance of *P. pelagicus*. This result is encouraging as it will help reduce the dependence of the aquaculture industry on fish oil and promote sustainable development of *P. pelagicus* culture.

# CHAPTER 8

## The effects of dietary arachidonic acid (20:4n-6) on survival, development and growth of *Portunus pelagicus* early juveniles

---

### 8.1 Introduction

The importance of essential fatty acids (EFA) for survival, development and growth of crustaceans has been extensively studied over past decades and their requirements are known to vary among species (Kanazawa et al., 1979b; Petriella et al., 1984; Reigh and Stickney, 1989; Xu et al., 1993; Merican and Shim, 1996; Glencross and Smith, 2001a; Gonzalez-Felix et al., 2003b; Liddy et al., 2004; Suprayudi et al., 2004; Wu et al., 2007). The EFA includes both the n-3 group fatty acids, such as linolenic acid (LNA; 18:3n-3), ecosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) and the n-6 group fatty acids, including linoleic acid (LOA; 18:2n-6) and arachidonic acid (ARA; 20:4n-6). These fatty acids are considered essential because of the limited capacity to synthesize them *de novo* from shorter chain fatty acids in crustaceans (Kanazawa et al., 1979b). Among these essential fatty acids, the highly unsaturated fatty acids (HUFA) have often shown greater nutritional values than the polyunsaturated fatty acids (PUFA) (Xu et al., 1994; Merican and Shim, 1997; Glencross et al., 2002a; Gonzalez-Felix et al., 2002b). For example, growth of juvenile black tiger prawn, *Penaeus monodon,* was significantly improved when they were fed a diet that contained DHA at 2.5% compared to those fed a diet supplemented with 3.0% LNA (Merican and Shim, 1997). However, within the HUFA, considerable research efforts have been dedicated to the importance and requirements of n-3

HUFA, especially DHA and EPA, to aquaculture species e.g. larvae of Chinese mitten crab, *Eriocheir sinensis* (Sui et al., 2007), juveniles of gilthead seabream, *Sparus aurata* (Ibeas et al., 1997) and juveniles of *Litopenaeus vannamei* (Gonzalez-Felix et al., 2002a). ARA, a type of n-6 HUFA conversely has attracted less attention (Xu et al., 2010) although lack of n-6 fatty acids in the diets have been clearly shown to negatively impact crustacean growth (Reigh and Stickney, 1989; Glencross and Smith, 1999).

Growth of crustaceans involves ecdysis or molting, a major physiological process during which animals need to extricate themselves from the old exoskeleton. This process is believed to be regulated by eicosanoids, a hormone derived from ARA (Gonzalez-Felix et al., 2003b). In brown tiger prawn, *Penaeus esculentus,* it has been reported that when injected with prostaglandin $E_2$, a type of eicosanoids, molt cycles were shortened with improved growth compared to those without prostaglandin treatment (Koskela et al., 1992). Adding algae that are rich in ARA to the culture water of mud crab larvae, *Scylla paramosain*, enriched rotifers, the live prey of the larvae and thus improved growth and molting frequency of the larvae (Nghia et al., 2001). In addition, in juveniles of the Chinese mitten crab, *E. sinensis,* larvae of the spiny lobster, *Jasus edwardsii,* and phyllosoma larvae of the western rock lobster, *Panulirus cygnus*, ARA was shown to be preferentially conserved and retained in the muscle when starved suggesting a crucial role as a structural lipid (Ritar et al., 2003; Liddy et al., 2004; Wen et al., 2006). In fish, eicosanoids derived from ARA have also been suggested to be more physiologically active than other eicosanoids derived from other HUFA and have critical roles in cellular signal transduction (Sargent et al., 1999). However, in comparison to EPA and DHA, the beneficial effects of ARA were relatively trivial. For

example, removing ARA from the diet of juvenile tiger prawn, *Penaeus monodon*, when the diet already contained high levels of other HUFA, showed no significant adverse effects on their growth rate and survival (Merican and Shim, 1996). Similarly, when ARA and DHA was supplemented at 1% of dry weight in diets of juvenile Chinese prawn, *Penaeus chinensis*, ARA was found to be less effective than DHA in improving their growth rate (Xu et al., 1994). The discrepancies between these studies probably reflects different needs for ARA among crustaceans and hence, calling for more species-specific study.

Additionally, there was an assumption that ARA requirements by aquaculture species might be met by the ARA already present in marine fish oil, a common ingredient in formulated diets, since ARA requirements is relatively low (Bell and Sargent, 2003; Xu et al., 2010). However, as the current utilization of marine fish oil for aquaculture feed is unsustainable and has led to substantially increased prices (Tacon and Metian, 2008), there have been increased efforts to replace fish oil with terrestrial oil (mainly plant oils) in the aquaculture feed production industry. Since, unlike fish oils, plant oils are often rich in monounsaturated fatty acids (MUFA) and PUFA but lack of HUFA (Merican and Shim, 1994; Kamarudin and Roustaian, 2002; Vasagam et al., 2005; Holme et al., 2007b), this has raised the question as to whether supplemental ARA is needed for diet formulation. To answer this question, quantitative studies of the ARA requirements of particular aquaculture species are called for. Thus, considering the importances of ARA as stated above, and experiment in Chapter 7 has managed to replace up to 50% fish oil the diet of *P. pelagicus*, this study was carried out to investigate the dietary ARA requirements of *P. pelagicus* early juveniles.

## 8.2 Materials and Methods

### *8.2.1 Source of crabs*

Broodstock capture and husbandry and larval culture protocol were described in Chapter 2 (Section 2.2.1).

### *8.2.2 Diet preparation*

Six iso-caloric and iso-lipidic diets were formulated to contain 40% purified ARA at 0.0 (control), 2.0, 4.0, 6.0, 8.0 and 10.0 g $kg^{-1}$ of the diet dry weight which was in addition to ARA already present in the fish oil used as an ingredient of these diets (Table 8.1). The formulation of these diets contained 40 g $kg^{-1}$ triglyceride with a combination of fish oil and soybean oil at 1:1 ratio which was found optimal for *P. pelagicus* early juveniles in previous Chapters 6 and 7 and macadamia oil was used to achieve iso-lipidicy of these diets. There were further two diets formulated to contain 40% purified ARA at 5.0 and 10.0 g $kg^{-1}$, respectively, however, in these two diets, no fish oil and soybean oil were used, only macadamia oil was added to provide iso-lipidicy of the diets. Macadamia oil was chosen because of its low content of PUFA and HUFA and thus could also provide further clues to the ARA supplementation requirement under conditions of negligible levels of PUFA and HUFA in the diets of *P. pelagicus* (Table 8.2).

Prior to diet preparation, fish meal was pulverized and defatted three times by vigorously mixing fish meal in a solvent mixture of chloroform and methanol (2:1, v/v) for 1 h (Folch et al., 1957). All dry and wet ingredients were then weighed on Sartorius TE2145 electronic balance (0.0001 g), and then mixed separately in an electric mixer before being

combined together to become a homogenous blend. Subsequently, agar, the diet binder used in this study, was prepared by dissolving it in hot water at above 80$^{o}$C (Sheen and Wu, 1999). As the solution became clear, it was allowed to cool to 40$^{o}$C when the diet ingredient mixture was added slowly to the agar solution and thoroughly mixed. The agar quickly gelatinized when temperature dropped to lower than 37$^{o}$C. The gelatinized diets were then cut to small pieces of approximately 2 mm$^{3}$ and stored in a -20$^{o}$C freezer until used.

**Table 8.1** Ingredients (g kg$^{-1}$ dry diet) of the experimental diets. Basal diet formulation based on Holme (2007a), Sheen and Wu (1999) and Genodepa (2004) with some modifications.

| *Basal mix* Ingredients | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 | Diet 6 | Diet 7 | Diet 8 |
|---|---|---|---|---|---|---|---|---|
| Defatted fish meal [1] | 500 | 500 | 500 | 500 | 500 | 500 | 500 | 500 |
| Vitamin mixture [3a] | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| Mineral Mixture [3b] | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| Choline chloride [2c] | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| DCP [2d] | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Agar [2g] | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 |
| Starch [2e] | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Cellulose [2f] | 44 | 44 | 44 | 44 | 44 | 44 | 44 | 44 |
| *Lipids* | | | | | | | | |
| Phospholipid [2a] | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 90 |
| Cholesterol [2b] | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Fish oil [2h] | 15 | 15 | 15 | 15 | 15 | 15 | - | - |
| Soybean oil [4] | 15 | 15 | 15 | 15 | 15 | 15 | - | - |
| Macadamia oil [5] | 10 | 8 | 6 | 4 | 2 | 0 | 35 | 30 |
| 40% purified Arachidonic Acid (ARA) [6] | 0 | 2 | 4 | 6 | 8 | 10 | 5 | 10 |

[1] Skretting Tasmania

[2] Sigma-Aldrich Pty Ltd [a]P3644, [b]C8667, [c]98% powder C7527, [d]dibasic calcium phosphate C4131, [e]S4126 (corn), [f]C8002 alpha, [g]A7002, [h]F8020 from menhaden

[3] Rabar Pty Ltd [a]ZZ600 DPI, each 1kg contains: vitamin A 2miu, vitamin D3 0.8miu, vitamin E 40g, vitamin K 2.02g, inositol 50g, vitamin B3 30.40g, vitamin B5 9.18g, vitamin B9 2.56g, vitamin B2 4.48g, vitamin B12 0.004g, biotin 0.1g, vitamin B6 4g, vitamin B1 3.4g, vitamin C 44.4g, para amino benzoic acid 20g, tixosil 5g, antioxidant 30g [b]ZZ603 DO 067 DPI, each 1kg contains: copper 1g, cobalt 100mg, magnesium 59.4mg, manganese 5g. iodine 800mg, selenium 20mg, iron 8mg, zinc 20g, aluminium 100mg, chromium 100mg

[4] CSD Grains Pty Ltd

[5] Macadamia (Australia) Ltd

[6] Cargill Pty Ltd

*8.2.3 Experimental design and set-up*

Newly molted C1 crabs used in this study were obtained as described in Chapter 2 (Section 2.2.2). They were placed individually into 750 mL circular culture vessels. The individual housing of all experimental crabs eliminated compounding effects of cannibalism and allowed precise recording of consecutive intermolt periods of each crab. Thirty healthy C1 crabs with average dry weight of 1.12 ± 0.05 mg, carapace width and carapace length of 3.38 ± 0.06 and 2.51 ± 0.03 mm, respectively, were used for each diet treatment and each culture unit was regarded as a replicate, hence a total of 240 crabs were used for the experiment. The culture units were randomly placed in water-bath with submerged heaters to maintain temperature at 28 ± 2$^{o}$C. A 100% water exchange was carried out every morning for all culture units before feeding. This was done by transferring crabs individually to new culture units while the previous units were sanitized with hypochlorite solution and dried overnight for next morning use. Molting and mortality were recorded during water exchange and crabs were then fed to satiation with designed diets. Salinity and pH was maintained at

30 ± 2‰ and 7.5 to 8.1 throughout the experiment while photoperiod was set at 14 h:10 h (light:dark) at all times.

Towards the end of the experiment, any crabs that were found molted to the crab stage 4 (C4) in the morning were removed from the culture in the afternoon for the measurement of carapace size (length and width) and dry weight. Sampling of crabs in the afternoon allowed sufficient time for newly molted crabs to harden their exoskeleton. Carapace measurements and dry weight determination were carried out as described in Chapter 3 (Section 3.2.3). The experiment was terminated when all crabs had either molted to the C4 stage or dead.

*8.2.4 Fatty acids analysis*

All experimental diets as well as the fish oil, soybean oil and macadamia oil used to formulate the diets were analyzed for fatty acid compositions. For the experimental diets, this was done by firstly extracting lipids from diet samples using the method of Folch et al. (1957). Lipid extracts and oil samples were then obtained by transesterified with 0.4 M KOH-methanol to produce fatty acid methyl esters (FAME). FAME were subsequently injected into an Agilent 6890 gas chromatograph fitted with an HP-5.5% Phenyl Methyl Siloam capillary column (30.0 mm x 25 mm, Agilent 19091J-413, USA) and substantiated by flame ionization detection (FID). The injector, detector and column temperature were set as described by Wu et al (2007). Peaks were identified by comparing the retention times with fatty acids standards (Sigma Chemical Co, St Louis, MO, USA) and individual fatty acid was quantified by the reference to the internal standard (19:0).

*8.2.5 Data and statistical analysis*

To assess growth rate during the duration of the experiment, specific growth rate (SGR) based on increments in dry weight, carapace width and length of the experimental crabs were calculated for each dietary treatment using the equation described in Chapter 3 (Section 3.2.5). Development time (days), dry weight (mg), carapace width (mm) and carapace length (mm) of newly molted C4 crabs as well as SGR data (% $day^{-1}$) were analyzed using one-way ANOVA after confirmation of normality and homogeneity of variance. Significant differences between treatments were determined using Tukey's test and the level of significant different was set at $p<0.05$. All statistics were performed using the SPSS statistic software, version 17.

## 8.3 Results

*8.3.1 Analyzed ARA contents and fatty acid compositions of experimental diets and oils*

The analyzed ARA contents as well as contents of other fatty acids of the experimental diets are shown in Table 8.3, while the fatty acid profiles of various oils used for diet formulation are shown in Table 8.2. The diet without ARA supplementation contained 0.17 g 100 $g^{-1}$ ARA which was likely to have come from fish oil as an ingredient of the diet. The diets formulated to contain 2.0, 4.0, 6.0, 8.0 and 10.0 g $kg^{-1}$ of 40% purified supplemental ARA had actual ARA contents of 0.93, 1.65, 2.4, 3.13 and 4.03 g 100 $g^{-1}$, respectively. The two diets that contained macadamia oil only instead of fish oil and soybean oil mixture, and supplemented with 5.0 and 10.0 g $kg^{-1}$ 40% purified ARA contained 2.15 and 3.86 g 100 $g^{-1}$ ARA, respectively (Table 8.3).

Among the three oils used for formulating the diets, macadamia oil contained a broader range of fatty acids compared to both soybean oil and fish oil although the most dominant fatty acids was the MUFA, oleic acid (18:1n-9) at 55.46 g 100 $g^{-1}$. In contrast, the soybean oil was dominated by the PUFA, linoleic acid (LOA; 18:2n-6) at 54.46 g 100 $g^{-1}$. Soybean oil contained an undetectable amount of HUFA, while macadamia oil contained a very low level of HUFA (0.52 g 100 $g^{-1}$). In contrast, fish oil, as expected, contained a high level of HUFA at 28.49 g 100 $g^{-1}$ (Table 8.2).

**Table 8.2** Fatty acid compositions (g 100 $g^{-1}$) of fish oil, soybean oil, macadamia oil and 40% purified arachidonic acid (ARA) used for diet formulation in the present study.

| Major fatty acids | Oil used for formulated diets | | | |
|---|---|---|---|---|
| | Fish oil | Soybean oil | Macadamia oil | ARA |
| 14:0 | 7.75 | - | 0.82 | 0.42 |
| 16:0 | 16.56 | 10.04 | 11.03 | 10.78 |
| 16:1n-7 | 11.01 | - | 16.00 | 0.28 |
| 18:0 | 2.90 | 3.84 | 4.96 | 7.09 |
| 18:1n-9 | 8.40 | 21.45 | 55.46 | 14.85 |
| 18:1n-7 | 2.86 | 1.38 | 2.11 | 0.81 |
| 18:2n-6 | 5.15 | 54.46 | 1.90 | 6.79 |
| 18:3n-3 | 1.26 | 8.29 | 3.34 | - |
| 18:3n-4 | 0.55 | - | 2.04 | - |
| 20:4n-6 | 0.91 | - | - | 41.19 |
| 20:5n-3 | 11.53 | - | 0.13 | 0.25 |
| 22:0 | - | 0.37 | 0.92 | - |
| 22:1 | - | - | 0.19 | - |
| 22:6n-3 | 11.84 | - | 0.39 | 0.34 |
| | | | | |
| ∑ SFA | 28.98 | 14.25 | 17.73 | 14.9 |
| ∑ MUFA | 26.54 | 23.00 | 73.57 | 16.55 |
| ∑ PUFA (≥18:2n) | 10.55 | 62.74 | 7.28 | 7.15 |
| ∑ HUFA (≥ 20:3n) | 28.49 | - | 0.52 | 45.33 |
| | | | | |
| ∑ n-3 | 31.85 | 8.30 | 3.86 | 0.85 |
| ∑ n-6 | 6.70 | 54.50 | 1.03 | 51.63 |
| n-3/n-6 | 4.77 | 0.15 | 2.03 | 0.02 |
| DHA/EPA | 1.03 | - | 3.00 | 1.36 |

*(∑) include minor fatty acids that are not shown in the Table

**Table 8.3** Fatty acids compositions (g 100 $g^{-1}$) of the experimental diets with different levels of supplemented 40% purified arachidonic acid (ARA) (g $kg^{-1}$).

| Major fatty acids | Contain mixture of fish oil, soybean oil (1:1) and macadamia oil | | | | | | Macadamia oil only | |
|---|---|---|---|---|---|---|---|---|
| | Supplemented ARA level | | | | | | | |
| | 0.0 | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | 5.0 | 10.0 |
| 14:0 | 1.44 | 1.51 | 1.39 | 1.43 | 1.50 | 1.43 | 0.38 | 0.37 |
| 14:1n7 | 0.47 | 0.42 | 0.85 | 0.27 | 0.28 | 0.45 | 0.46 | 0.37 |
| 15:0 | 0.16 | 0.10 | 0.15 | 0.15 | 0.14 | 0.15 | 0.04 | 0.03 |
| 16:0 | 15.9 | 16.18 | 16.35 | 15.96 | 16.57 | 16.51 | 13.43 | 13.92 |
| 16:1n7 | 3.66 | 3.30 | 3.07 | 2.63 | 2.48 | 2.21 | 6.18 | 5.62 |
| 16:1n5 | 0.10 | 0.07 | 0.17 | 0.08 | 0.06 | 0.06 | 0.05 | 0.03 |
| 16:2n4 | 0.18 | 0.13 | 0.19 | 0.15 | 0.16 | 0.16 | 0.09 | 0.08 |
| 16:3n4 | 0.19 | 0.18 | 0.20 | 0.20 | 0.21 | 0.20 | 0.08 | 0.08 |
| 18:0 | 3.86 | 6.51 | 3.49 | 5.58 | 3.98 | 3.63 | 5.57 | 7.99 |
| 18:1n9 | 16.72 | 13.51 | 15.34 | 12.93 | 12.60 | 13.09 | 27.18 | 23.16 |
| 18:1n7 | 1.74 | 1.35 | 1.64 | 1.2 | 1.39 | 1.45 | 1.82 | 1.49 |
| 18:2n6 | 42.17 | 43.67 | 42.75 | 43.86 | 45.07 | 44.47 | 32.33 | 34.48 |
| 18:3n6 | 0.02 | 0.07 | 0.11 | 0.15 | 0.21 | 0.21 | 0.10 | 0.19 |
| 18:3n3 | 5.30 | 5.67 | 5.08 | 5.03 | 5.37 | 5.59 | 3.39 | 3.57 |
| 18:3n4 | 0.52 | 0.40 | 0.47 | 0.47 | 0.47 | 0.45 | 0.03 | 0.30 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 18:4n3 | 0.33 | 0.21 | 0.29 | 0.26 | 0.27 | 0.23 | 0.28 | 0.25 |
| 20:1n9 | 0.49 | 0.36 | 0.38 | 0.30 | 0.25 | 0.25 | 1.24 | 0.97 |
| 20:1n7 | 0.53 | 0.42 | 0.42 | 0.40 | 0.33 | 0.30 | 1.00 | 0.84 |
| **20:4n6** | **0.17** | **0.93** | **1.65** | **2.40** | **3.13** | **4.03** | **2.15** | **3.86** |
| 20:4n3 | 0.24 | 0.19 | 0.31 | 0.23 | 0.20 | 0.13 | 0.17 | 0.11 |
| 20:5n3 | 1.88 | 1.64 | 1.68 | 1.81 | 1.89 | 1.70 | 0.58 | 0.65 |
| 22:2n6 | 0.15 | 0.28 | 0.10 | 0.24 | 0.29 | 0.14 | 0.05 | 0.12 |
| 22:5n3 | 0.34 | 0.31 | 0.44 | 0.32 | 0.28 | 0.34 | 0.62 | 0.14 |
| 22:6n3 | 1.82 | 1.61 | 1.58 | 1.75 | 1.74 | 1.67 | 1.09 | 1.01 |
| | | | | | | | | |
| ∑SFA | 21.36 | 24.30 | 21.38 | 23.12 | 22.19 | 21.72 | 19.42 | 22.31 |
| ∑MUFA | 23.71 | 19.43 | 21.87 | 17.81 | 17.39 | 17.81 | 37.93 | 32.48 |
| ∑PUFA (≥18:2n) | 48.16 | 50.09 | 49.41 | 49.75 | 51.41 | 50.86 | 35.9 | 38.66 |
| ∑HUFA (≥ 20:3n) | 4.45 | 4.68 | 5.72 | 6.51 | 7.24 | 7.87 | 4.61 | 5.77 |
| | | | | | | | | |
| ∑n-3 | 9.91 | 9.63 | 9.44 | 9.4 | 9.75 | 9.66 | 6.13 | 5.73 |
| ∑n-6 | 42.51 | 44.9 | 45.51 | 46.65 | 48.7 | 48.8 | 34.6 | 38.6 |
| n-3/n-6 | 0.23 | 0.21 | 0.21 | 0.20 | 0.20 | 0.20 | 0.18 | 0.15 |
| DHA/EPA | 0.97 | 0.98 | 0.94 | 0.97 | 0.92 | 0.98 | 1.88 | 1.55 |

*(∑) include minor fatty acids that are not shown in Table

The fatty acid profiles of the experimental diets largely reflected the relative levels of inclusion of the three oils as well as ARA supplementation (Table 8.3). For example, the levels of palmitoleic acid (16:1n-7), oleic acid (18:1n-9) and gonodoic acid (20:1n-9) substantially increased in the two diets that contained macadamia oil only as macadamia oil contained higher levels of these fatty acids, which in turn largely resulted in substantially higher MUFA levels of these diets. These two diets contain significantly less polyunsaturated fatty acids (PUFA) and highly unsaturated fatty acids (HUFA) than other diets, especially in regarding to linoleic acid (LOA; 18:2n-6), linolenic acid (LNA; 18:3n-3), ecosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). The levels of saturated fatty acids (SFA) in all diets were, however, relatively similar. Furthermore, it appeared that inclusions of supplemental ARA at all levels in the diets with fish oil inclusion did not significantly alter the n-3 to n-6 fatty acid ratios.

