Lipid nutrition of early life history of two commercially important tropical crustaceans, the blue swimmer crab (*Portunus pelagicus*) and the ornate rock lobster (*Panulirus ornatus*), with emphasis on highly unsaturated fatty acids

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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/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001).

The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee.

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Statement of contribution of others

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Abstract

The blue swimming crab, *Portunus pelagicus* and ornate rock lobster, *Panulirus ornatus* are two commercially important crustacean species of high market value with wild populations distributed throughout the tropical and sub-tropical Indo-Pacific region. As the wild populations of *P. pelagicus* and *P. ornatus* have been over-exploited, there is an increasing interest in the development of their aquaculture to meet the rising market demand. However, one of the major challenges for closed-life cycle production of *P. pelagicus* and *P. ornatus* is the development of a commercially viable hatchery technology. Comprehensive and quantitative understanding of larval nutrition is very important for the development of a reliable hatchery technology of *P. pelagicus* and *P. ornatus*. However, basic knowledge of the nutritional requirements and physiology is very limited for the early life stage of both species.

It is well known that lipids play a crucial role on the survival, growth and development of crustacean larvae. To further knowledge in this area a series of experiments were initially conducted to understand the ontogenetic changes of key lipid composition, including lipid classes and fatty acids, during the embryonic and larval development. This was extended to measure changes of lipid composition during the starvation of newly hatched larvae of *P. pelagicus* and *P. ornatus*. From this foundation, four experiments were designed to assess dietary optimum 20:4n6 (ARA) levels and the 22:6n3/20:5n3 (DHA/EPA) ratios for *P. pelagicus* and *P. ornatus* larvae based on enriched *Artemia*, which is one of the commonly used hatchery feeds for marine crustacean larvae. Experiments included enriched *Artemia* containing different levels of dietary fatty acids which were fed to newly hatched larvae of *P. pelagicus* and *P. ornatus*. In these experiments the survival, development, growth and fatty acid composition of larvae were closely monitored to identify the dietary optimum HUFA composition.

Chapter 2 and 3 were conducted to investigate the changes of lipid class and fatty acid composition during the embryonic development of *P. pelagicus* and *P. ornatus*. For both species, there are significant increases of egg volume and moisture content while the total lipid content decreased dramatically. However, different trends were found between the lipid class profiles of *P. pelagicus* and *P. ornatus*, including the percentage of phospholipids (PL), free fatty acids (FFA) and cholesterol (CHO) (%total lipid). The principal fatty acids were 16:0, 16:1n7, 18:1n9, 18:1n7, 20:4n6 (ARA), 20:5n3 (EPA) and 22:6n3 (DHA) in the eggs of both species. Despite of embryonic stages, significantly higher DHA levels and DHA/EPA ratios were found in the eggs of *P. ornatus* compared to *P. pelagicus*. However the eggs of *P.*

pelagicus contained higher ARA level than that of *P. ornatus*. During embryonic development, ARA, EPA and DHA were relatively conserved for *P. pelagicus* while 18:1n9, 18:2n6, ARA and EPA were relatively conserved for *P. ornatus*. These differences indicate that the two crustacean species have different lipid dynamics during their embryonic development.

Chapter 4 and 5 were designed to investigate the ontogenetic changes of growth and lipid composition during larval development of both species. Although individual biomass and body size increased significantly, the specific growth rate (SGR%/day) of body weight generally decreased significantly during the larval development of both species. The lipid class profile of early-mid Stages phyllosoma were dominated by PL (>85% total lipids) for *P. ornatus* while the significant lower PL levels were detected in the late stage *P. pelagicus* larvae compared the newly hatched *P. pelagicus* larvae and all early-mid Stages *P. ornatus* larvae. Generally, the fatty acid composition of larvae reflects that of their larvae diet. However, ARA and DHA were preferentially accumulated by later larval stage of *P. pelagicus* while early-mid Stage (II-V) phyllosoma preferentially sequestered and accumulated a higher proportion of ARA, EPA and DHA compared to the levels that present in their diet. These data indicate that larval *P. pelagicus* and *P. ornatus* have an ability to preferentially sequester, store or modify their dietary lipid composition to support normal growth and development.

Chapter 6 and 7 were designed to examine the effects of starvation on survival, biomass and lipid composition and to explain the preferential conservation of important fatty acids for newly hatched larvae of *P. pelagicus* and *P. ornatus.* The results showed that during starvation, significant decreasing trends were detected on individual body dry weight, total lipids (%dry weight) or per larva (μg/larva) for the newly hatched larvae of *P. pelagicus* and *P. ornatus.* During the starvation, more than 50% of PL was utilized for the larvae of both species, indicating the oxidation of membrane structural lipids. The higher reductions were found on EPA and DHA than other fatty acids during the starvation of *P. pelagicus* larvae while both DHA and ARA were highly conserved for newly hatched *P. ornatus* phyllosoma. These results suggested that the HUFA requirement of larval *P. pelagicus* is lower and different to early Stage *P. ornatus* phyllosoma.

Chapter 8 and 9 were designed to investigate the basis for the relative conservation of ARA during the starvation of newly hatched larvae of both species. Investigations included revealing the dietary optimum ARA contents for the survival, development and growth of *P. pelagicus* and *P. ornatus* larvae as supplied through the feeding enriched *Artemia* as larval food. For larval *P. pelagicus*, dietary ARA level significantly affected not only survival, development and

growth of the larvae, but also the occurrence of moulting death syndrome (MDS) as well as the ratio of chela length/carapace length (CHL/CL) of zoea IV. For the early Stage *P. ornatus* larvae, dietary ARA levels did not significantly affect survival, but significant differences were detected on development time and growth among the different treatments. The results suggest that optimal ARA level is approximately 6.27 mg/g DW (2.97% of total fatty acids) in the enriched *Artemia* for *P. pelagicus* larvae, and around 3.69 mg/g DW (1.90% total fatty acids) for early Stage *P. ornatus* phyllosoma.