*8.3.2 Survival, development and growth*

The overall survival of *P. pelagicus* from newly settled C1 to C4 stage was relatively similar for all diet treatments, ranging from 50.0% to 76.7%. Crabs fed the diet without ARA supplementation and 60 g $kg^{-1}$ ARA supplementation achieved the highest survival of 76.7%. The two diets incorporating macadamia oil only, instead of fish and soybean oil mixture, and with 40% purified ARA inclusion level at 5.0 and 10.0 g $kg^{-1}$ (analyzed ARA content: 2.15 and 3.86 g 100 $g^{-1}$, respectively) yielded very similar survival of 63.3% and 60.0% (Fig. 8.1).

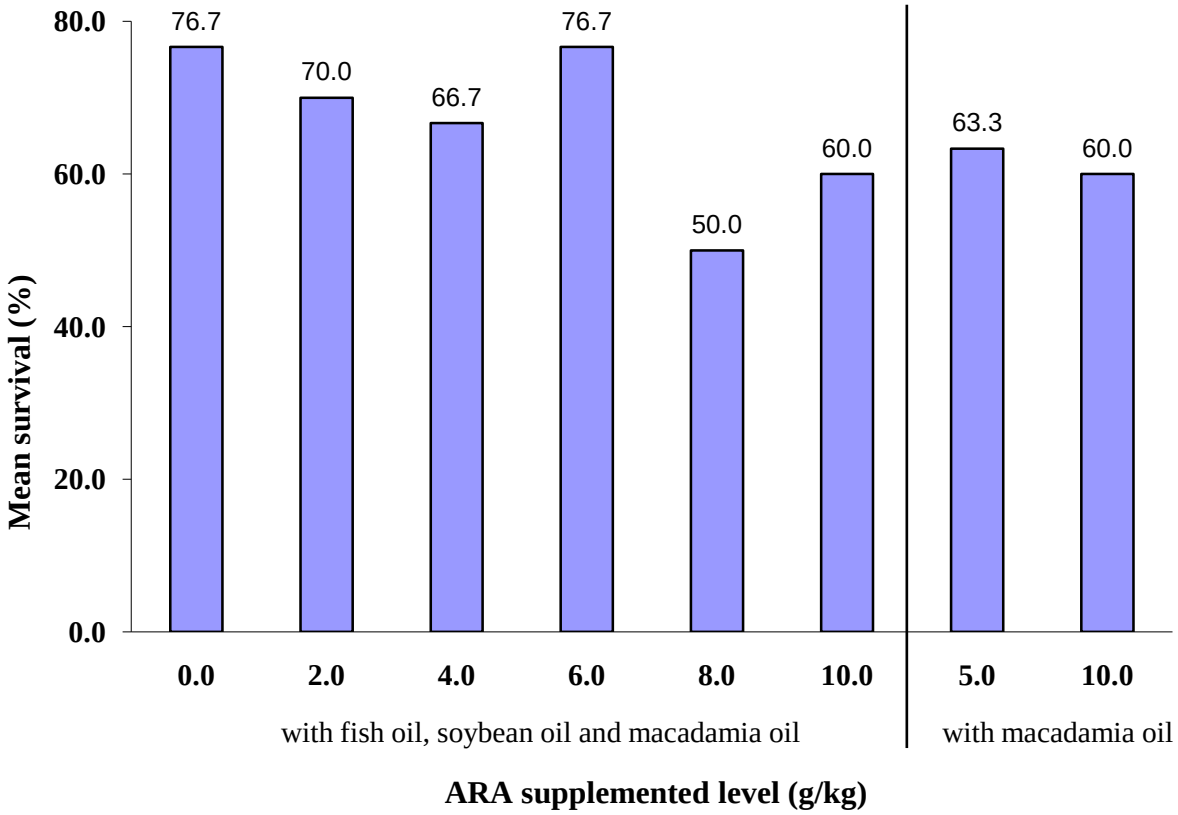



**Figure 8.1** Mean survival (%) from newly settled crab stage 1 to the crab stage 4 of *Portunus pelagicus*, fed diets containing different levels of arachidonic acid (ARA; 20: 4n-6).

Among all diet treatments, crabs fed the diet without ARA supplementation also had the fastest development time from C1 to C4 (18.5 ± 0.4 days) (Table 8.4). However, no significant difference was detected for either intermolt duration at each crab stage or for the cumulative development time from C1 to C4 among all diet treatments ($p>0.05$). In contrast to survival and development, the growth of early *P. pelagicus* juveniles was significantly affected by diets composition. The highest growth, in term of dry weight and carapace width and length of newly molted C4 crabs, came from the diet treatment in which crabs were fed the diet without ARA supplementation (analyzed ARA content: 0.17 g 100 $g^{-1}$) (Table 8.5). Higher ARA contents in the diets appeared to negatively impact on growth and SGR and both the dry weight of the C4 crabs as well as SGR (dry weight) of the diet treatment without ARA supplementation, which contained the lowest analyzed ARA level at 0.17 g 100 $g^{-1}$, were significantly higher ($p<0.01$) than all other treatments. No significant difference in dry weight or SGR (dry weight) was detected among the rest of the treatments ($p<0.05$) (Table 8.5).

**Table 8.4** The mean (± SE) intermolt period for each crab stage and mean cumulative development time from C1 to C4 (in days) of *Portunus pelagicus* fed semi-purified diets supplemented with different levels of arachidonic acid (ARA). No significant differences were detected between any treatments for all development time ($p>0.05$).

| Supplementation level of 40% purified ARA (g $kg^{-1}$) | C1 to C2 | C2 to C3 | C3 to C4 | Cumulative development time C1 to C4 |
|---|---|---|---|---|
| Diets contained mixture of fish oil, soybean oil (1:1) and macadamia oil | | | | |
| 0.0 | 4.7 ± 0.2 | 6.5 ± 0.2 | 7.5 ± 0.2 | 18.5 ± 0.4 |
| 2.0 | 4.9 ± 0.2 | 6.7 ± 0.3 | 8.0 ± 0.7 | 19.3 ± 0.9 |
| 4.0 | 5.8 ± 0.8 | 6.8 ± 0.3 | 8.3 ± 0.5 | 19.7 ± 0.8 |
| 6.0 | 4.5 ± 0.2 | 7.1 ± 0.3 | 8.5 ± 0.5 | 19.9 ± 0.7 |
| 8.0 | 4.1 ± 0.2 | 6.8 ± 0.2 | 7.7 ± 0.3 | 18.6 ± 0.5 |
| 10.0 | 5.1 ± 0.2 | 7.0 ± 0.3 | 8.1 ± 0.5 | 19.5 ± 0.8 |
| Diets contained macadamia oil only | | | | |
| 5.0 | 5.1 ± 0.3 | 7.7 ± 0.3 | 9.3 ± 0.5 | 21.0 ± 0.8 |
| 10.0 | 5.1 ± 0.3 | 7.6 ± 0.4 | 7.5 ± 0.4 | 19.2 ± 0.7 |

**Table 8.5** Mean (± SE) dry weight, carapace width and length and specific growth rate (SGR) of newly molted 4$^{th}$ stage juvenile *Portunus pelagicus* that had been fed the diets containing different levels of ARA since the day settled as the first stage crabs.

| Supplementation level of 40% purified ARA (g kg$^{-1}$) | Newly molted C4 crabs | | | Specific growth rate (SGR) | | |
|---|---|---|---|---|---|---|
| | Dry weight (mg) | Carapace width (mm) | Carapace length (mm) | Dry weight (% day$^{-1}$) | Carapace width (% day$^{-1}$) | Carapace length (% day$^{-1}$) |
| Diets contained mixture of fish oil, soybean oil (1:1) and macadamia oil | | | | | | |
| 0.0 | 8.06 ± 0.20$^{a}$ | 8.26 ± 0.11$^{a}$ | 4.60 ± 0.07$^{a}$ | 10.65 ± 0.13$^{a}$ | 4.82 ± 0.07$^{a}$ | 3.25 ± 0.08$^{a}$ |
| 2.0 | 6.99 ± 0.28$^{b}$ | 7.90 ± 0.13$^{ab}$ | 4.51 ± 0.07$^{a}$ | 9.44 ± 0.21$^{b}$ | 4.39 ± 0.08$^{ab}$ | 3.01 ± 0.08$^{ab}$ |
| 4.0 | 6.60 ± 0.39$^{b}$ | 7.42 ± 0.12$^{bc}$ | 4.12 ± 0.05$^{bc}$ | 8.96 ± 0.23$^{b}$ | 3.99 ± 0.09$^{bc}$ | 2.51 ± 0.06$^{cd}$ |
| 6.0 | 6.34 ± 0.24$^{b}$ | 7.01 ± 0.14$^{c}$ | 4.01 ± 0.10$^{c}$ | 8.68 ± 0.19$^{b}$ | 3.65 ± 0.10$^{c}$ | 2.33 ± 0.12$^{d}$ |
| 8.0 | 6.64 ± 0.17$^{b}$ | 7.64 ± 0.11$^{ab}$ | 4.42 ± 0.04$^{a}$ | 9.57 ± 0.15$^{b}$ | 4.37 ± 0.07$^{ab}$ | 3.03 ± 0.05$^{ab}$ |
| 10.0 | 6.33 ± 0.31$^{b}$ | 7.67 ± 0.18$^{ab}$ | 4.37 ± 0.07$^{ab}$ | 8.84 ± 0.25$^{b}$ | 4.19 ± 0.12$^{b}$ | 2.83 ± 0.09$^{bc}$ |
| Diets contained macadamia oil only | | | | | | |
| 5.0 | 6.35 ± 0.30$^{b}$ | 7.57 ± 0.15$^{bc}$ | 4.31 ± 0.06$^{ab}$ | 8.78 ± 0.33$^{b}$ | 4.09 ± 0.15$^{b}$ | 2.74 ± 0.10$^{bc}$ |
| 10.0 | 6.46 ± 0.31$^{b}$ | 7.52 ± 0.11$^{bc}$ | 4.30 ± 0.09$^{abc}$ | 9.09 ± 0.25$^{b}$ | 4.17 ± 0.08$^{b}$ | 2.79 ± 0.08$^{bc}$ |

*Different superscripts within a column indicate significant differences ($p<0.05$)

## 8.4. Discussion

In finfish, dietary arachidonic acid (ARA) has been demonstrated to promote survival and growth, improve adaptation to salinity changes in osmotic condition, enhance gamete production and larval quality, and promote better immune function (Bell and Sargent, 2003). For example, in larvae of the gilthead seabream, *Sparus aurata*, optimal level of dietary ARA has been shown to improve growth and survival, and resistance of larval fish to handling and osmotic stress (Koven et al., 2001). Similarly, salinity adaptation of Atlantic salmon, *Salmo salar*, was suggested to be linked to EPA/ARA ratio in the gill tissues as increased ARA level led to increased prostaglandins production which is responsible for salinity adaptation (Bell et al., 1997). However, for crustaceans, the importance of dietary ARA is less studied.

Among studies conducted on crustaceans, it was revealed that in the presence of other essential fatty acids (EFA), i.e. LOA, LNA, DHA and EPA, growth of tiger prawn, *P. monodon*, juveniles, was inversely related to higher ARA supplemental levels in their diet (Glencross and Smith, 2001b). In the present study, similar negative effects of dietary ARA higher than 0.17 g 100 $g^{-1}$ on growth of *P. pelagicus* juveniles was clearly demonstrated. In a study on juvenile Pacific white shrimp, *L. vannamei*, however, when fish oil was replaced by heterotrophic algal sources rich in DHA and ARA, no detrimental effects on final weight gain, survival and food efficiency rate of the juvenile prawns were detected (Patnaik et al., 2006). In contrast, for the mud crab, *S. serrata* juveniles, provision of ARA and DHA simultaneously in their diet reduced growth rates compared to those fed ARA and DHA supplied separately at 0.2% of diet dry weight (Sheen and Wu, 2003). For crustaceans, it has been suggested that between dietary EFA, in particular HUFA, high competitive interactions

exist (Glencross et al., 2002a). In a review by Sargent et al. (1999), three types of competitive interactions: competitions in fatty acid biosynthesis; competitions in eicosanoids formation; and action and competitions in phospholipid biosynthesis.

Eicosanoids are hormone-like compounds produced by HUFA to act in their immediate vicinity and they have significant biological functions, such as promoting molting and spawning in crustaceans (Sargent et al., 1999; Sheen and Wu, 1999; Tocher, 2003). In mammals and fish, EPA is believed to often compete with ARA for producing eicosanoids or for esterification spots with phospholipid in cellular membranes. These competitive interactions are determined by the ratio of ARA to EPA in the membranes, which is directly correlated to their dietary intake (Sargent et al., 1999; Tocher, 2003). An elevated level of ARA in the diet may alter such a balance. This may be particularly relevant as previous results from this study has found that a dietary n-3 and n-6 fatty acid profile resulting from incorporation of fish oil and soybean oil (1:1 ratio) for a total triglyceride level of 40 g $kg^{-1}$, was optimal for *P. pelagicus* early juveniles (Chapters 6 and 7). The 1:1 fish oil to soybean oil ratio has been adopted as the basal diet formulation in this chapter. It is also worth considering possible negative effects of excessive levels of dietary HUFA due to increased ARA level as this may make the diets more susceptible to oxidation, which could result in the diets becoming toxic to crustaceans (Yu and Sinnhuber, 1976; Gurr and Harwood, 1991).

It was interesting to note that provision of 40% purified ARA at levels of 5.0 and 10.0 g $kg^{-1}$ (analyzed ARA content at 2.15 and 3.86 g 100 $g^{-1}$, respectively), in the diets when macadamia oil was used to replace fish and soybean oil, survival, development and growth

achieved were similar to that of the crabs fed the diets with different levels of ARA supplementation plus fish oil and soybean mixture. As expected, the analyzed results showed that the later contained substantially higher levels of n-3 and n-6 essential fatty acids (EFA), including LOA, LNA, EPA and DHA. Although it obviously requires future experiments to verify, this may suggest possible compensatory values of ARA to the lower levels of these EFA for *P. pelagicus*. This study also clearly demonstrated that the requirement of ARA by *P. pelagicus* early juveniles is very low. ARA around 0.2 100 $g^{-1}$ in the diet is probably sufficient to support good survival, development and growth of young crabs and this normally could be met by the level of ARA present naturally in fish oil as a diet ingredient. This is an advantage for *P. pelagicus* aquaculture as it could incur significant cost savings in formulated feed production for the crab. However, it is worth noting that fish oil differs in its quality and this could potentially influence the level of ARA needed. Moreover, Glencross and Smith (2001) suggested that the requirement for ARA by crustaceans may be related to the particular stage of their life cycle and the significance of dietary ARA may more relevant to reproductive performance than growth. For example, in domesticated *P. monodon* broodstock, ARA supplementation of 5.0 g $kg^{-1}$ has been shown to increase the percentage of spawning females, number of spawning per female and number of eggs per spawning (Coman et al., 2009). The *P. pelagicus* used in present study were newly settled juveniles far from reaching sexual maturity. Therefore, their requirement for ARA may be much lower than later stages. Further investigations on the ARA requirements during puberty or the adult stage are therefore warranted for *P. pelagicus* to evaluate if ARA supplementation in addition to fish oil already incorporated in the diet is needed as at these stages.

# CHAPTER 9

## The effects of different dietary phospholipid and triglyceride ratios on survival, development, growth and stress resistance of *Portunus pelagicus* early juveniles

---

### 9.1 Introduction

Polar and neutral lipids are classified by their ability to associate with water i.e. polar lipids are hydrophilic lipids while neutral lipids are hydrophobic lipids. Both polar and neutral lipids possessed a glycerol back bone where fatty acids esterified (Gurr and Harwood, 1991). Polar lipids are primarily composed of phospholipid while neutral lipids often composed of triglyceride (Tocher, 2003). The nutritional values of phospholipid (PL) added to the diets of aquaculture species vary depending on the purity and types of PL used, such as phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE). Phospholipid can also be derived from various sources, including egg, bovine, soybean and marine fish. In aquaculture, the most commonly used dietary phospholipid is lecithin derived from soybean (Kanazawa et al., 1985; Chen, 1993; Coutteau et al., 1996; Camara et al., 1997; Thongrod and Boonyaratpalin, 1998; Coutteau et al., 2000). For triglyceride, their inclusion is often in the form of animal or plant oils, e.g. fish oil, corn oil or soybean oil. The dietary inclusion of triglyceride must consider factors includes source of the oil, addition of only one oil or a mixture of two or more oils, the ratio of two or more oils used and, most fundamentally, the fatty acid profile(s) of the oil(s) (D'Abramo and Sheen, 1993; Lim et al., 1997; Bell et al., 2001; Glencross et al., 2002d; Gonzalez-Felix et al., 2002b; Kamarudin and Roustaian, 2002; Ando et al., 2004; Coman et al., 2009).

Phospholipid and components of triglyceride, e.g. highly unsaturated fatty acids (HUFA) and polyunsaturated fatty acids (PUFA), cannot be synthesized *de novo* by crustaceans (Kanazawa et al., 1979b; Kanazawa et al., 1985). Dietary supply of PL and triglyceride in crustacean diets is therefore necessary as crustaceans require both of them as important source for essential fatty acids, choline, inositol and phosphate, which play crucial roles to the performance of farmed species (Oliva-Teres, 2000; L.Gonzalez-Felix et al., 2002; Michael et al., 2007; Tocher et al., 2008). Due to their importance for crustaceans, over the past decades, substantial efforts have been made to determine the optimum levels of dietary supplemental PL (Kean et al., 1985; Coutteau et al., 1996; Camara et al., 1997; Paibulkichakul et al., 1998) and triglyceride for cultured species (Sheen, 1997; Sheen and Wu, 1999; Glencross et al., 2002b; Gonzalez-Felix et al., 2002b). Past research in this area evolved from considering dietary PL and triglyceride requirements separately to determining optimal ratio of the two by addressing them simultaneously (Briggs et al., 1994; Kontara et al., 1997; Glencross et al., 1998; Gonzalez-Felix et al., 2002a; Wu et al., 2007).

Although both are considered essential, neutral lipids have been suggested to have relatively lower nutritional values than phospholipid as fatty acids in the polar lipid fraction are more beneficial for promoting survival and growth of crustaceans (Coutteau et al., 1996; Camara et al., 1997; Wold et al., 2007). Phospholipids are also known to assist in lipid metabolism by enhancing absorption of ingested fats, e.g. triglyceride, by either acting as an important part of lipid transporting vehicle, lipoprotein, or by increasing their solubility during digestion (Coutteau et al., 1997; Glencross et al., 1998; Yepiz-Plascencia et al., 2000). Increased polarity of lipid molecules enhances lipid assimilation perhaps by acting as a

surfactant for lipid emulsification (Conklin et al., 1980; Glencross and Smith, 1997). The effectiveness of acylsarcosyltaurine, an emulsifactant, was shown to increase when phospholipid is presence (Lester et al., 1975).

However, PL currently used in aquaculture is normally of terrestrial based, i.e. derived from soybean. They therefore, lack essential HUFA, such as ecosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (ARA; 20:4n-6) (Coutteau et al., 2000). These essential fatty acids are not only important for promoting good survival and growth of crustaceans, but are the precursors for eicosanoids, the molting hormones (Koskela et al., 1992). Therefore, the dietary supply of neutral lipids as an important source of fatty acids becomes crucial for crustaceans. For example, in mud crab, *Scylla serrata*, it has been reported that when a neutral lipid-free diet was fed to crabs, reduced molting frequency and growth resulted, which was linked to a lack of eicosanoids (Sheen and Wu, 1999). The present study, therefore, evaluated the relative nutritional values of dietary phospholipid and triglyceride as well as the effects of different dietary ratios of the two on the performance of *P. pelagicus* early juveniles.

## 9.2. Materials and methods

### *9.2.1 Source of crabs*

Broodstock capture and husbandry and larval culture protocol as were described in Chapter 2 (Section 2.2.1).

*9.2.2 Diet preparation*

Five iso-energetic and iso-lipidic diets were formulated to contain polar lipids (phospholipid) and neutral lipids (triglyceride mixture) at ratio of 1:0, 2:1, 1:1, 1:2 and 0:1, respectively. Phospholipid (Type IV-S, P 3644) used in this study was obtained from Sigma-Aldrich (Australia). Information provided by the manufacturer showed that the PL were derived from soybean with a purity >90% and contained 55% phosphatidylcholine (PC) and 20% phosphatidylethanolamine (PE). Triglyceride mixture used in this study is a combination of menhaden fish oil (F 8020) obtained from Sigma-Aldrich (Australia) and soybean oil supplied by CSD Grains Pty Ltd. Both of these oils were mixed at 1:1 ratio which was found optimal in Chapter 7.

To prepare the diets, fish meal was pulverized and sieved through 100 μm mesh before being added to a chloroform/methanol solution and mixed vigorously by magnetic stirrer (2:1, v:v) (Folch et al., 1957). The solution was then filtered with the aid of a vacuum pump and this defatting process was repeated three times to ensure maximum removal of lipids from the fish meal. All dry and wet ingredients of the diets were then mixed well in separate mixing bowls before the two were combined together and throughout blended in an electric mixer. Subsequently, agar, the binder used in this study, was dissolved in 100 mL distilled water heated to $\geq 80^{o}$C. As the agar solution turned clear, it was allowed to cool down to $40^{o}$C when the prior mentioned diet mixture was added slowly to the agar solution and the diet mixture quickly becomes gelatinized when temperature reduced to $37^{o}$C. The gelatinized diets was then cut to small pieces of approximately 2 $mm^3$ and stored in a freezer at $-20^{o}$C until used.

**Table 9.1** Formulation of experimental diets (g $kg^{-1}$) with different phospholipid and triglyceride (PL:TG) ratios. The analyzed contents of phospholipid and triglyceride of the diets are shown at the lower part of the Table. Diet formulation was modified from Holme (2007a), Sheen and Wu (1999) and Genodepa (2004).

| Ingredients | Dietary phospholipid : triglyceride (PL:TG) | | | | |
|---|---|---|---|---|---|
| | 1:0 | 2:1 | 1:1 | 1:2 | 0:1 |
| Defatted fish meal[1] | 500 | 500 | 500 | 500 | 500 |
| Cholesterol[2c] | 10 | 10 | 10 | 10 | 10 |
| Phospholipid[2d] | 150 | 100 | 75 | 50 | 0 |
| Triglyceride mixture; fish oil[2a]:soybean oil[4]; 1:1 | 0 | 50 | 75 | 100 | 150 |
| Vitamin mixture[3a] | 40 | 40 | 40 | 40 | 40 |
| Mineral mixture[3b] | 40 | 40 | 40 | 40 | 40 |
| Choline chloride[2e] | 10 | 10 | 10 | 10 | 10 |
| DCP[2f] | 6 | 6 | 6 | 6 | 6 |
| Agar[2g] | 120 | 120 | 120 | 120 | 120 |
| Starch[2h] | 80 | 80 | 80 | 80 | 80 |
| Cellulose[2i] | 44 | 44 | 44 | 44 | 44 |
| *Analyzed* | | | | | |
| Phospholipid | 110.8 | 100.6 | 91.5 | 62.3 | 5.4 |
| Triglyceride | 3.1 | 37.0 | 52.6 | 83.2 | 130.3 |

[1] Skretting Tasmania b Sigma-Aldrich Pty Ltd

[2] Sigma-Aldrich Pty Ltd [a]from menhaden F8020 [b]C8267 [c]C8667 [d]P3644 [e]98% powder C7527 [f]dibasic calcium phosphate C4131 [g]A7002 [h]S4126 (corn) [i]C8002 alpha

[3] Rabar Pty Ltd [a]ZZ600 DPI, each 1kg contains: vitamin A 2miu, vitamin D3 0.8miu, vitamin E 40g, vitamin K 2.02g, inositol 50g, vitamin B3 30.40g, vitamin B5 9.18g, vitamin B9 2.56g, vitamin B2 4.48g, vitamin B12 0.004g, biotin 0.1g, vitamin B6 4g, vitamin B1 3.4g, vitamin C 44.4g, para amino benzoic acid 20g, tixosil 5g, antioxidant 30g [b]ZZ603 DO 067 DPI, each 1kg contains: copper 1g, cobalt 100mg, magnesium 59.4mg, manganese 5g. iodine 800mg, selenium 20mg, iron 8mg, zinc 20g, aluminium 100mg, chromium 100mg

[4] CSD Grains Pty, Ltd

*9.2.3 Experimental design and set up*

First stage C1 crabs used in the experiment were obtained as described in Chapter 2 (Section 2.2.2). They were placed individually in 750 mL circular culture vessels to start the experiment. The mean (± SE) initial dry weight of the C1 crabs was 0.84 ± 0.01 mg, while the mean carapace width and length was 3.16 ± 0.06 and 2.52 ± 0.07 mm, respectively. There were 30 crabs in each dietary treatment, bringing the total culture units to 150. The individual housing of the crabs was necessary to eliminate any compounding effects of cannibalism and to allow precise recording of intermolt periods for each crab. Throughout the experiment, a 100% water exchange was carried out daily in the morning for all culture units during which crabs were transferred individually to new culture vessels and any molts and mortality were recorded. After the water exchange, all surviving crabs were fed to satiation with designed diets and the vessels used from the previous day were sanitized in hypochlorite solution and dried overnight for next morning use. Throughout the experiment, temperature and salinity were monitored daily and kept at 28 ± 1$^{o}$C and 30 ± 2‰ , respectively, while photoperiod was set at 14:10 (light: dark).

Towards the end of the experiment, any crabs that were found molted to the 4$^{th}$ stage crabs (C4) in the morning were removed from the culture in the afternoon for measurement of carapace size and dry weight. Sampling of crabs in the afternoon allowed sufficient time for newly molted crabs to harden their exoskeleton. Carapace measurements and dry weight determination were carried out as described in Chapter 3 (Section 3.2.3). The experiment was terminated when all crabs had either molted to the C4 stage or had died.

To test if dietary different ratios of PL and triglyceride resulted in variations in osmotic shock resistance of the crabs, ten randomly selected C4 crabs from each dietary treatment were subject to a sudden salinity reduction from 30‰ to 7‰. All crabs used for this test were newly molted but fully calcified C4 crabs and they were transferred directly from a salinity of 30‰ to 7‰ and retained at 7‰ for 540 minutes (9 hours). The choice of 7‰ salinity for the osmotic shock test was based on a previous study on *P. pelagicus* salinity tolerant by Romano and Zeng (2006). From the point that osmotic shock started, mortalities of the crabs were monitored every 10 minutes for the first hour but every 20 minutes for the following 8 hours. Crabs were considered dead if they did not responded to gentle probes. Crabs from the diet treatment with PL:TG ratio at 0:1 was excluded from the osmotic shock test because of low survival to C4 of the treatment.

*9.2.4 Analysis of phospholipid, triglyceride and fatty acid profiles of experimental diets*

To analyze phospholipid (g $kg^{-1}$) and triglyceride (g $kg^{-1}$) contents, as well as fatty acid compositions (g 100 $g^{-1}$), of the experimental diets, total lipid was extracted using chloroform: methanol (2:1, v/v) based on the method described by Folch et al. (1957). Phospholipids in the lipid aliquot were separated from neutral lipids using petroleum:methanol (1:1, v/v) by dissolving them in the solution (Folch et al., 1957). The solvents were volatized using a stream of nitrogen to determine residue from the petroleum fraction hence determining the weight of total lipid (PL and neutral lipids). The lipid fractions were subsequently separated and quantified using an Iatroscan MK-6s TLC-FID analyzer (Iatron Laboratories Inc., Tokyo, Japan) to determine triacylglycerides content (TG) (Wu et al., 2007; Wu et al., 2010b).

Fatty acid compositions of the oils and phospholipid used for diet formulation as well the experimental diets were analyzed following transesterification of lipid with 0.4 M KOH-methanol to produce fatty acid methyl esters (FAME). FAME were then injected into an Agilent 6890 gas chromatograph fitted with an HP-5.5% Phenyl Methyl Siloam capillary column (30.0 m x 25 mm, Agilent 19091J-413, USA) and substantiated by flame ionization detection (FID). The injector, detector and column temperature were set as described by Wu et al. (2007). Peaks were identified by comparing the retention times with fatty acids standards (Sigma Chemical Co, St Louis, MO, USA).

*9.2.5 Data and statistical analysis*

To assess growth rate over the culture period, specific growth rate (SGR) based on increments in crab dry weight, carapace width and length were calculated for each diet treatment using the equation described in Chapter 3 (Section 3.2.5). Development time (days), dry weight (mg), carapace width (mm) and carapace length (mm) of newly molted C4 crabs as well as SGR data (% $day^{-1}$) were analyzed using one-way ANOVA after confirmation of normality and homogeneity of variances. Significant differences between treatments were determined using Tukey's test and the level of significance different was set at $p<0.05$. All statistics was performed using the SPSS statistic software, version 17.

**9.3 Results**

*9.3.1 Diet phospholipid, triglyceride and fatty acid contents*

The analyzed contents of phospholipid and triglyceride are shown in Table 9.1 while fatty acid compositions of the triglyceride and phospholipid used for diet formation, and

those of experimental diets, are shown in Tables 9.2 and 9.3, respectively. Diets formulated to have PL:TG ratios of 1:0, 2:1, 1:1, 1:2 and 0:1, actually contained PL at 110.8, 100.6, 91.5, 62.3 and 5.4 g $kg^{-1}$, respectively, and triglyceride at 3.1, 37.0, 52.6, 83.2 and 130.3 g $kg^{-1}$, respectively (Table 9.1). Overall, the ratios of phospholipid (PL) and triglyceride (TG) obtained from analysis were close to formulation values.

The phospholipid used was derived from soybean and contained moderate levels of saturated fatty acids (SFA) at 29.60 g 100 $g^{-1}$ and a high level of PUFA at 61.76 g 100 $g^{-1}$, of which palmitic acid (16:0) and linoleic acid (LOA: 18:2n-6) were the main contributors (Table 9.2). The soybean oil used for diet formation contained similarly high level of PUFA (62.74 g 100 $g^{-1}$) but had a with higher level of monounsaturated fatty acids (MUFA) (23.00 g 100 $g^{-1}$). The fish oil contained a more diverse/balanced fatty acid profiles with similar SFA, MUFA and HUFA level at 28.98, 26.54 and 28.49 g 100 $g^{-1}$, respectively. Fish oil also contained other essential fatty acids, i.e. LOA (18:2n-6), linolenic acid (LNA; 18:3n-3), EPA (20:5n-3), DHA (22:6n-3) and ARA (20:4n-6) (Table 9.2).

Fatty acid compositions of the experimental diets generally reflected the fatty acid compositions of the phospholipid and triglyceride supplemented in the diets. For example, diets supplemented with phospholipid only, contained the highest level of LOA, while diets supplemented with higher ratios of triglyceride (PL:TG ratios at 0:1 and 2:1) contained higher levels of EPA and DHA. In addition, with the exception of diets supplemented with phospholipid only, the DHA and EPA ratios of the diets were generally quite similar (Table 9.3).

**Table 9.2** Fatty acid compositions (g 100 $g^{-1}$) of phospholipid and triglyceride mixture used for formulating with varying phospholipid to triglyceride ratios (PL:TG).

| Major fatty acids | Phospholipid | Fish oil | Soybean oil |
|---|---|---|---|
| 14:0 | 0.15 | 7.75 | - |
| 16:0 | 23.44 | 16.56 | 10.04 |
| 16: 1n-7 | 0.22 | 11.01 | - |
| 18:0 | 5.67 | 2.90 | 3.84 |
| 18: 1n-9 | 5.15 | 8.40 | 21.45 |
| 18: 1n-7 | 1.36 | 2.86 | 1.38 |
| 18: 2n-6 | 54.99 | 5.15 | 54.45 |
| 18: 3n-3 | 6.76 | 1.26 | 8.29 |
| 18: 3n-4 | - | 0.55 | - |
| 20: 4n-6 | - | 0.91 | - |
| 20:5n-3 | - | 11.53 | - |
| 22:0 | - | - | 0.37 |
| 22:6n-3 | - | 11.84 | - |
| | | | |
| ∑ SFA | 29.60 | 28.98 | 14.25 |
| ∑ MUFA | 7.83 | 26.54 | 23.00 |
| ∑ PUFA (≥18:2n) | 61.76 | 10.55 | 62.74 |
| ∑ HUFA (≥20:3n) | - | 28.49 | - |
| | | | |
| ∑ n-3 | 6.88 | 31.85 | 8.30 |
| ∑ n-6 | 58.21 | 6.70 | 54.50 |
| n-3/n-6 | 0.12 | 4.77 | 0.15 |
| DHA/EPA | - | 1.03 | - |

*(∑) include minor fatty acids that are not shown in the Table

**Table 9.3** Fatty acid compositions (g 100 $g^{-1}$) of experimental diets containing different phospholipid and triglyceride mixture ratios (PL:TG).

| | Experimental diets | | | | |
|---|---|---|---|---|---|
| Major fatty acids | Diet 1 (PL:TG =1:0) | Diet 2 (PL:TG = 2:1) | Diet 3 (PL:TG = 1:1) | Diet 4 (PL:TG = 1:2) | Diet 5 (PL:TG = 0:1) |
| C14:0 | 0.25 | 1.9 | 2.7 | 3.84 | 3.5 |
| C14:1n7 | 0.6 | 0.39 | 0.38 | 0.27 | 0.41 |
| C16:0 | 29.5 | 16.38 | 16.22 | 14.26 | 16.36 |
| C16:1n7 | 0.37 | 2.82 | 3.9 | 5.76 | 5.1 |
| C18:0 | 2.63 | 3.69 | 4.31 | 4.74 | 3.79 |
| C18:1n9 | 8.74 | 12.42 | 13.2 | 14.19 | 14.01 |
| C18:2n6 | 47.56 | 45.63 | 39.08 | 27.34 | 35.29 |
| C18:3n6 | 0.05 | 0.11 | 0.13 | 0.36 | 0.12 |
| C18:3n3 | 5.15 | 5.68 | 5.38 | 4.7 | 4.84 |
| C18:3n4 | 0.35 | 0.65 | 0.9 | 1.38 | 1.08 |
| C18:4n3 | - | 0.23 | 0.19 | 0.14 | 0.21 |
| C20:1n9 | - | 0.18 | 0.22 | 0.31 | 0.26 |
| C20:1n7 | - | 0.31 | 0.41 | 0.78 | 0.49 |
| C20:4n6 | - | 0.2 | 0.26 | 0.43 | 0.28 |
| C20:3n3 | - | 0.08 | 0.08 | 0.08 | 0.06 |
| C20:4n3 | - | 0.29 | 0.43 | 0.72 | 0.5 |

| | | | | | |
|---|---|---|---|---|---|
| C20:5n3 | 0.27 | 2.61 | 3.46 | 5.77 | 4.27 |
| C22:2n6 | - | 0.22 | 0.2 | 0.31 | 0.24 |
| C22:5n3 | - | 0.48 | 0.63 | 1.11 | 0.74 |
| C22:6n3 | 0.7 | 2.37 | 2.87 | 4.93 | 3.4 |
| | | | | | |
| ∑ SFA | 32.49 | 22.13 | 23.45 | 23.29 | 23.92 |
| ∑ MUFA | 11.42 | 17.74 | 19.96 | 23.11 | 22.33 |
| ∑ PUFA | 53.11 | 52.29 | 45.69 | 34.22 | 41.57 |
| ∑ HUFA | 0.97 | 6.03 | 7.73 | 13.31 | 9.25 |
| | | | | | |
| ∑ n-3 | 6.12 | 11.74 | 13.04 | 17.45 | 14.02 |
| ∑ n-6 | 47.61 | 46.16 | 39.67 | 28.71 | 35.93 |
| n-3/n-6 | 0.13 | 0.25 | 0.33 | 0.61 | 0.39 |
| DHA/EPA | 2.59 | 0.91 | 0.83 | 0.85 | 0.80 |

*(∑) include minor fatty acids that are not shown in Table

*9.3.2 Crab survival, development and growth*

The highest survival of 83.3% was recorded for crabs fed the diet with a PL:TG ratio of 1:1. This is followed by survival of 80.0%, 76.7% and 70.0% for crabs fed the diets with PL:TG ratios of 1:2, 1:0 and 2:1, respectively. The lowest survival of 40.0% was recorded for crabs fed the diet supplemented with triglyceride only (PL:TG at 0:1) (Figure 9.1).

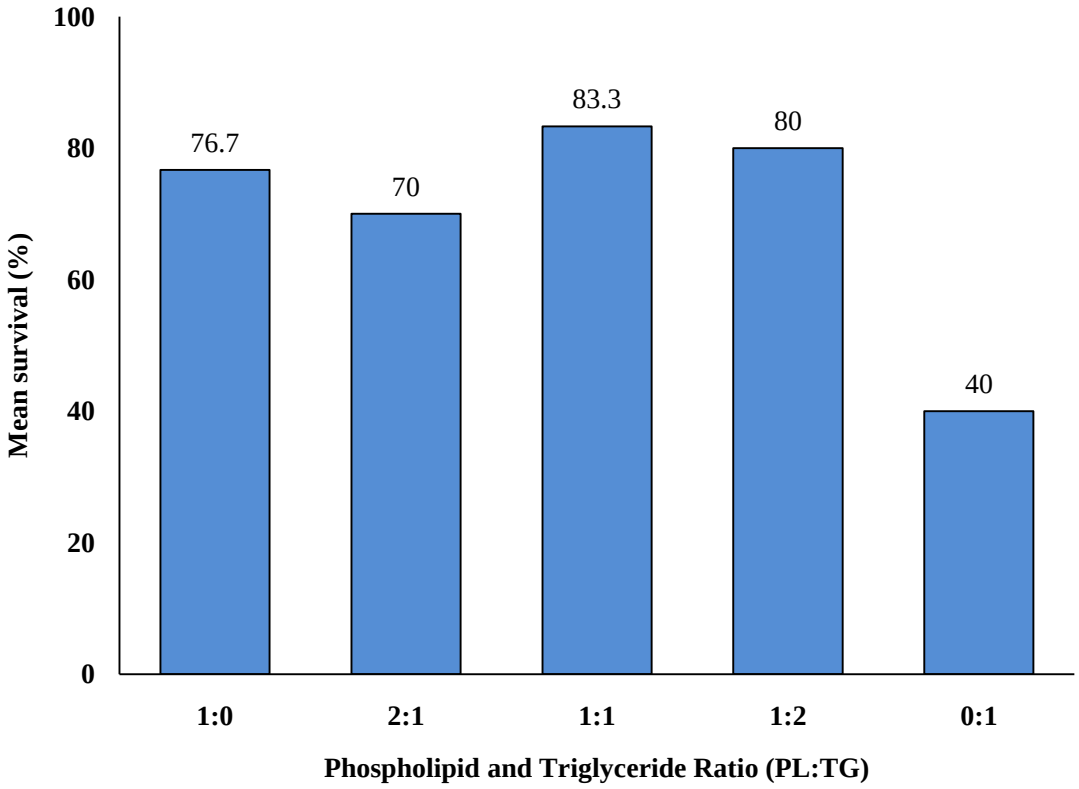



**Figure 9.1** Mean survival (%) of *Portunus pelagicus* from the newly settled first stage crabs to the fourth crabs stage (C4) when fed iso-nitrogenous and iso-lipidic semi-purified diets containing different ratios of phospholipid and triglyceride mixture (PL:TG).

No significant difference in development time was found among treatments for the first molt from C1 to C2 (Table 9.4). However, from C2 onwards, the differences in intermolt durations of crabs fed different diets became significant ($p<0.05$). Crabs fed the diet with a PL:TG ratio of 0:1 had the longest intermolt durations for C2 and C3 stages and for the cumulative development time from C1 to C4; the differences were highly statistically significant ($p<0.01$) (Table 9.4). Crabs fed the diet with a PL:TG ratio of 1:2 also had significantly longer development times for the C3 stage and the cumulative development time from C1 to C4, when compared to crabs fed diets with PL:TG ratios of 1:0 and 1:1 ($p<0.05$) (Table 9.4). However, no significant difference in development time was found among crabs fed the diets with PL:TG ratios of 1:0, 2:1 and 1:1 at all stages ($p>0.05$).

**Table 9.4** Mean (± SE) development time for each crab stage and cumulative development time from C1 to C4 stage (in days) of *Portunus pelagicus* fed semi-purified diets with different phospholipid and triglyceride mixture ratios (PL:TG).

| Dietary phospholipid to triglyceride mixture ratio (PL : TG) | C1 to C2 | C2 to C3 | C3 to C4 | Cumulative development time C1 to C4 |
|---|---|---|---|---|
| 1 : 0 | 4.0 ± 0.2 | 5.1 ± 0.2$^{a}$ | 6.3 ± 0.3$^{ab}$ | 15.2 ± 0.5$^{a}$ |
| 2 : 1 | 4.2 ± 0.1 | 5.4 ± 0.2$^{a}$ | 6.3 ± 0.2$^{ab}$ | 15.4 ± 0.3$^{ab}$ |
| 1 : 1 | 4.3 ± 0.1 | 5.1 ± 0.2$^{a}$ | 5.8 ± 0.2$^{a}$ | 15.1 ± 0.3$^{a}$ |
| 1 : 2 | 4.1 ± 0.1 | 5.9 ± 0.2$^{ab}$ | 6.8 ± 0.2$^{b}$ | 16.7 ± 0.3$^{b}$ |
| 0 : 1 | 4.2 ± 0.1 | 6.7 ± 0.3$^{b}$ | 8.3 ± 0.5$^{c}$ | 18.8 ± 0.8$^{c}$ |

$^{abc}$ Different superscripts within a column indicate significant differences ($p<0.05$)

There was a clear trend that crabs fed the diets containing higher PL to TG ratios had greater dry weights and large carapace size as newly molted C4 crabs, and the differences between treatments were often statistically significant ($p<0.05$) (Table 9.5). The same trend was also shown for specific growth rates (SGR) based on dry weight, carapace width and carapace length, with crabs fed the diet with a PL:TG ratio of 1:0 having the highest SGR for dry weight ($15.44 \pm 0.48$ % $day^{-1}$), carapace width ($6.46 \pm 0.10$ % $day^{-1}$) and carapace length ($3.96 \pm 0.11$ % $day^{-1}$), and crabs fed the diet with a PL:TG ratio of 0:1 had the lowest SGR for dry weight ($9.13 \pm 0.66$ % $day^{-1}$), carapace length ($4.45 \pm 0.14$ % $day^{-1}$) and carapace length ($2.47 \pm 0.09$ % $day^{-1}$). The SGR (dry weight, carapace width and carapace length) for diet treatments with higher or same PL to TG ratios (PL:TG at 1:0, 2:1 and 1;1) were significantly higher than those for the diet treatments with lower PL to TG ratios (i.e. PL:TG at 1:2 and 0:1), while SGR of the diet treatment with a PL:TG ratio of 1:2 were significantly higher than those of the diet treatment with PL:TG ratio at 0:1 ($p<0.05$) (Table 9.5). However, no significant differences in SGR were found among the diet treatments with PL:TG ratios of 1:0, 2:1and 1:1 ($p<0.05$) (Table 9.5).

**Table 9.5** Mean (± SE) dry weight, carapace width and length, and specific growth rate (SGR) of newly molted 4$^{th}$ stage juvenile *Portunus pelagicus* fed diets containing different phospholipid and triglyceride mixture ratios (PL:TG).

| Dietary phospholipid and triglyceride mixture (PL:TG) | Newly molted C4 crabs | | | Specific growth rate (SGR) | | |
|---|---|---|---|---|---|---|
| | Dry weight (mg) | Carapace width (mm) | Carapace length (mm) | Dry weight (% day$^{-1}$) | Carapace width (% day$^{-1}$) | Carapace length (% day$^{-1}$) |
| 1 : 0 | 9.02 ± 0.65$^{a}$ | 8.47 ± 0.12$^{a}$ | 4.60 ± 0.08 $^{a}$ | 15.44 ± 0.48$^{a}$ | 6.46 ± 0.10$^{a}$ | 3.96 ± 0.11$^{a}$ |
| 2 : 1 | 7.21 ± 0.31$^{ab}$ | 8.04 ± 0.07$^{ab}$ | 4.53 ± 0.07$^{a}$ | 13.89 ± 0.27$^{a}$ | 6.06 ± 0.06$^{a}$ | 3.80 ± 0.08$^{a}$ |
| 1 : 1 | 6.90 ± 0.43$^{ab}$ | 7.96 ± 0.16$^{ab}$ | 4.45 ± 0.08$^{a}$ | 13.81 ± 0.43$^{a}$ | 6.10 ± 0.14$^{a}$ | 3.76 ± 0.12$^{a}$ |
| 1 : 2 | 5.46 ± 0.35$^{bc}$ | 7.73 ± 0.18$^{bc}$ | 4.35 ± 0.08$^{a}$ | 11.07 ± 0.43$^{b}$ | 5.34 ± 0.15$^{b}$ | 3.26 ± 0.10$^{b}$ |
| 0: 1 | 5.05 ± 0.69$^{c}$ | 7.33 ± 0.20$^{c}$ | 4.01 ± 0.07$^{b}$ | 9.13 ± 0.66$^{c}$ | 4.45 ± 0.14$^{c}$ | 2.47 ± 0.09$^{c}$ |

$^{abc}$ Different superscripts within a column indicate significant differences ($p<0.05$)

*9.3.3 Osmotic shock*

Mortality of C4 crabs over a 9 h period following sudden salinity drop from 30 to 7‰ is shown in Fig. 9.2. The diet treatment with a PL:TG ratio of 0:1 was excluded from the test because too few crabs made to C4. For all diet treatments, mortality began 3 h after osmotic shock. However, for crabs fed the diets with PL:TG ratios of 1:2 and 1:1, mass mortalities ensued in the subsequent 2 h, resulting in total mortality after 6 h for the crabs fed diet with a PL:TG ratio at 1:2. A few crabs from the diet treatment with a PL:TG ratio of 1:1 survived a bit longer but total mortality occurred after 9 h. In contrast, crabs from both dietary treatments with higher PL to TG ratios (PL:TG at 1:0 and 2:1) still had 50% crabs survival after 9 h when the test concluded (Fig. 9.2).

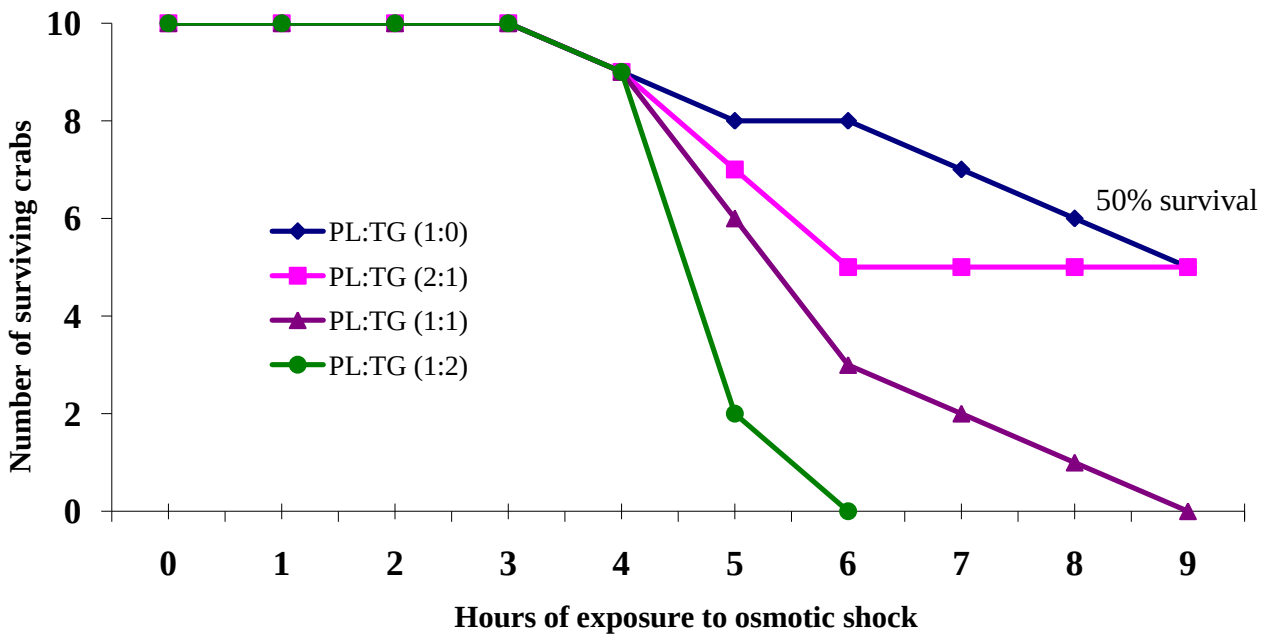



**Figure 9.2** Survival of newly molted 4$^{th}$ stage juvenile of *Portunus pelagicus* subjected to sudden salinity change from 30‰ to 7‰ over a 9 h period. The crabs were fed diets containing different ratios of phospholipid and triglyceride mixture (PL:TG) since the day settled as the first stage crabs.

## 9.4 Discussion

The appropriate dietary levels of phospholipid and triglyceride have long been a subject of investigation for crustacean nutritionist due to their important roles in promoting survival and growth of cultured crustaceans (Gonzalez-Felix et al., 2002a; Sheen and Wu, 2003; Gong et al., 2007). In the present study, development and growth of early juveniles of the blue swimmer crab, *P. pelagicus* fed diets containing various ratios of PL to TG demonstrated that dietary PL are considerably more effective in promoting growth than the triglyceride mixture. For development time, addition of triglyceride at level higher than PL in the diets of *P. pelagicus* prolonged their development time from the C1 to C4 stage, illustrating the superiority of PL compared to triglyceride.

Based on past studies on the dietary lipid requirements of crustaceans, optimal dietary neutral lipid (mixture of marine and plant oil, or marine oil only) inclusion is generally within the range of 2 to 10% of diet dry weight. Inclusion of these oils in crustacean diets above the optimal level often resulted in reduced growth (Castell and Covey, 1976; Davis and Robinson, 1986; Sheen and D'Abramo, 1991; Sheen and Wu, 1999). The impaired growth rate has been suggested to be caused by nutrient imbalances and related to toxic products of lipid oxidation at excessively high levels, or due to excessive dietary energy (Akiyama et al., 1992; D'Abramo, 1997). However, in the present study, the energy level of all experimental diets was formulated to be very similar (iso-nitrogenous and iso-lipidic), thus any significant adverse effects observed in some diet treatments (e.g. diet treatments with PL:TG ratios of 0:1 and 1:2), is clearly not due to the level of total lipids supplemented in the diets (160 g $kg^{-1}$; including inclusion of cholesterol), but rather, the high levels of

triglyceride. In comparison to neutral lipids, polar lipids have been suggested to be better sources of energy and essential fatty acids due to their higher digestibility (Coutteau et al., 1997). For example, in juveniles of the black tiger prawn, *Penaeus monodon*, the apparent digestibility of neutral lipids supplied in the form of natural and/or enriched oil and free fatty acids, sharply reduced when the level of dietary neutral lipids exceeded 135 g $kg^{-1}$ (Glencross et al., 2002b).

PL have been shown to improve the digestion and utilization of neutral lipids and increase the growth rate of various crustaceans (Kanazawa et al., 1985; Coutteau et al., 1996; Gong et al., 2001; Vasagam et al., 2005). PL are known to assist neutral lipids transportation in crustaceans; as an important component of lipoprotein, PL help transporting triglyceride from gut to hepatopancreas and subsequently to other tissues (Teshima et al., 1986a). For juveniles of *P. monodon*, it has been reported that dietary PL at a level of 2.7% diet dry weight is optimal for digestion of dietary neutral lipids at 8.5% of diet dry weight. Increased PL to 4% in the diet appeared to reduce the apparent digestibility of the neutral lipids although the difference was not significant (Glencross et al., 1998). In the present study, inclusion of neutral lipids in the form of triglyceride above 50% of the supplemented lipid in the diets of *P. pelagicus* was found to significantly reduce development and growth of the crabs. A previous study has shown that the rate of fatty acids binding to the phospholipids vesicles during transportation depends on the quantity of the fatty acids, in addition to the concentration of fatty acids monomers and specific fatty acid chain (Hamilton, 1998).

The active components of PL in improving growth and survival of crustaceans have been suggested to include phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and fatty acid components of the phospholipid (Coutteau et al., 1997). Among these lipid compositions, PC has been found to be the most active constituent for enhancing feed utilization and biological functions of crustaceans (Kanazawa et al., 1985; Michael et al., 2007). For fatty acid compositions of PL, among major terrestrial sources, e.g. chicken egg phosphatidylcholine (PC), soybean PC, bovine brain phosphatidylethanolamine (PE) and ovine brain phosphatidylserine (PS), soybean PC was found to be the most beneficial in improving growth and survival of the Kuruma prawn, *Marsupenaeus japonicus* (Kanazawa et al., 1985) due to it containing high levels of polyunsaturated fatty acids, dominantly linoleic acid (LOA; 18:2n-6) and linolenic acid (LNA; 18:3n-3). The present study also used PL derived from soybean, which contained relatively high PC at 55% PC and more than 60% PUFA. Though it is not a purely purified product, it is available at a substantially lower price. It therefore suits the objective of the present study to understand lipid nutrition of *P. pelagicus* with the ultimate aim of developing a practical formulated feed for the culture of the crab species.

Interestingly, despite the soybean derived PL used in the present study containing low amounts of highly unsaturated fatty acids (HUFA), the development and growth rate of crabs from the diet treatment containing PL only, and without triglyceride supplementation (PL:TG ratio at 1:0), were not negatively affected. This might be partially attributed to the existence of essential fatty acids in the tissues and cells of *P. pelagicus* conserved from the larval stages into the first stage crabs. Furthermore, all experimental diets used in this study have

been formulated to contain cholesterol at an optimal level (Chapter 3), hence their molt may be supported by the optimal production of ecdysteroids, the molting hormones derived from cholesterol (Skinner, 1985).

It is worth noting that unlike neutral lipids, the high level of PL inclusion in the diets was found not to negatively impact survival, development and growth of *P. pelagicus* early juveniles. Similar results have been reported for other decapod crustaceans, such as clawed lobster, *Homarus* sp. (Conklin et al., 1980) and post larvae of *P. monodon* (Paibulkichakul et al., 1998). It was further found that high levels of dietary PL supplementation significantly improved the ability of crabs to resist osmotic shock, indicating high dietary PL levels enhanced osmoregulatory ability of *P. pelagicus.* However, further studies are warranted to confirm this as the improved tolerance to salinity shock could actually reflect the overall better physiological condition of *P. pelagicus* fed the diets containing high levels of PL as these crabs generally had shorter developmental times and higher growth rates. In summary, this experiment demonstrated the superiority of dietary polar lipids in the form of phospholipid to neutral lipids supplied in the form of triglyceride. However, further prolonged study will help to determine whether the effects of supplementation of PL only is sufficient for grow out of *P. pelagicus* or whether use of higher purity PL and higher levels of HUFA in the diets, may yield better results.

# CHAPTER 10

## General Discussion and Conclusion

---

### 10.1 General discussion

As reviewed in Chapter 1, the blue swimmer crab, *Portunus pelagicus*, has worldwide markets and the crab can be sold in various forms including hard shell crabs, soft shell crabs and various pasteurized crab meat products. A recent study has also confirmed that *P. pelagicus* is highly nutritional for human consumption, containing low levels of cholesterol and lipid but high levels of 'good' fatty acids (Wu et al., 2010a). The popularity and market demand for *P. pelagicus* is expected to increase and because current markets have nearly exclusive reliance on fisheries and the heavy pressure it exerted on natural stock, aquaculture of *P. pelagicus* needs to be developed to provide an alternative supply to the market and to relieve fisheries pressure. However, a lack of information on the culture requirements of *P. pelagicus* limits industry development and no attempt has been made so far to formulate a species-specific diet for *P. pelagicus*. It is well acknowledged that a good understanding of basic nutritional requirements of *P. pelagicus* is a necessary first step for the development of specifically formulated diets for this promising aquaculture species. This study focused on lipid nutrition of *P. pelagicus* early juveniles and it covered the major lipid constituents. Lipids can be classified as either polar lipids (phospholipid) or neutral lipids (triglyceride, wax) and they are needed in diets for crustaceans because lipids play important roles in many physiological processes and are a source of concentrated energy (Glencross and Smith, 1997; Glencross et al., 1998; Sheen and Wu, 1999).

Despite the existence of literature on the lipid nutrition of penaeid prawn species, past studies have shown that the requirements of lipids and its constituents, by crustaceans, is highly species-specific (Xu et al., 1993; Camara et al., 1997; D'Abramo, 1997; Coutteau et al., 2000; Gong et al., 2001; Gonzalez-Felix et al., 2003b; Vasagam et al., 2005; Holme et al., 2007b; Zhou et al., 2007; Coman et al., 2009). On this basis, this comprehensive study on the lipid nutrition of *P. pelagicus* consisted of a total of 9 experiments (Chapter 2 to Chapter 9) to determine the level of major dietary lipid constituents needed for optimal culture performance of *P. pelagicus*. The major outcomes are summarized in Table 10.1. The key interactive effects of some lipid constituents were also examined and the potential practical uptake of the results of this study was taken into consideration when designing the experiments and particularly in the choice of diet ingredients used in formulating experimental diets. The major outcomes of the study in a broader context, as well as results and common issues that are not fitted to be addressed in separated chapters will be discussed in this chapter.

Firstly, based on the results from all feeding experiments, it was apparent that the intermolt period of *P. pelagicus* from C1 to C2 stage was not significantly affected by the various dietary treatments tested. This may imply that during the first a few days of the experiments, the newly settled first crab stage *P. pelagicus* may still be able to utilize nutrient reserves accumulated from previous larval stages and development is therefore relatively unaffected by diet composition. This is more plausible when considering that the intermolt period of first stage crabs is relatively short, being only between 2 to 4 days. Furthermore, for all feeding experiments, although some diets were deficient in particular important lipid

constituents, such as cholesterol, phospholipid and essential fatty acids, all diets contained protein and carbohydrate that could be used to generate energy for fueling the molting process. The situation is different in starved *P. pelagicus*, where no starved C1 *P. pelagicus* were able to molt to C2 stage despite a high capacity to resistance starvation (i.e. survival as high as 97% after 7 days of starvation) (Chapter 2). Starvation is likely to exert more immediate and significant impacts on the conservation of nutrients because during starvation, some nutrients need to be catabolized first to provide metabolic energy. Considering the likely residual effects of nutrients accumulated during the larval stages and the results of Chapter 2 which demonstrated high resistance of *P. pelagicus* early juveniles to 'nutritional stress', from experiment 3 (Chapter 4) onwards, the culture period used in experiments was extended to C4 stage.

For the results of several diet experiments, the specific growth rate (SGR; % $day^{-1}$) was found to be more sensitive than just measurements of dry weight, carapace width and carapace length of the crab. SGRs are more relevant parameters to aquaculture practice because in aquaculture; it is not only absolute increases in biomass or size that matters, but the time to achieve such increases is also important. As growth in crustaceans only occurs immediately following molt when crustaceans are able to increase their body size while the exoskeleton is still soft (Sheen and Wu, 1999), all experiments were conducted over a duration required for crabs to reach a certain developmental stage rather than culturing for a pre-determined duration. This ensured that data obtained were more reliable and also made comparison among trials possible.

In Chapter 2, the effects of starvation and re-feeding on *P. pelagicus* were assessed with the initial intention of investigating fatty acid utilization patterns in tissues under the experimental conditions. The results however, also showed that newly settled C1 *P. pelagicus* had relatively high resistance to starvation considering their small size and the fact that they had just undergone major morphological changes (metamorphosis) from megalopae to the juvenile crab stage, which involved formation of new tissues and organ development, which requires high metabolic energy (Roustaian et al., 2001). More interestingly, *P. pelagicus* also showed significant compensatory growth effects after being re-fed following 7 days of starvation. This could be significant for aquaculture industries where it is to not feed the animals due to water quality issues, disease outbreaks and to reduce production cost (Wu and Dong, 2002a; Figueiredo et al., 2008). Fatty acid analysis of the experimental crab tissues confirmed the importance of highly unsaturated fatty acids (HUFA) as the majority of HUFA were comparatively retained during starvation while polyunsaturated fatty acids (PUFA) were utilized more heavily.

From Chapter 3 onwards, the subsequent experiments focused on determining the requirements and optimal dietary levels of major lipid constituents for *P. pelagicus* early juveniles. The first experiment, described in Chapter 3, was on the requirements for cholesterol. Compared to other sterols, cholesterol is known to have higher dietary values in promoting growth and survival of crustaceans (Teshima and Kanazawa, 1983), which is linked to its important functions as cell constituent and as a metabolic precursor to sex and molting hormones, including adrenal corticoids, bile acids and vitamin D (Teshima et al., 1989; Olsen, 1998; Sheen, 2000). A dietary cholesterol inclusion level of 10.0 g $kg^{-1}$ was

found to be best for *P. pelagicus* while a higher inclusion level of 15 g $kg^{-1}$ showed signs of sterol metabolism disorder as notably higher incidents of molt death syndrome (MDS) were recorded, suggesting *P. pelagicus* early juveniles may not be able to utilize such a high level of dietary cholesterol.

The dietary requirement and optimal level of dietary phospholipid (PL) was established in Chapter 4. To maximize the applied potential of this study, this experiment opted to use lower purity (PL content > 90%; but with 55% purified phosphatidylcholine (PC) and 20% phosphatidylethanolamine (PE)) but much cheaper PL instead of costly, high purified PC used in some previous studies (Camara et al., 1997; Coutteau et al., 2000; Holme et al., 2007a) to formulated the experimental diets. The results demonstrated that dietary PL not only effectively improved survival, development and growth of *P. pelagicus* but also increased their resistance to osmotic shock (Chapter 4 and 9). Although not proven directly with this study, the substantially increased resistance to low salinity (7‰) when *P. pelagicus* fed diets containing high levels of dietary PL implied an important role of PL in enhancing the osmoregulatory ability of *P. pelagicus.* However, unlike the results obtained for the cholesterol experiment, in which molt death syndrome (MDS) was obvious during molting when crabs were fed diets with 15.0 g $kg^{-1}$ cholesterol, in this experiment, MDS appeared randomly across all diet treatments. However upon closer inspection, a trend of high mortality occurring during the first 48 h immediately following the molt was clear among crabs fed the diet deficient in supplemental PL. Compare to the dietary PL requirements reported for other crustaceans (Chen and Jenn, 1991; Thongrod and Boonyaratpalin, 1998; Holme et al., 2007a), the optimal level needed by *P. pelagicus* (100 g $kg^{-1}$ diet dry weight) is

substantially higher, which may be partially attributed to the used of less purified PL in the present study. However, even when the difference in PL purity is taken into consideration; the dietary requirement for PL by *P. pelagicus* is still relatively high.

Cholesterol absorption and functions have been reported to be closely related to phospholipid (Teshima et al., 1986b) and compensatory effects between dietary cholesterol and phospholipid (PL) have been reported for several crustaceans, including juveniles of the Pacific white shrimp, *Litopenaeus vannamei* (Gong et al., 2000b) and megalopae of the mud crab, *Scylla serrata* (Holme et al., 2007a). The interactive effects of cholesterol and PL are suggested to be linked to the role of PL in lipid transportation and improved digestion in crustaceans (Teshima and Kanazawa, 1980; Leger, 1985). For example, Teshima and Kanazawa (1986) demonstrated that in postlarvae of *Penaeus japonicus,* cholesterol was retained longer in the midgut glands compare to when PL were included in the postlarvae diets. The capacity of PL to increase absorption of cholesterol in hemolymph has been shown to be dependent on the degrees saturation of the fatty acids incorporated in the PL (Jiang et al., 2001). Thus, in investigating the interactive effects of PL and cholesterol in Chapter 5, two experiments were conducted and the diets used were not made iso-lipidic as in Chapter 4, but were made iso-energetic instead by using corn starch.

In the first experiment, it was demonstrated that addition of cholesterol in the diet of *P. pelagicus* could reduce the level of PL needed to achieve similar survival. Interestingly, interaction effects were not detected for both development and growth data although the importance of both PL and cholesterol was clearly shown. Based on the results of the two

experiments reported in Chapter 5, when the lower purity PL was used, the supplemental level of PL at 90 g $kg^{-1}$ and cholesterol at 10.0 g $kg^{-1}$ gave the best results and these levels were used in subsequent experiments.

With the establishment of the optimal levels of dietary cholesterol and PL needed for *P. pelagicus,* the study move on to investigate requirements of neutral lipids and three experiments were executed with results presented in Chapter 6, 7 and 8. These three experiments were designed to investigate both the level of total neutral lipids and the balance of important fatty acids in the diets of *P. pelagicus*. Neutral lipids, usually in the form of oils added to the diets, contain mostly fatty acids in triglyceride bond and are the major provider of essential fatty acids (Tocher, 2003). Using a fish oil and corn oil mixture at a ratio of 2:1, various inclusion levels of triglyceride were tested in the diets of *P. pelagicus*, which contained cholesterol and PL at optimized levels based on the results of previous experiments (Chapter 5). The results demonstrated that *P. pelagicus* early juveniles required fairly low amounts of dietary triglyceride, a range of 20 to 40 g $kg^{-1}$, was sufficient with further increases above 40 g $kg^{-1}$ appearing to have detrimental effects such as reduced developmental and growth rates of the crabs.

Crustaceans are known to be unable to elongate and desaturase polyunsaturated (PUFA) or synthesis highly unsaturated fatty acids (HUFA) from shorter chain fatty acids, thus HUFA need to be included in their diets (Kanazawa et al., 1979a; Kanazawa et al., 1979b). Among many types of oils, fish oil is deemed the ‘superior’ oil because it contains high levels of PUFA and HUFA and this has led to increased dependency of the aquafeed

and aquaculture industries on marine oils (Merican and Shim, 1996). The high consumption and heavy reliance of the industries on the unsustainable nutrient source is alarming and it is predicted that the production of marine fish oil would not be able to cope with rapid growth of aquaculture industry (FAO, 2006; Tacon and Metian, 2008). Therefore, much efforts has been dedicated in replacing fish oil with terrestrial based oil for the manufacture of aquafeeds (Bell et al., 2001; Kamarudin and Roustaian, 2002; Vasagam et al., 2005; Holme et al., 2007b; Peng et al., 2008). Chapter 7 depicted the results in which it was found that up to 50% of the fish oil in the diets of *P. pelagicus* could be replaced by soybean oil. Moreover, the 1:1 ratio of fish oil to soybean oil in the diets often supported improved development and growth rates of juvenile *P. pelagicus* than the diet formulated with fish oil only; probably reflecting the needs of *P. pelagicus* for n-6 fatty acids that are abundant in soybean oil.

The results of fatty acid analysis of fish oil and soybean oil used for diet formulation in Chapter 7 showed that among the HUFA of the fish oil, arachidonic acid (ARA; 20:4n-6) was very low, while in soybean oil, despite being high in n-6 fatty acids, ARA content was negligible. This raised concerns about whether ARA supplied in the diets was sufficient for *P. pelagicus* and the subsequent experiment (Chapter 8) was designed to investigate whether addition of extra ARA on top of the level existing in the mixture of fish oil and soybean oil (1:1 ratio) in diets of *P. pelagicus* would further benefit them. The results clearly showed that further addition of ARA was unnecessary.

The final experiment was conducted to investigate the effects of different dietary polar lipids (phospholipid) to neutral lipids (triglyceride) ratios on survival, development and

growth of *P. pelagicus*. As it was established in Chapter 4 that inclusion of PL improved *P. pelagicus* resistance to osmotic shock, a similar stress test was performed at the end of this study to evaluate whether provision of high level of dietary triglyceride (i.e. neutral lipids) with optimal n-3 to n-6 levels might achieve similar results. Surprisingly, the results demonstrated that, within the time frame of the experiment, provision of triglyceride was not necessary when PL were supplemented at a high level of 150 g $kg^{-1}$. In contrast, the diet containing triglyceride only (without PL) proved detrimental to *P. pelagicus*, which could perhaps be attributed to the crucial function of PL as lipid transporter for fatty acids and cholesterol (Chapter 5) as well as PL's roles in assisting lipid digestion (Coutteau et al., 1997; Teshima, 1997; Glencross et al., 1998). Better resistance to osmotic shock by crabs fed the diets contained high PL compared to those fed the diets containing high proportion of triglyceride again highlighted the importance of PL in promoting osmoregulatory ability of *P. pelagicus.* This results showed the potential of manipulation of diet ingredients to improve the ability of *P. pelagicus*, a species known to be a weak osmoregulator (Romano and Zeng, 2006), to cope with osmotic stress that may be encountered during culture.

**Table 10.1** Schematic overview of the result and implications of this study.

| Experiment | Main result/recommendation |
|---|---|
| **Chapter 2**<br>Survival, development, growth and fatty acid compositions of *Portunus pelagicus*, early juveniles, subject to starvation and re-feeding. | • The point-of-no-return (PNR) for the first stage crabs of *P. pelagicus* is suggested to be between 7-10 days.<br>• Following starvation, re-fed crabs appeared to prioritize development (molting) over growth and quickly recovered their fatty acid profile.<br>• Compared to other groups of fatty acids, highly unsaturated fatty acids (HUFA) appeared to be preferably retained during starvation, indicating important biological roles. |
| **Chapter 3**<br>Survival, development and growth of *Portunus pelagicus* early juveniles fed diets containing various levels of cholesterol. | • *P. pelagicus* fed the diet supplemented with 10.0 g $kg^{-1}$ cholesterol obtained the highest survival, growth and shortest development time.<br>• Although molt death syndrome (MDS) occurred in all diet treatments, the incidents was the highest among crabs fed the diet supplemented with highest level of cholesterol of 15 g $kg^{-1}$. |
| **Chapter 4**<br>The influence of dietary phospholipid on survival, development, growth and stress resistance of *Portunus pelagicus* early juveniles. | • With the purity of PL used in the present study, addition of PL at 30 g $kg^{-1}$ significantly improved survival; however, the shortest development time and best growth were achieved when the dietary PL reached 100 g $kg^{-1}$ and beyond.<br>• High levels of dietary PL effectively improved resistance to osmotic shock. |
| **Chapter 5**<br>Evaluation of dietary phospholipid and cholesterol requirements for *Portunus pelagicus* early juveniles. | • A compensative relationship between dietary cholesterol and PL was suggested based on survival data of *P. pelagicus*<br>• Dietary PL and cholesterol levels significantly affected development time and SGR for carapace size.<br>• Dietary cholesterol supplementation at 10 g $kg^{-1}$ and PL at 90 g $kg^{-1}$ is recommended for *P. pelagicus*. |

| | |
|---|---|
| **Chapter 6**<br>Assessment of survival, development and growth of *Portunus pelagicus* early juveniles, fed semi-purified diets supplemented with different levels of triglyceride. | • Overall, the highest survival, growth and shortest development time was achieved when *P. pelagicus* were fed the diet with triglyceride supplemented at 40 g $kg^{-1}$.<br>• Addition of triglyceride above 40 g $kg^{-1}$ in the diets appeared to reduce crab growth. |
| **Chapter 7**<br>The effects of dietary soybean oil to fish oil ratios on survival, development and growth of *Portunus pelagicus* early juveniles. | • The dietary soybean oil to fish oil ratio of 1:1 was found not to affect culture performance of *P. pelagicus*, suggesting in the formulation of diets, fish oil could be replaced up to 50% by soybean oil for *P. pelagicus*.<br>• Compared to the diet formulated with fish oil only, higher growth rate was actually achieved when *P. pelagicus* were fed the diet with fish and soybean oil mixture (1:1), it implied *P. pelagicus* preferred a balance of dietary n-3 and n-6 fatty acids. |
| **Chapter 8**<br>The effects of dietary arachidonic acid (20: 4n-6) on survival, development and growth of *Portunus pelagicus* early juveniles. | • A low level of arachidonic acid (ARA) at 0.17 g 100 $g^{-1}$ contained in the fish oil was found sufficient for *P. pelagicus.*<br>• Additional ARA supplementation on top that already contained in the fish oil in the diets (0.17 g 100 $g^{-1}$) was found to negatively affected growth rate of *P. pelagicus.* |
| **Chapter 9**<br>The effects of different dietary phospholipid and triglyceride ratios on survival, development, growth and stress resistance of *Portunus pelagicus* early juveniles | • Growth and development time of *P. pelagicus* were not negatively affected when the diets contained higher levels of PL than triglyceride.<br>• Dietary PL appeared to have higher nutritional value to *P. pelagicus* than triglyceride. |

## 10.2 Future directions

The farming of *P. pelagicus* is still in its infancy with very limited commercial production. Among the bottlenecks that limit its expansion is a lack of information on *P. pelagicus* nutritional requirements. While this study is a first step in this direction, focusing on lipid nutrition of *P. pelagicus* juveniles and it potential application in formulated feed development for this species, further research is needed to investigate other aspects of nutrition and feed development for this species, starting with the major nutrients such as protein/amino acids, vitamins and minerals as well as physical properties of formulated diet particles, including texture, size, shape and water stability.

Feeding management is essential in aquaculture as feeds and feeding represent the largest operating costs in most farming operations (FAO, 2006). A potential way to reduce the feeding cost is to take advantage of the process known as compensatory growth, defined as accelerated growth rate after re-feeding following food deprivation, as has been shown in some crustaceans (Wu et al., 2001; Singh and Balange, 2007; Stumpf et al., 2010). This study already demonstrated the ability of *P. pelagicus* to achieve compensatory growth after 7 days of food deprivation, however, the compensatory growth was achieve only after one cycle of starvation. Repetitive starvation and feeding study would be beneficial in determining the maximum potential of *P. pelagicus* to accelerate growth rate or the limitation that they may have. In this study, analysis of the fatty acid compositions of *P. pelagicus* during starvation gave clear indication of preferential fatty acids to be conserved or utilized, however, due to small size of the crabs, the analysis was only able to be conducted on whole crabs. Fatty acid

analysis of the muscle and hepatopancreas separately would give more information on the reserved and utilized fatty acids.

Phospholipid on its own is such a large component of the lipids and more detailed study of its dietary significance will add more information on the requirements and utilization of PL for *P. pelagicus*. As the *P. pelagicus* industry is far from established and not much investment is directed towards industry, experiments on PL in this study emphasized on practicality and affordability in diet development for *P. pelagicus*. Thus the next step in PL nutritional study should be conducted using purified levels of specific PL, such as PC, PE and phosphatidylinositol (PI). Study has shown that the requirements of PL for post larval *Marsupenaeus japonicus* differed when it was incorporated as purified PC (1.5%) or soybean lecithin (6.5%) (Camara et al., 1997). Two of the chapters (Chapter 4 and 9) in this thesis also strongly demonstrated the beneficial effects of PL in surviving osmotic shock over 7 and 9 h. To understand the underlying mechanism of PL in osmoregulatory ability of *P. pelagicus*, two-way study involving different levels of salinity and different levels of dietary PL should be conducted over long-term culture periods.

Changes in dietary lipid level have been suggested to influence the digestibility of other nutrients (Glencross et al., 2002b). For example, increasing lipid levels in the diet of the abalone, *Haliotis laevigata* has been shown to adversely effect their digestibility of dietary amino acids and total nitrogen. As Chapter 6 demonstrated that the inclusion of triglyceride above an optimal level appeared to reduce growth rate of *P. pelagicus*, further

research should be expended to clarify the link between the amount of lipid provided in the diet of *P. pelagicus* and their digestibility to other nutrients.

In summary, once an ‘optimized’ diet has been developed for *P. pelagicus*, it will provide a major stimulus for expansion of aquaculture of this species. This thesis has addressed one of the important nutritional requirements for *P. pelagicus*, lipids, and extrapolation of data from this study could be a guideline for more robust scientific development in *P. pelagicus* nutritional study. Through continuous research like this, the ultimate goal of a sustainable and profitable *P. pelagicus* aquaculture industry can be manifested.

**Bibliography:**


Abruhonsa, F.A., Kittaka, J., 1997. Effect of starvation on the first larvae of *Homarus americanus* (Decapoda, Nephropidae) and phyllasomas of *Jasus verreauxi* and *Jasus edwardsii* (Decapoda, Panuliridae) Bulletin of Marine Science 61, 73-80.

Ackman, R.G., 1967. Characteristics of the fatty acid composition and biochemistry of some fresh-water fish oils and lipids in comparison with marine oils and lipids. Comparative Biochemistry and Physiology 22, 907-922.

Ackman, R.G., 1998. Comparisons of lipids in marine and freshwater organism. In: Lipids in freshwater ecosystem. Arts, M.T., Wainman, B.C. (Eds.). Springer-Verlag, New York, pp. 263-288.

Akiyama, D.M., Dominy, W.G., Lawrence, A.L., 1992. Penaied shrimp nutrition. In: Marine shrimp culture: Principles and practices. Fast, A.W., Lester, L.J. (Eds.). Elsevier, Amsterdam, The Netherlands, pp. 535-568.

Ali, M., Nicieza, S., Wootton, R.J., 2003. Compensatory growth in fishes: a response to growth depression. Fish and Fisheries 4, 147-190.

Anderson, T., Silva, S.D., 2003. Nutrition. In: Aquaculture; Farming Aquatic Animals and Plants. Lucas, J., Southgate, P. (Eds.). Blackwell Publishing, Victoria, Australia, pp. 146-171.

Ando, Y., Samoto, H., Murayama, Y., 2004. Positional distribution of DHA and EPA in triacyl-sn-glycerols (TAG) of Artemia franciscana nauplii enriched with fish oils ethyl esters and TAG. Aquaculture 233, 321-335.

Anger, K., 2001. The molting cycle. In: The biology of decapod crustaceans larvae. Vonk, R. (Ed.). A.A. Balkema, Helgoland, pp. 81-107.

Baker, J.L., Kumar, M., 1994. Review of the blue crab (*Portunus pelagicus*) fishery in South Australia. SARDI Research Report. Volume. Publisher, Urrbrae. pp 59.

Barclay, M.C., Dall, W., Smith, D.M., 1983. Changes in lipid and protein during starvation and the moulting cycle in the tiger prawn, *Penaeus esculentus* Haswell. Journal of Experimental Marine Biology and Ecology 68, 229-244.

Barnes, R.d., 1987. Invertebrate zoology. Harcourt Brace Jovanovich College, Fort Worth, TX, pp 893.

Baum, N.A., Conklin, D.E., Chang, E.S., 1990. Effect of dietary lecithin in combination with casein or crab protein on cholesterol uptake and transport in the lobster *Homarus americanus*. Journal of the World Aquaculture Society 21, 277-287.

Bell, J.G., Sargent, J.R., 2003. Arachidonic acid in aquaculture feeds: Current status and future opportunities. Aquaculture 218, 491-499.

Bell, J.G., Tocher, D.R., Farndale, B.M., Cox, D.I., McKinney, R.W., Sargent, J.R., 1997. The effect of dietary lipids on polyunsaturated fatty acid metabolism in Atlantic Salmon (*Salmo salar*) undergoing parr-smolt transformation. Lipids 32, 515-525.

Bell, J.G., McEvoy, J., Tocher, D.R., McGhee, F., Campbell, P.J., Sargent, J.R., 2001. Replacement of Fish Oil with Rapeseed Oil in Diets of Atlantic Salmon (*Salmo salar*) Affects Tissue Lipid Compositions and Hepatocyte Fatty Acid Metabolism. Journal of Nutrition. 131, 1535-1543.

Bell, M.V., Henderson, R.J., Sargent, J.R., 1986. The role of polyunsaturated fatty acids in fish. Comparative Biochemistry and Physiology 4, 711-719.

Boonyaratpalin, M., 1998. Nutrition of *Penaeus merguiensis* and *Penaeus indicus*. Reviews in Fisheries Science 6, 69 - 78.

Bosworth, B.G., Wolters, W.R., 1995. Compensatory growth in juvenile red swamp crawfish, *Procrambarus clarkii* In: Eighth International Symposium on Astacology. Romaire, R.P. (Ed.) Lousiana State University Baton Rouge, Lousiana, pp. 648-656.

Bowser, P.R., Rosemark, R., 1981. Mortalities of cultured lobsters, *Homarus sp*, associated with a molt death syndrome. Aquaculture 23, 11-18.

Briggs, M.R.P., Jauncey, K., Brown, J.H., 1988. The cholesterol and lecithin requirements of juvenile prawn (*Macrobrachium rosenbergii*) fed semipurified diets. Aquaculture 70, 121-129.

Briggs, M.R.P., Brown, J.H., Fox, C.J., 1994. The effect of dietary lipid and lecithin levels on the growth, survival, feeding efficiency, production and carcass composition of post-larval *Penaeus monodon*. Aquaculture and Fisheries Management 25, 279-294.

Brown, M.S., Goldstein, J.L., 1978. General Scheme for regulation of cholesterol metabolism in mammalian cells. In: Disturbances in Lipid and Lipoprotein Matabolism. Dietschy, J.M., Jr., A.M.G., Ontko, J.A. (Eds.). Waverly Press Inc, Bethesda, Maryland, pp. 173-179.

Camara, M.R., Coutteau, P., Sorgeloss, P., 1997. Dietary phosphatidylcholine requirements in larval and postlarval *Penaeus japonicus* Bate. Aquaculture Nutrition 3, 39-48.

Campbell, N.A., Reece, J.B., Mitchell, L.G., 1999. Biology 5th Edition. Benjamin/Cummings, Menlo Park, California, pp 1171.

Castell, J.D., Covey, J.F., 1976. Dietary Lipid Requirements of Adult Lobsters, *Homarus americanus* (M.E.). Journal of Nutrition. 106, 1159-1165.

Castine, S., Southgate, P.C., Zeng, C., 2008. Evaluation of four dietary protein sources for use in microbound diets fed to megalopae of the blue swimmer crab, *Portunus pelagicus*. Aquaculture 281, 95-99.

Catacutan, M.R., 1991. Growth and fatty acid composition of *Penaeus monodon* juveniles fed various lipids. The Israeli Journal of Aquaculture - Bamidgeh 43, 47-56.

Chaiyawat, M., Eunggrasamee, I., Raksakulthai, N., 2009. Meat quality of blue swimming crab (*Portunus pelagicus*, Linnaeus 1758) fattened with different diets. Kasetsart Journal of Natural Science 43, 132-142.

Chande, A.L., Mgaya, Y.D., 2004. Food habits of the blue swimming crab, *Portunus pelagicus* along the coast of Dar es Salaam, Tanzania. Western Indian Ocean Journal of Marine Science 3, 37-42.

Chang, E.S., 1985. Hormonal Control of Molting in Decapod Crustacea. American Zoologist 25, 179-185.

Chen, H., Hwang, G., 1992. Estimation of dietary riboflavin required to maximise tissue riboflavin concentration in juvenile shrimp (*Penaeus monodon*). Journal of Nutrition 122, 2474-2478.

Chen, H.Y., 1993. Requirements of marine shrimp *Penaeus mondon* juveniles for phosphatidylcholine and cholesterol. Aquaculture 109, 165-176.

Chen, H.Y., Jenn, J.S., 1991. Combined effects of dietary phosphatidylcholine and cholesterol on the growth, survival and body composition of marine shrimp *Penaeus penicillatus*. Aquaculture 96, 167-178.

Chen, J.-C., Liu, P.-C., Lin, Y.-T., 1989. Culture of *Penaeus monodon* in an intensified system in Taiwan. Aquaculture 77, 319-328.

Cheng, Z.J., Hardy, R.W., 2004. Protein and lipid sources affect choelsterol concentration of juvenile Pacific white shrimp, *Litopenaeus vannamei* (Boone). Journal of Animal Science 82, 1136-1145.

Chiu, Y.L., Davie P., Chan W., Ng P., 2005. What is the real *Portunus pelagicus*? *Unpublished report.* Department of Biological Science, National University of Singapore, Lower kent Ridge, Singapore.

Clifford, H.C., Brick, R.W., 1983. Nutritional physiology of the freshwater shrimp *Macrobrachium rosenbergii* (de man)-I. Substrate metabolism in fasting juvenile shrimp. Comparative Biochemistry and Physiology Part A: Physiology 74, 561-568.

Coman, G., Arnold, S., Barclay, M., Smith, D., 2009. Effect of arachidonic acid supplementation on reproductive performance of tank-domesticated *Penaeus monodon*. Aquaculture Nutrition 17, 141-151.

Comoglio, L.I., Gaxiola, G., Roque, A., Cuzon, G., Amin, O., 2004. The effect of starvation on refeeding, digestive enzyme activity, oxygen consumption and ammonia excretion in juvenile white shrimp, *Litopenaeus vannamei*. Journal of Shellfish Research 23, 243-249.

Conklin, D.E., D'Abramo, L.R., Bordner, C.E., Baum, N.A., 1980. A successful purified diet for the culture of juvenile lobsters: The effect of lecithin. Aquaculture 21, 243-249.

Coutteau, P., Camara, M.R., Sorgeloos, P., 1996. The effect of different levels and sources of dietary phosphatidylcholine on the growth, survival, stress resistance, and fatty acid composition of postlarval *Penaeus vannamei*. Aquaculture 147, 261-273.

Coutteau, P., Kontara, E.K.M., Sorgeloos, P., 2000. Comparison of phosphatidylcholine purified from soybean and marine fish roe in the diet of postlarval *Penaeus vannamei* Boone. Aquaculture 181, 331-345.

Coutteau, P., Geurden, I., Camara, M.R., Bergot, P., P.Sorgeloos, 1997. Review on the dietary effects of phospholipids in fish and crustacean larviculture. Aquaculture 155, 149-164.

Cuzon, G., 1998. Nutritional Review of *Penaeus stylirostris*. Reviews in Fisheries Science 6, 129-141.

D'Abramo, L.R., 1997. Triacylglcerols and Fatty Acids In: Crustacean Nutrition. Advances in World Aquaculture. Abramo, L.R.D., Conklin, D.E., Arigama, D.M. (Eds.). World Aquaculture Society, Baton Rouse, Lousiana, pp. 71-86.

D'Abramo, L.R., Sheen, S.-S., 1993. Polyunsaturated fatty acid nutrition in juvenile freshwater prawn *Macrobrachium rosenbergii*. Aquaculture 115, 63-86.

D'Abramo, L.R., Castell, J.D., 1997. Research methodology. In: Crustacean nutrition. Advances is World Aquaculture. D'Abramo, L.R., Conklin, D.E., Akiyama, D.M. (Eds.). World Aquaculture Society, Baton Rouge, Louisiana, pp. 3-25.

D'Abramo, L.R., Bordner, C.E., Conklin, D.E., 1982. Relationship between dietary phosphatidylcholine and serum cholesterol in the lobster *Homarus sp*. Marine Biology 67, 231-235.

D'Abramo, L.R., Bordner, C.E., Conklin, D.E., Baum, N.A., 1981. Essentiality of dietary phosphatidylcholine for the survival of juvenile lobster. Journal of Nutrition 111, 425-431.

D'Abramo, L.R., Bordner, C.E., Conklin, D.E., Baum, N.A., 1984. Sterol requirement of juvenile lobsters, *Homarus sp*. Aquaculture 42, 13-25.

D'Abramo, L.R., Wright, J.S., Wright, K.H., Bordner, C.E., Conklin, D.E., 1985. Sterol requirement of cultured juvenile crayfish, *Pacifastacus leniusculus*. Aquaculture 49, 245-255.

D.o.F, Western Australia, 2005. Western Australia Fisheries Legislation. Perth, Department of Fisheries Western Australia, http://www.fish.wa.gov.au/docs/pub/LegislationHow/gateway.php?0006. Accessed on 7th September 2010

Dall, W., 1974. Indices of nutritional state in the western rock lobster, *Panulirus longipes* (Milne Edwards). I. Blood and tissue constituents and water content. Journal of Experimental Marine Biology and Ecology 16, 167-180.

Dall, W., Smith, D.M., 1986. Oxygen consumption and ammonia-N excretion in fed and starved tiger prawns, *Penaeus esculentus* Haswell. Aquaculture 55, 23-33.

Dall, W., Chandumpai, A., Smith, D.M., 1992. Fatty acid composition of organs and tissues of the tiger prawn *Penaeus esculentus* during the moulting cycle and during starvation. Marine Biology 113, 45-55.

Davis, D.A., Robinson, E.H., 1986. Estimation of dietary lipid requirement of white crayfish *Procambarus acutus*. Journal of the World Aquaculture Society 17, 37-43.

Deshimaru, O., Kuroki, K., Yone, Y., 1979. The composition and level of dietary lipid appropriate for the growth of prawn. Bulletin of Japanese Society of Scientific Fisheries 45, 591-594.

DPI&F, 2005. Crab farming: a new opportunity for Australia aquaculture. In: Shelley, C. (Ed.) Department of Primary Industries and Fisheries, Queensland, Brisbane, pp. 16.

DPI&F, 2008. Annual status report 2008: Blue swimmer crab fishery. Fargher, B. (Ed.). Department of |Primary Industries and Fisheries, Brisbane. pp 15.

Duerr, E.O., Walsh, W.A., 1996. Evaluation of cholesterol additions to soyabean meal-based diet for juvenile pacific white shrimp, *Penaeus vannamei* (Boone), in an outdoor growth trial. Aquaculture Nutrition 2, 111-116.

Edgar, G.J., 1990. Predator-prey interactions in seagrass beds. II. Distribution and diet of the blue manna crab *Portunus Pelagicus* linnaeus at Cliff Head, Western Australia. Journal of Experimental Marine Biology and Ecology 139, 23-32.

Edwards, P., Tuan, L.A., Allan, G.L., 2004. A survey of marine trash fish and fish meal as aquaculture feed ingredients in Vietnam. Edwards, P., Tuan, L.A., Allan, G.L. (Eds.). ACIAR Working Paper No. 57. ACIAR, Canberra. pp 56.

FAO, 2006. State of world aquaculture 2006 in FAO Fisheries Technical Paper. Rome, FAO Fisheries and Aquaculture Department, http://www.fao.org/docrep/009/a0874e/a0874e00.htm. Accessed on 5th June 2010

FAO, 2008. *Eriocheir sinensis*: Cultured Aquatic Species Information Programme. Rome, FAO Fisheries and Aquaculture Department, http://www.fao.org/fishery/culturedspecies/Eriocheir_sinensis/en. Accessed on 5th of July 2010

FAO, 2010. FAO Fisheries Department, Fisheries Information, Data and Statistic Unit. Fishstat Plus: Universal software for fishery statistical time series. *Portunus pelagicus* 1950-2009. http://www.fao.org/figis/servlet/SQServlet?ds=Capture&k1=SPECIES&k1v=1&k1s=2629&outtype=html. Acessed on 21st April 2011.

Fielder, D.R., Eales, A.J., 1972. Observation on courtship, mating and sexual maturity in *P. pelagicus* (L., 1766) (Crustacea, Portunidae). Journal of Natural History 6, 273-277.

Figueiredo, J., Penha-Lopes, G., Narciso, L., Lin, J., 2008. Effect of starvation during late megalopa stage of *Mithraculus forceps* (Brachyura: Majidae) on larval duration, synchronism of metamorphosis, survival to juvenile, and newly metamorphosed juvenile size. Aquaculture 274, 175-180.

Folch, J., Lee, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animals tissue. Journal of Biological Chemistry 226, 477-509.

Fountoulaki, E., Alexis, M.N., Nengas, I., Venou, B., 2003. Effects of dietary arachidonic acid (20:4n-6) on growth, body composition, and tissue fatty acid profile of gilthead seabream fingerlings (*Sparus aurata* L.). Aquaculture 225, 309-323.

Geurden, I., Marion, D., Charlon, N., Coutteau, P., Bergot, P., 1998. Comparison of different soybean phosphalipidic fractions as dietary supplements for common carp, *Cyprinus carpio*, larvae. Aquaculture 161, 225-235.

Gilman, C.I., Leusch, F.D.L., Carl Breckenridge, W., MacLatchy, D.L., 2003. Effects of a phytosterol mixture on male fish plasma lipoprotein fractions and testis P450scc activity. General and Comparative Endocrinology 130, 172-184.

Glencross, B.D., Smith, D.M., 1997. Comparison of triacylglycerols, esterified and free fatty acids as neutral lipid sources in the diet of the prawn *Penaeus monodon*. Aquaculture 159, 67-85.

Glencross, B.D., Smith, D.M., 1999. The dietary linoleic and linolenic fatty acids requirements of the prawn *Penaeus monodon* Aquaculture Nutrition 5, 53-63.

Glencross, B.D., Smith, D.M., 2001a. Optimising the dietary levels of eicosapentaenoic and docosahexaenoic essential fatty acids for the prawn, *Penaeus monodon* Aquaculture Nutrition 7, 101-112.

Glencross, B.D., Smith, D.M., 2001b. A study of the arachidonic acid requirements of the giant tiger prawn, *Penaeus monodon*. Aquaculture Nutrition 7, 59-69.

Glencross, B.D., Smith, D.M., Williams, K.C., 1998. Effect of dietary phospholipids on digestion of neutral lipid by the prawn *Penaeus monodon*. Journal of the World Aquaculture Society 29, 365-369.

Glencross, B.D., Smith, D.M., Thomas, M.R., Williams, K.C., 2002a. Optimising the essential fatty acids in the diet for weight gain of the prawn, *Penaeus monodon*. Aquaculture 204, 85-99.

Glencross, B.D., Smith, D.M., Thomas, M.R., Williams, K.C., 2002b. The effects of dietary lipid amount and fatty acid composition on the digestibility of lipids by the prawn, *Penaeus monodon*. Aquaculture 205, 157-169.

Glencross, B.D., Smith, D.M., R., T.M., C., W.K., 2002c. Optimising the essential fatty acids in the diet for weight gain of the prawn, *Penaeus monodon* Aquaculture 204, 85-99.

Glencross, B.D., Smith, D.M., Tonks, M.L., Tabrett, S.J., Williams, K.C., 2002d. The effect of dietary n-3 and n-6 fatty acid balance on the growth of the prawn *Penaeus monodon*. Aquaculture Nutrition 8, 43-51.

Gong, H., Lawrence, A.L., Jiang, D.-H., Gatlin, D.M., 2000a. Lipid nutrition of juvenile Litopenaeus vannamei: II. Active components of soybean lecithin. Aquaculture 190, 325-342.

Gong, H., Lawrence, A.L., Jiang, D.-H., Castille, F.L., Gatlin, D.M., 2000b. Lipid nutrition of juvenile *Litopenaeus vannamei* I. Dietary cholesterol and de-oiled soy lecithin requirements and their interaction. Aquaculture 190, 305-324.

Gong, H., Lawrence, A.L., III, D.M.G., Jiang, D.-H., Zhang, F., 2001. Comparison of different types and levels of commercial soybean lecithin supplemented in semipurified diets for juvenile *Litopenaeus vannamei* Boone. Aquaculture Nutrition 7, 11-17.

Gong, H., Jiang, D.-H., Lightner, D.V., Collins, C., Brock, D., 2004. A dietary modification approach to improve the osmoregulatory capacity of *Litopenaeus vannamei* cultured in the Arizona desert. Aquaculture Nutrition 10, 227-236.

Gong, H., Lawrence, A.L., III, D.M.G., Jiang, D.-H., Zhang, F., 2007. Comparison of different types and levels of comercial soybean lecithin supplemented in semipurified diets for juvenile *Litopenaeus vannamei* Boone. Aquaculture Nutrition 7, 11-17.

Gonzalez-Baro, M.d.R., Pollero, R.J., 1998. Fatty Acid Metabolism of *Macrobrachium borellii*: Dietary Origin of Arachidonic and Eicosapentaenoic Acids. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 119, 747-752.

Gonzalez-Felix, M.L., M.Gatlin, D., Lawrence, A.L., Perez-Velazquez, M., 2002a. Effect of dietary phospholipid on essential fatty acid requirements and tissue lipid composition of *Litopenaeus vannamei* juveniles. Aquaculture 207, 151-167.

Gonzalez-Felix, M.L., Gatlin, D.M., Lawrence, A.L., Perez-Velazquez, M., 2002b. Effect of various dietary lipid levels on quantitative essential fatty acid requirements of juvenile pacific white shrimp *Litopenaeus vannamei*. Journal of World Aquacuture Society 33, 330-340.

Gonzalez-Felix, M.L., Lawrence, A.L., Gatlin, D.M., Perez-Velazquez, M., 2002c. Growth, survival and fatty acid composition of juvenile *Litopenaeus vannamei* fed different oils in the presence and absence of phospholipids. Aquaculture 205, 325-343.

Gonzalez-Felix, M.L., Lawrence, A.L., Gatlin, D.M., M.Perez-Velazquez, 2003a. Nutritional evaluation of fatty acid for the open thelycum shrimp, *Litopenaeus vannamei*: I. Effect of dietary linoleic and linolenic acid at different concentrations and ratios on juvenile shrimp growth, survival and fatty acid composition. Aquaculture Nutrition 9, 105-113.

Gonzalez-Felix, M.L., Gatlin, D.M., Lawrence, A.L., M.Perez-Velazquez, 2003b. Nutritional evaluation of fatty acids for the open thelycum shrimp, *Litopenaeus vannamei*: II. Effect of dietary n-3 and n-6 polyunsaturated and highly unsaturated fatty acids on juvenile shrimp growth, survival and fatty acid composition. Aquaculture Nutrition 9, 115-122.

Grieneisen, M.L., Warren, J.T., Gilbert, L.I., 1993. Early steps in ecdysteroid biosynthesis: Evidence for the involvement of cytochrome P-450 enzymes. Insect Biochemistry and Molecular Biology 23, 13-23.

Guary, J.-C., Kayama, M., Murakami, Y., 1975. Variations saisonnières de la composition en acides gras chez *Penaeus japonicus* (Crustacea: Decapoda). Marine Biology 29, 335-341.

Gurr, M.I., Harwood, J.L., 1991. Lipid Biochemistry: An introduction. Chapman and Hall, London, pp. 406.

Gurr, M.I., Harwood, J.L., Frayn, K.N., 2002a. Lipids in cellular structure. In: Lipid Biochemistry: An introduction 5th Edition. Blackwell Science, London, pp. 320.

Gurr, M.I., Harwood, J.L., Frayn, K.N., 2002b. Fatty acids structure and metabolism. In: Lipid Biochemistry: An introduction 5th Edition. Blackwell Science, London, pp. 320.

Hac-Wydro, K., Wydro, P., Jagoda, A., Kapusta, J., 2007. The study on the interaction between phytosterols and phospholipids in model membranes. Chemistry and Physics of Lipids 150, 22-34.

Halver, J.E., 1989. Fish Nutrition. Academic Press, New York, pp. 239-345.

Hamasaki, K., Takeuchi, T., Sekiya, S., 1998. Dietary value for larval swimming crab *Portunus trituberculatus* of marine rotifer *Brachionus rotundiformis* culture with several feeds. Nippon Suisan Gakkaishi 64, 841-846.

Hamilton, J.A., 1998. Fatty acid transport: difficult or easy? Journal of Lipid Research 39, 467-481.

Heide, A., Foss, A., Stefansson, S.O., Mayer, I., Norberg, B., Roth, B., Jenssen, M.D., Nortvedt, R., Imsland, A.K., 2006. Compensatory growth and fillet crude composition in juvenile Atlantic halibut: Effects of short term starvation periods and subsequent feeding. Aquaculture 261, 109-117.

Henderson, R.J., Bell, M.V., Sargent, J.R., 1985. The conversion of polyunsaturated fatty acuds to prostaglandins by tissue homogenates of the turbot *Scophthalmus maximus* L. . Journal of Experimental Marine Biology and Ecology 85, 93-99.

Hernández-Vergara, M.P., Rouse, D.B., Olvera-Novoa, M.A., Davis, D.A., 2003. Effects of dietary lipid level and source on growth and proximate composition of juvenile redclaw (*Cherax quadricarinatus*) reared under semi-intensive culture conditions. Aquaculture 223, 107-115.

Hernandez, P.V., Olvera-Novoa, M.A., Rouse, D.B., 2004. Effect of dietary cholesterol on growth and survival of juvenile redclaw crayfish *Cherax quadricarinatus* under laboratory conditions. Aquaculture 236, 405-411.

Hien, T.T.T., Hai, T.N., Phuong, N.T., Ogata, H.Y., Wilder, M.N., 2005. The effects of dietary lipid sources and lecithin on the production of giant freshwater prawn *Macrobrachium rosenbergii* larvae in the Mekong Delta region of Vietnam. Fisheries Science 71, 279-286.

Hilton, J.W., Harrison, K.E., Slinger, S.J., 1984. A semi-purified test diet for Macrobrachium rosenbergii and the lack of need for supplemental lecithin. Aquaculture 37, 209-215.

Holme, M.-H., Zeng, C., Southgate, P.C., 2006. The effects of supplemental dietary cholesterol on growth, development and survival of mud crab, *Scylla serrata*, megalopa fed semi-purified diets. Aquaculture 261, 1328-1334.

Holme, M.-H., Southgate, P.C., Zeng, C., 2007a. Assesment of dietary lecithin and cholesterol requirements of mud crab, *Scylla serrata*, megalopa using semi-purified microbound diets. Aquaculture Nutrition 13, 413-423.

Holme, M.-H., Southgate, P.C., Zeng, C., 2007b. Survival, development and growth response of mud crab, *Scylla serrata*, megalopae fed semi-purified diets containing various fish oil:corn oil ratios. Aquaculture 269, 427-435.

Holme, M.-H., Brock, I., Southgate, P.C., Zeng, C., 2009. Effects of starvation and feeding on lipid class and fatty acid profile of late stage mud crab, *Scylla serrata*, larvae. Journal of the World Aquaculture Society 40, 493-504.

Ibeas, C., Izquierdo, M.S., Lorenzoa, A., 1994. Effect of different levels of n-3 highly unsaturated fatty acids on growth and fatty acid composition of juvenile gilthead seabream (*Sparus aurata*). Aquaculture 127, 177-188.

Ibeas, C., Cejas, J.R., Gomez, T., Hernandez, A.L., 1997. Influence of eicosapentaenoic to docosahexaenoic acid ratio (EPA/DHA) of dietary lipids on growth and fatty acid composition of gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 150, 91-102.

Jiang, Y., Noh, S.K., Koo, S.I., 2001. Egg phosphatidylcholine decreases the lymphatic absorption of cholesterol in rats. Journal of Nutrition 131, 2358-2363.

Johnston, D., Harris, D., Caputi, N., Thomson, A., 2011. Decline of a blue swimmer crab (*Portunus pelagicus*) fishery in Western Australia - History, contributing factors and future management strategy. Fisheries Research 109, 119-130.

Jose, J., Menon, N.G., 2005. Growth of the blue swimmer crab, *Portunus pelagicus* (Linnaeus, 1758) (Decapoda, Brachyura) in captivity. Crustaceana 78, 1-18.

Kamarudin, M.S., Roustaian, P., 2002. Growth and fatty acid composition of freshwater prawn, *Macrobrachium rosenbergii* larvae fed diets containing various ratios of cod liver oil-corn oil mixture. Journal of Applied Ichtyology 18, 148-153.

Kanazawa, A., 1980. Nutritional requirements and artificial diets of kuruma shrimp, *Penaeus japonicus*. In: Ninth Joint Meeting of the UJNR Aquaculture Panel, Crustacean Culture. Sindermann, C.J. (Ed.) Kyoto, Japan, pp. 3-13.

Kanazawa, A., Teshima, S., Ono, K., 1979a. Relationship between essential fatty acids requirements of aquatic animals and the capacity for bioconversion of linolenic acid to highly unsaturated fatty acids. Comparative Biochemistry and Physiology 63B, 295-298.

Kanazawa, A., Teshima, S., Tokiwa, S., 1979b. Biosynthesis of fatty acids from palmitic acid in the prawn, *Penaeus japonicus*. Memoirs of the Faculty of Fisheries of Kagoshima University 28, 17-20.

Kanazawa, A., Teshima, S., Sakamoto, M., 1985. Effects of dietary lipids, fatty acids, and phospholipids on growth and survival of prawn (*Penaeus japonicus*) larvae. Aquaculture 50, 39-49.

Kanazawa, A., Tanaka, N., Teshima, S.I., Kashiwada, K.I., 1971. Nutritional requirements of prawn: II. Requirements for sterols. Bulletin of Japanese Society of Scientific Fisheries 37, 211-215.

Kangas, M.I., 2000. Synopsis of the biology and exploitation of the blue swimmer crab, *Portunus pelagicus* Linnaeus, in Western Australia. Fisheries Research Report 121. Fisheries Western Australia, pp. 1-20.

Kean, J.C., Castell, J.D., Boghen, A.G., D'Abramo, L.R., Conklin, D.E., 1985. A re-evaluation of the lecitihin and cholesterol requirements of juvenile lobster (*Homarus americanus*) using crab protein-based diets. Aquaculture 47, 143-149.

Klinbunga, S., Khetpu, K., Khamnamtong, B., Menasveta, P., 2007. Genetic Heterogeneity of the blue swimming crab (*Portunus pelagicus*) in Thailand determined by AFLP analysis. Biochemical Genetics 45, 725-736.

Kontara, E.K.M., Coutteau, P., P.Sorgeloos, 1997. Effect of dietary phospholipid on requirement and incorporation of n-3 highly unsaturated fatty acids in postlarval *Penaeus japonicus* Bate. Aquaculture 158, 305-320.

Koolman, J., 1982. Ecdysone metabolism. Insect Biochemistry 12, 225-250.

Koskela, R.W., Greenwoog, J.G., Rothlisberg, P.C., 1992. The influence of prostaglandin $E_2$ and the steroid hormones, 17 alpha-hydroxyprogesterone and 17 beta-estradiol on moulting and ovarian development in the tiger prawn, *Penaeus esculentus*, Hastwell, 1879 (Crustacea: Decapoda). Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 101A, 295-299.

Koven, W., Barr, Y., Lutzky, S., Ben-Atia, I., Weiss, R., Harel, M., Behrens, P., Tandler, A., 2001. The effect of dietary arachidonic acid (20:4n-6) on growth, survival and resistance to handling stress in gilthead seabream (Sparus aurata) larvae. Aquaculture 193, 107-122.

Koven, W.M., Kissil, G.W., Tandler, A., 1989. Lipid and n-3 requirement of *Sparus aurata* larvae during starvation and feeding. Aquaculture 79, 185-191.

Koven, W.M., S., K., Tandler, A., Kissil, G.W., Sklan, D., 1993. The effect of dietary lecithin and lipase, as a function of age, on n-9 fatty acid incorporation in the tissue lipids of *Sparus aurata* larvae. Fish Physiology and Biochemistry 10, 357-364.

Kumar, M., Ferguson, G., Xiao, Y., Hooper, G., Venema, S., 2000. Studies on reproductive biology and distribution of the blue swimmer crab (*Portunus pelagicus*) in South Australian waters. SARDI Research Report 47. South Australia Research Development Institute, Urrbare. pp. 32.

Kumar, M.S., 1997. Proceedings of the First National Workshop on Blue Swimmer Crab, *Portunus pelagicus*. SARDI Research Report 16. South Australia Research Development Institute, Adelaide. pp 129.

L.Gonzalez-Felix, M., M.Gatlin, D., L.Lawrence, A., Perez-Velazquez, M., 2002. Effect of dietary phospholipid on essential fatty acid requirements and tissue lipid composition of *Litopenaeus vannamei* juveniles. Aquaculture 207, 151-167.

Lachaise, F., Carpentier, G., Somme, G., Colardeau, J., Beydon, P., 1989. Ecdysteroid synthesis by crab Y-Organs. The Journal Of Experimental Zoology 252, 283-292.

Leger, C., 1985. Digestion, absorption and transport of lipids. In: Nutrition and Feeding in Fish. Cowey, C.B., Mackie, A.M., Bell, J.G. (Eds.). Academic Press Inc., London, pp. 299-331.

Lestang, S.d., Hall, N.G., Potter, I.C., 2003. Reproductive biology of the blue swimmer crab (*Portunus pelagicus*, Decapoda:Portunidae) in five bodies of water on the west coast of Australia. Fishery Bulletin 101, 745-757.

Lester, R., Carey, M.C., Little, J.M., S.R.Dowd, 1975. Crustacean intestinal detergent promotes sterol solubilisation. Science 189, 1098-1100.

Li, Z.-H., Wang, J.-X., Sales, J., Li, P., Chen, D.-Q., 2009. Effect of intermittent stravation on growth and some antioxidant indexes of *Macrobrachium nipponense* (De Haan). Aquaculture Research 40, 526-532.

Liddy, G.C., M.Nelson, M., D.Nichols, P., F.Phillips, B., B.Maguire, G., 2004. The lipid composition of early stage western rock lobster (*Panulirus cygnus*) phyllosoma: importance of polar lipid and essential fatty acids. Journal of Shellfish Research 23, 265-273.

Lim, C., Ako, H., Brown, C.L., Hahn, K., 1997. Growth response and fatty acid composition of juvenile *Penaeus vannamei* fed different sources of dietary lipid. Aquaculture 151, 143-153.

Limbourn, A.J., Nichols, P.D., 2009. Lipid, fatty acid and protein content of late larval to early juvenile stages of the western rock lobster, *Panulirus cygnus*. Comparative

Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 152, 292-298.
Limbourn, A.J., Babcock, R.C., Johnston, D.J., Nichols, P.D., Knot, B., 2008. Post-settlement energy reserves in *Panulirus cygnus*: experimental effects of starvation on survival and nutritional condition. Marine Biology 153, 445-456.
Meagher, T.D., 1971. Ecology of the crab *Portunus pelagicus* (Crustacea: Portunidae) in south western Australia. University of Western Australia. p. 232.
Mercer, L.P., 1982. The quantitative nutrient-response relationship. Journal of Nutrition 112, 560-566.
Merican, Z., Shim, K.F., 1994. Lipid and fatty acid utilization in adult *Penaeus monodon* fed diets supplemented with various oils. Aquaculture 123, 335-347.
Merican, Z., Shim, K.F., 1997. Quantitative requirements of linolenic and docosahexaenoic acid for juvenile Penaeus monodon. Aquaculture 157, 277-295.
Merican, Z.O., Shim, K.F., 1995. Apparent digestibilty of lipid and fatty acids in residual lipids of meals by adult *Penaeus monodon*. Aquaculture 147, 275-291.
Merican, Z.O., Shim, K.F., 1996. Qualitative requirements of essential fatty acids for juvenile Penaeus monodon. Aquaculture 147, 275-291.
Michael, F.R., Teshima, S., Koshio, S., Ishikawa, M., Uyan, O., Ren, T., 2007. Effect of two choline sources on the performance of postlarval *Marsupenaeus japonicus* bate. Aquaculture Nutrition 13, 59-64.
Morais, S., Conceição, L.E.C., Rønnestad, I., Koven, W., Cahu, C., Zambonino Infante, J.L., Dinis, M.T., 2007. Dietary neutral lipid level and source in marine fish larvae: Effects on digestive physiology and food intake. Aquaculture 268, 106-122.
Mourente, G., Tocher, D.R., 1992. Effects of weaning onto a pelleted diet on docosahexaenoic acid (22:6n-3) levels in brain of developing turbot (*Scophthalmus maximus*) Aquaculture 105, 363-377.
Mourente, G., Rodriguez, A., 1997. Effects of salinity and dietary DHA (22:6n-3) content on lipid composition and performance of *Penaeus kerathurus* larvae. Marine Biology 128, 289-298.
NACA, FAO, 2009. Reducing the dependence on the utilzation of trash fish/low value fish as feed for aquaculture of marine finfish in the Asian region. In: FAO/NACA Inception Planning Workshop. NACA & FAO, Krabi, Thailand, pp. 147.
Nakatsuji, T., Lee, C.-Y., Watson, R.D., 2009. Crustacean molt-inhibiting hormone; structure, function and cellular mode of action. Comparative Biochemistry and Physiology, Part A 152, 139-148.
New, M.B., 1976. A review of dietary studies with shrimp and prawns. Aquaculture 9, 101-144.
New, M.B., Wijkstrom, U.N., 2002. Use of fish meal and fish oil in aquafeeds. Further thoughts on the fish meal trap. In: FAO Fisheries Circulation No. 975. FAO, Rome, pp. 61.
Nghia, T.T., Wille, M., Sorgeloss, P., 2001. Influence of the content and ratio of essential HUFA's in the live food on larviculture success of the mud crab (*Scylla paramamosain*). In: Larvi 01 - Fish and Shellfish Larviculture Symposium European Aquaculture Society, Special Publication. Hendry, C.I., Stappen, G.V., Wille, M., Sorgeloos, P. (Eds.) Oostende, Belgium, pp. 430-433.

NRC, 1983. Nutrient requirements of warm water fishes and shell-fishes. In: Nutrient requirements of domestic animal. National Academy Press, Washington, D.C., USA, pp. 102.

O'Sullivan, D., Savage, J., 2008. Soft shell crabs produce in Queensland. In, Austasia Aquaculture 22. Turtle Press Pty. Ltd., Tasmania. pp. 6-9.

Oliva-Teres, A., 2000. Recent advances in European sea bass and gilthead sea bream nutrition. Aquaculture International 8, 471-492.

Olsen, Y., 1998. Lipids and essential fatty acids in aquatic food webs; what can freshwater ecologist learn from mariculture? In: Lipids in freshwater ecosystem. Arts, M.T., Wainman, B.C. (Eds.). Springer-Verlag, New York. pp. 161-191.

Paibulkichakul, C., Piyatiratitivorakul, S., Kittakoop, P., Viykarn, V., Fast, A.W., Mensveta, P., 1998. Optimal dietary levels of lecithin and cholesterol for black tiger prawn *Penaeus monodon* larvae and postlarvae. Aquaculture 167, 273-281.

Palacios, E., 2007. Salinity stress test and its relation to future performance and different physiological responses in shrimp postlarvae. Aquaculture 268, 123-135.

Palacios, E., Bonilla, A., Ange´lica Pe´rez, Racotta, I.S., Civera, R., 2004. Influence of highly unsaturated fatty acids on the responses of white shrimp (*Litopenaeus vannamei*) postlarvae to low salinity. Aquaculture 219, 201-215.

Passano, L.M., 1960. Molting and its control. In: The physiology of crustacea. Waterman, T.H. (Ed.). Academic Press Inc, New York, pp. 473-536.

Paterson, B., Mann, D., Kelly, B., Barchiesi, M., 2007. Limb-loss in pond-reared blue swimmer crabs *Portunus pelagicus* (L.): effect on growth in an indoor shedding system. Aquaculture Research 38, 1569-1579.

Paterson, B.D., 2009. Advances in the culture of crabs In: New technologies in aquaculture; Improving production efficiency, quality and environmental management. Burnell, G., Allan, G. (Eds.). Woodhead Publishing Ltd, Cambridge, UK, pp. 843-865.

Patnaik, S., Samocha, T., Davis, D., Bullis, R., Browdy, C., 2006. The use of HUFA-rich algal meals in diets for *Litopenaeus vannamei*. Aquaculture Nutrition 12, 395-401.

Pedrazzoli, A., Molina, C., Montoya, N., Townsend, S., León-Hing, A., Parades, Y., Calderón, J., 1998. Recent Advances on Nutrition Research of *Penaeus vannamei* in Ecuador. Reviews in Fisheries Science 6, 143-151.

Peng, S., Chen, L., Qin, J.G., Hou, J., yu, N., Long, Z., Ye, J., Sun, X., 2008. Effects of replacement of dietary fish oil by soybean oil on growth performance and liver biochemical composition in juvenile black seabream, *Acanthoparus schlegeli*. Aquaculture 276, 154-161.

Petriella, A.M., Muller, M.I., Fenucci, J.L., Saez, M.B., 1984. Influence of dietary fatty acids and cholesterol on the growth and survival of the argentine prawn, *Artemesia longinaris* Bate. Aquaculture 37, 11-20.

Phillips, K.M., Ruggio, D.M., Toivo, J.I., Swank, M.A., Simpkins, A.H., 2002. Free and Esterified Sterol Composition of Edible Oils and Fats. Journal of Food Composition and Analysis 15, 123-142.

Phlippen, M.K., Webster, S.G., Chung, J.S., Dircksen, H., 2000. Ecdysis of decapod crustaceans is associated with a dramatic release of crustacean cardioactive peptide into the haemolymph. Journal of Experimental Biology 203, 521-536.

Piedad-Pascual, F., 1985. Lecithin requirements of *Penaeus monodon* juveniles. In: First International Conference on the Culture of Penaieds Prawns/Shrimps. Takaki, Y.,

Primavera, J.H., Llobera, J.A. (Eds.) Southeast Asian Fisheries Development Center, Aquaculture Department, Manila, Philippines, Iiloilo, Philiphines, pp. 181.

Piedecausa, M.A., Mazón, M.J., García García, B., Hernández, M.D., 2007. Effects of total replacement of fish oil by vegetable oils in the diets of sharpsnout seabream (*Diplodus puntazzo*). Aquaculture 263, 211-219.

Poston, H.A., 1990. Effect of Body Size on Growth, Survival, and Chemical Composition of Atlantic Salmon Fed Soy Lecithin and Choline. The Progressive Fish-Culturist 52, 226-230.

Potter, I.C., Chrystal, P.J., Loneragan, N.R., 1983. The biology of the blue manna crab *P. pelagicus* in an Australian estuary. Marine Biology 78, 75-85.

Read, G.H.L., 1981. The response of *Penaeus indicus* (Crustacea: Penaeidea) to purified and compounded diets of varying fatty acid composition. Aquaculture 24, 245-256.

Rees, J.F., Cure, K., Piyatiratitivorakul, S., Sorgeloos, P., Menasveta, P., 1994. Highly unsaturated fatty acid requirements of *Penaeus monodon* postlarvae: an experimental approach based on Artemia enrichment. Aquaculture 122, 193-207.

Reigh, R.C., Stickney, R.R., 1989. Effects of purified fatty acids on the fatty acid composition of freshwater shrimp, *Macrobrachium rosenbergii* Aquaculture 77, 157-174.

Ritar, A.J., Dunstan, G.A., Crear, B.J., Brown, M.R., 2003. Biochemical composition during growth and starvation of early larval stages of culture spiny lobster (*Jasus edwarsii*) phyllosoma. Comparative Biochemistry and Physiology 136A, 353-370.

Rodríguez, C., Pérez, J.A., Díaz, M., Izquierdo, M.S., Fernández-Palacios, H., Lorenzo, A., 1997. Influence of the EPA/DHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. Aquaculture 150, 77-89.

Rodríguez, C., Pérez, J.A., Badía, P., Izquierdo, M.S., Fernández-Palacios, H., Hernández, A.L., 1998. The n-3 highly unsaturated fatty acids requirements of gilthead seabream (Sparus aurata L.) larvae when using an appropriate DHA/EPA ratio in the diet. Aquaculture 169, 9-23.

Romano, N., Zeng, C., 2006. The effects of salinity on the survival, growth and haemolymph osmolality of early juvenile blue swimmer crabs, *Portunus pelagicus*. Aquaculture 260, 151-162.

Romano, N., Zeng, C., 2008. Blue swimmer crabs, emerging species in Asia. In: Global Aquaculture Advocate 11. Global Aquaculture Alliance, Missouri, USA. pp. 34-36.

Roustaian, P., Kamaruddin, M.S., Omar, H., Saad, C.R., Ahmad, M.H., 2001. Biochemical changes in freshwater prawn *Macrobrachium rosenbergii* during larval development. Journal of the World Aquaculture Society 32, 53-59.

Roy, L.A., Davis, D.A., Saoud, I.P., 2006. Effects of lecithin and cholesterol supplementation to practical diets for *Litopenaeus vannamei* reared in low salinity waters. Aquaculture 257, 446-452.

Salhi, M., Hernández-Cruz, C.M., Bessonart, M., Izquierdo, M.S., Fernández-Palacios, H., 1999. Effect of different dietary polar lipid levels and different n-3 HUFA content in polar lipids on gut and liver histological structure of gilthead seabream (*Sparus aurata*) larvae. Aquaculture 179, 253-263.

Sanchez-Paz, A., Garcia-Carreno, F., Muhlia-Ahmazan, A., Peregrino-Uriarte, A.B., Hernandez-Lopez, J., Yepiz-Plascencia, G., 2006. Usage of energy reserves in

crustaceans during starvation: Status and future directions. Insect Biochemistry and Molecular Biology 36, 241-249.
Saraswathy, G., Sugumar, A., Selvan, U.R., Neethiselvan, N., 2006. Comparison of meat quality of *Portunus pelagicus* and *Portunus sanguinolentus*. Fishery Technology 43, 168-175.
Sargent, J.R., Henderson, R.J., Tocher, D.R., 2002. The lipids. In: Fish Nutrition. Halver, J., Hardy, E. (Eds.). Academic Press, Elsevier, San Diego, California, USA, pp. 181-257.
Sargent, J.R., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J., Tocher, D., 1999. Lipid nutrition of marine fish during early development: Current status and future direction. Aquaculture 179, 217-229.
Scandol, J., Rowling, K., Graham, K., 2008. Blue Swimmer Crab (*Portunus pelagicus*). NSW Department of Primary Industries, Cronulla, pp 33-36.
Sheen, S.-S., 2000. Dietary cholesterol requirement of juvenile mud crab *Scylla serrata*. Aquaculture 189, 277-285.
Sheen, S.-S., D'Abramo, L.R., 1991. Response of juvenile freswater prawn, *Macrobrachium rosenbergii*, to different levels of a cod liver oil/corn oil mixture in a semi purified diet. Aquaculture 93, 121-134.
Sheen, S.-S., Wu, S.-W., 1999. The effects of dietary lipid levels on the growth response of juvenile mud crab *Scylla serrata*. Aquaculture 175, 143-153.
Sheen, S.S., 1997. Lipid supplementation of semi-purified diets for *Penaeus chinensis* juvenile. Journal of the Fisheries Society of Taiwan 24, 235-242.
Sheen, S.S., Wu, S.W., 2003. Essential Fatty Acid Requirements of Juvenile Mud Crab, *Scylla serrata* (Forskal, 1775) (Decapoda, Scyllaridae). Crustaceana 75, 1387-1401.
Sheen, S.S., Chen, S.J., Huang, Y.S., 1994a. Effect of dietary lipid levels on the growth response of tiger prawn, *Penaeus monodon*. Journal of the Fisheries Society of Taiwan 21, 205-213.
Sheen, S.S., Liu, P.C., Chen, S.N., Chen, J.C., 1994b. Cholesterol requirement of juvenile tiger shrimp *Penaeus monodon*. Aquaculture 125, 131-137.
Shieh, H.S., 1969. The biosynthesis of phospholipids in the lobster *Homarus americanus* Comparative Biochemistry and Physiology 30, 679-684.
Shinkarenko, L., 1979. Development of the larval stages of the blue swimming crab *Portunus pelagicus* L. (Portunidae : Decapoda : Crustacea). Australian Journal of Marine and Feshwater Research 30, 485-503.
Shudo, K., Nakamura, K., Ishikawa, S., Kitabayashi, K., 1971. Studies on the growth-promoting effects of both squid liver oil and cholesterol. Bulletin of Tokkai Regional Fisheries Research Lab 65, 129-137.
Singh, R.K., Balange, A.K., 2007. Compensatory growth and changes in nutrient composition in post-larvae of giant prawn, *Macrobrachium rosenbergii* following starvation. Journal of Applied Aquaculture 19, 39-49.
Skinner, D.M., 1985. Molting and regeneration. In: The biology of crustacea. Bliss, D.E., Mantel, L.H. (Eds.). Academic Press, New York, pp. 43-146.
Smith, D.M., Tabrett, S.J., Barclay, M.C., 2001. Cholesterol requirement of subadult black tiger shrimp *Penaeus monodon* (Fabricius). Aquaculture Research 2001, 399-405.
Smith, E.L., Hill, R., Lehman, I.R., Lefkowitz, R.J., Handler, P., White, A., 1983. Principles of biochemistry: General aspect. McGraw and Hill, Singapore. pp. 886.

Smith, H., 1982. Blue crabs in South Australia - their status, potential and biology. SAFIC 6, 6-9.

Spaziani, E., Kater, S., 1973. Uptake and turnover of cholesterol-14C in Y-organs of the crab *Hemigrapsus*, as a function of the molt cycle. Comparative Endocrinology 20, 534-549.

Steffens, W., 1989. Fat requirement. In: Principles of Fish Nutrition. Ellis Horwood, New York, pp 139-145.

Stuck, K.C., Watts, S.A., Wang, S.Y., 1996. Biochemical responses during starvation and subsequent recovery in postlarval white shrimp, *Penaeus vannamei*. Marine Biology 125, 33-45.

Stumpf, L., Calvo, N.S., Pietrokovsky, S., López Greco, L.S., 2010. Nutritional vulnerability and compensatory growth in early juveniles of the "red claw" crayfish *Cherax quadricarinatus*. Aquaculture 304, 34-41.

Sui, L., Wille, M., Cheng, Y., Sorgeloos, P., 2007. The effect of dietary n-3 HUFA levels and DHA/EPA ratios on growth, survival and osmotic stress tolerance of Chinese mitten crab *Eriocheir sinensis* larvae. Aquaculture 273, 139-150.

Suprayudi, M.A., Takeuchi, T., Hamasaki, K., 2004. Essential fatty acids for larval mud crab *Scylla serrata*: implications of lack of the ability to bioconvert C18 unsaturated fatty acids to highly unsaturated fatty acids. Aquaculture 231, 403-416.

Tackaert, W., Abelin, P., Leger, P., Sorgeloos, P., 1992. Stress resistance as a criterion to evaluate quality of postlarval shrimp reared under different feeding procedures. . In: Simposio Brasileiro Sobre Cultivo de Camarao MCR Aquacultura, Joao Pessoa, PB, Brazil, pp. 393-403.

Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospoect Aquaculture 285, 146-158.

Takeuchi, T., 2000. A review of studies on the effect of dietary n-3 highly unsaturated fatty acids on larval swimming crab *Portunus trituberculatus* and mud crab *Scylla tranquebarica.* In: Proceedings of JSPS-DGHE International Symposium on Fisheries Science in Tropical Area. Bogo, Indonesia, pp. 244-247.

Teshima, S., 1997. Phospholipids and Sterols. In: Crustacean Nutrition. Advances in World Aquaculture. Abramo, L.R.D., Conklin, D.E., Arigama, D.M. (Eds.). World Aquaculture Society, Baton Rouse, Louisiana, pp. 85-107.

Teshima, S., Kanazawa, A., 1980. Transport of dietary lipids and role of serum lipoproteins in the serum of prawn. Bulletin of Japanese Society of Scientific Fisheries 46, 51-55.

Teshima, S., Kanazawa, A., 1983. Digestibilty of dietary lipids in prawn. Bulletin of Japanese Society of Scientific Fisheries 49, 963-966.

Teshima, S., Kanazawa, A., 1986. Nutritive value of sterols for the juvenile prawn. Bulletin of Japanese Society of Scientific Fisheries 52, 1417-1422.

Teshima, S., Kanazawa, A., Kakuta, Y., 1986a. Role of dietary phospholipids in the transport of 14C tripalmitin in the prawn. Bulletin of Japanese Society of Scientific Fisheries 52, 519-524.

Teshima, S., Kanazawa, A., Kakuta, Y., 1986b. Effects of dietary phospholipids on lipids transport in the juvenile prawn. Bulletin of Japanese Society of Scientific Fisheries 52, 159-163.

Teshima, S., Kanazawa, A., Koshio, S., Kondo, N., 1989. Nutritive value of sitosterol for prawn, *Penaeus japonicus*. Nippon Suisan Gakkaishi 55, 153-157.

Teshima, S., Ishikawa, M., Koshio, S., Kanazawa, A., 1997. Assessment of cholesterol requirements in the prawn, *Penaeus japonicus*. Aquaculture Nutrition 3, 247-253.

Thongrod, S., Boonyaratpalin, M., 1998. Cholesterol and lecithin requirement of juvenile banana shrimp, *Penaeus merguiensis*. Aquaculture 161, 315-321.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11, 107-184.

Tocher, D.R., Bendiksen, E.A., Campbell, P.J., Bell, J.G., 2008. The role of phospholipids in nutrition and metabolism of teleost fish. Aquaculture 280, 21-34.

Tsvetnenko, E., Brown, J., Glencross, B.D., Evans, L.H., 2001. Measures of condition in dietary studies on Western rock lobster post -puereli. In: International Symposium on Lobster Health Management. Adelaide, pp. 675.

Van Veldhoven, P.P., Casteels, M., Mannaerts, G.P., Baes, M., 2001. Further insights into peroxisomal lipid breakdown via alpha- and beta-oxidation. Biochemical Society Transaction 29, 292-298.

Vanstone, C.A., Raeini-Sarjaz, M., Parsons, W.E., Jones, P.J., 2002. Unesterified plant sterols and stanols lower LDL-cholesterol concentrations equivalently in hypercholesterolemic persons. American Journal of Clinical Nutrition 76, 1272-1278.

Vasagam, K.P.K., Ramesh, S., Balasubramaniam, T., 2005. Dietary value of different vegetable oil in black tiger shrimp *Penaeus monodon* in the presence and absence of soy lecithin supplementation: Effect of growth, nutrient digestibility and body composition. Aquaculture 250, 317-327.

Walker, T., 2006. Coral Coast Mariculture claws a market for soft shell culture. In: Austasia Aquaculture 16. Turtle Press Pty. Ltd., Tasmania. pp. 6-7.

Ward, L.R., Carter, C.G., 2009. An evaluation of the nutritional value of alternative lipid sources to juvenile southern rock lobster, *Jasus edwardsii*. Aquaculture 296, 292-298.

Wassenberg, T.J., Hill, B.J., 1987. Feeding by the sand crab *Portunus pelagicus* on material discarded from prawn trawlers in Moreton Bay, Australia. Marine Biology 95, 387-393.

Watanabe, T., 2002. Strategies for further development of aquatic feeds. Fisheries Science 68, 242-252.

Wen, X., Chen, L., Ku, Y., Zhou, K., 2006. Effect of feeding and lack of food on the growth, gross biochemical and fatty acid composition of juvenile crab, *Eriocheir sinensis*. Aquaculture 252, 598-607.

West, T.L., Costlow, J.D., 1987. Size regulation in larvae of the crustacean *Balanus eburneus*. Marine Biology 96, 47-58.

Wijngaarden, D.V., 1967. Modified rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Analytical Chemistry 39, 848-849.

Williams, L.E., 2002. Queensland's fisheries resources. Current condition and recent trends. Department of Primary Industries, Brisbane, pp 119-127.

Williams, M.J., 1982. Natural food and feeding in the commercial sand crab *Portunus pelagicus* Linnaeus, 1766 (Crustacea : Decapoda : Portunidae) in Moreton Bay, Queensland. Journal of Experimental Marine Biology and Ecology 59, 165-176.

Wiseman, J., 1991. The chemical characteristics and nutritive value of fats. In: The Bulletin 785. National Renderers Association Inc, London. pp. 8-11.

Wold, P.-A., Hoehne-Reitan, K., Cahu, C.L., Infante, J.Z., Rainuzzo, J., Kjørsvik, E., 2007. Phospholipids vs. neutral lipids: Effects on digestive enzymes in Atlantic cod (*Gadus morhua*) larvae. Aquaculture 272, 502-513.

Wu, L., Dong, S., 2002a. The effects of repetitive 'starvation-and-refeeding' cycles on the compensatory growth response in chinese shrimp, *Fenneropenaeus chinensis* (Osbeck, 1765) (Decapoda, Penaiedae). Crustaceana 74, 1225-1239.

Wu, L., Dong, S., 2002b. Compensatory growth responses in juvenile chine shrimp, *Fenneropenaeus chinensis* at different temperature. Journal of Crustacean Biology 22, 511-520.

Wu, L., Dong, S., Wang, F., Tian, X., Ma, S., 2001. The effect of previous feeding regimes on the compensatory growth response in Chinese shrimp, *Fenneropenaeus chinensis*. Journal of Crustacean Biology 21, 559-565.

Wu, R.S.S., 1995. The environmental impact of marine fish culture: Towards a sustainable future. Marine Pollution Bulletin 31, 159-166.

Wu, X., Cheng, Y., Sui, L., Zeng, C., Southgate, P.C., Yang, X., 2007. Effect of dietary supplementation of phospholipids and highly unsaturated fatty acids on reproductive performance and offspring quality of Chinese mitten crab, *Eriocheir sinensis* (H. Milne-Edwards), female broodstock. Aquaculture 273, 602-613.

Wu, X., Zhou, B., Cheng, Y., Zeng, C., Wang, C., Feng, L., 2010a. Comparison of gender differences in biochemical composition and nutritional value of various edible parts of the blue swimmer crab. Journal of Food Composition and Analysis 23, 154-159.

Wu, X., Chang, G., Cheng, Y., Zeng, C., Southgate, P., Lu, J., 2010b. Effects of dietary phospholipid and highly unsaturated fatty acid on the gonadal development, tissue proximate composition, lipid class and fatty acid composition of precocious Chinese mitten crab, Eriocheir sinensis. Aquaculture Nutrition 16, 25-36.

Xu, H., Ai, Q., Mai, K., Xu, W., Wang, J., Ma, H., Zhang, W., Wang, X., Liufu, Z., 2010. Effects of dietary arachidonic acid on growth performance, survival, immune response and tissue fatty acid composition of juvenile Japanese seabass, *Lateolabrax japonicus*. Aquaculture 307, 75-82.

Xu, X., Ji, W., Castell, J.D., O'Dor, R., 1993. The nutritional value of dietary n-3 and n-6 fatty acids for the Chinese prawn (*Penaeus chinensis*). Aquaculture 118, 277-285.

Xu, X.L., Ji, W.J., Castell, J.D., O'Dor, R.K., 1994. Essential fatty acid requirement of the Chinese prawn, *Penaeus chinensis*. Aquaculture 127, 29-40.

Yepiz-Plascencia, G., Vargas-Albores, F., Higuera-Ciapara, I., 2000. Penaeid shrimp hemolymph lipoproteins. Aquaculture 191, 177-189.

Yu, T.C., Sinnhuber, R.O., 1976. Growth response of rainbow trout (Salmo gairdneri) to dietary [omega]3 and [omega]6 fatty acids. Aquaculture 8, 309-317.

Zar, J.H., 1999. Multisample hypothesis: The analysis of variance. In: Biostatistical analysis. Ryu, T. (Ed.). Prentice-Hall Inc., New Jersey, pp. 177-207.

Zhang, P., Zhang, X., Li, J., Gao, T., 2009. Starvation resistance and metabolic response to food deprivation and recovery feeding in *Fenneropenaeus chinensis* juveniles. Aquaculture International 17, 159-172.

Zhou, Q.-C., Tan, B.-P., Mai, K.-S., Liu, Y.-J., 2004. Apparent digestibility of selected feed ingredients for juvenile cobia *Rachycentron canadum*. Aquaculture 241, 441-451.

Zhou, Q.-C., LI, C.-C., Liu, C.-W., Chi, S.-Y., Yang, Q.-H., 2007. Effects of dietary lipid sources on growth and fatty acid composition of juvenile shrimp, *Litopenaeus vannamei*. Aquaculture Nutrition 13, 222-229.