Although previous studies have shown dietary DHA/EPA ratios are very important for crustaceans, no available information could be found for its importance in the larvae of *P. pelagicus* and *P. ornatus*. Therefore, two experiments were conducted to investigate the dietary optimum DHA/EPA ratios for *P. pelagicus* and *P. ornatus* larvae. There were five treatments with newly hatched larvae fed enriched *Artemia* contained different DHA/EPA ratios. Although no significant difference was found on the survival of early stage larvae for both species, the highest survival was found on the treatment fed enriched *Artemia* contained medium DHA/EPA ratio until megalopal stage of *P. pelagicus*. The dietary DHA/EPA ratios also significantly affected the development time and growth for both species. The dietary optimum DHA/EPA ratios were estimated at ca. 0.53 and > 1.64 for *P. pelagicus* larvae and early Stage phyllosoma of *P. ornatus*, respectively, which clearly indicated the early Stage phyllosoma of the ornate rock lobster had higher DHA/EPA ratio requirement than *P. pelagicus* larvae. These findings indicated that the optimization of the fatty acid composition of hatchery food will be an effective means to improve seed quality and quantity for both species.

The current project utilized an integrated methodology approach to study fatty acid nutrition of *P. pelagicus* and *P. ornatus* larvae. The embryonic and larval development experiments related to ontogenetic changes of key lipid composition were targeted to obtain fundamental information, followed by growth trails to identify the specific fatty acids requirement or ratios. Fatty acid analysis was used to gain a deep understanding of underlying mechanisms. The findings have significantly enhanced our understanding of fatty acid nutrition for *P. pelagicus* and *P. ornatus* larvae, which will facilitate the further improvement of hatchery technology and formulated diets for the larval culture of both species.

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Chapter 1

General introduction

1.1 Larval culture of the blue swimmer crab, *Portunus pelagicus* and the ornate rock lobster, *Panulirus ornatus* and fatty acid nutrition

The blue swimmer crab (*Portunus pelagicus*) and the ornate rock lobster (*Panulirus ornatus*) are two commercially important species in the tropical regions of the Indo-Pacific (Smith et al., 2009a; Wu et al., 2010d). Because of their large size, high meat yield and delicate flavour, there is an increasing demand for both *Portunus pelagicus and Panulirus ornatus* supply worldwide, which has led to a growing interest in *P. pelagicus* and *P. ornatus* aquaculture (Murugan et al., 2005; Romano and Zeng, 2008). However, mass production of artificial seed is a key prerequisite for the future development of aquaculture for both species.

Larval development of *P. pelagicus* includes four zoeal instars and one megalopal stage before settling as the first stage crab (Josileen and Menon, 2004b; Andres et al., 2010). Laboratory reared larvae under optimal conditions requires approximately 12-14 days to reach the first crab stage (C1) and the larvae readily accepted food only after 2-4 hour of post-hatching (Romano and Zeng, 2008), which are beneficial characteristics for mass hatchery production (Castine et al., 2008). While larval culture of *P. pelagicus* generally has better survival than that of other commercially important portunid crabs, such as the mud crab *Scylla* spp. and the blue crab, *Callinectes sapidus* (Zmora et al., 2005; Nghia et al., 2007), high incidences of moulting death syndrome (MDS) during the period when zoea IV metamorphoses to megalopa is still commonly observed (Soundarapandian et al., 2007; Castine et al., 2008), which signficantly impacts the final yield of seed production. Previous research has shown that unbalanced dietary highly unsaturated fatty acids (HUFA) could lead to low larval survival as well as a high incidence of MDS in larval culture of the mud crab, *S. serrata* (Suprayudi et al., 2004c; Dan and Hamasaki, 2011) and the swimming crab, *Portunus trituberculatus* (Takeuchi et al., 1999a; Takeuchi et al., 1999b; Arai et al., 2004). For *P. pelagicus*, larval nutrition, particular fatty acid nutrition, has also been suggested as a major contributor to incidents of unsuccessful larval rearing (Maheswarudu et al., 2008).

The larval cycle of *P. ornatus*, is complex, consisting of eleven distinct morphological pelagic planktonic phyllosoma stages, a transitional planktonic post-larval puerulus stage, then the first instar benthic juvenile (also known as the benthic post-puerulus) (Smith et al., 2009a; Limbourn and Nichols, 2009). Of the nine species of Palinurid lobsters in Australia, *P. ornatus*

is the fastest growing species and importantly has the shortest larval phase among rock lobsters (Phillips et al., 1992). Nevertheless the larval phase is long, in relation to commercially farmed crustaceans such as penaeid prawns as it takes 4 to 6 months to complete larval development (Johnston et al., 2009). As a result, the hatchery production of lobsters is more difficult compared to other crustaceans because of their prolonged larval phase (Takeuchi and Murakami, 2007). Despite several years of research, the commercial hatchery production of *P. ornatus* has not progressed significantly due to poor survival of the larvae to juvenile which together with the extended larval phase (Johnston et al., 2008; Smith et al., 2009a). The current bottleneck in the aquaculture production of all Palinurid marine lobster species is the lack of ability to rear large quantities larvae, from egg through the multiple planktonic phyllosoma stages to puerulus and the benthic juvenile stages. Successful larval production has been achieved for *P. ornatus* in Australia between 2006 and 2010, but only at small scales (Smith et al., 2009a; O'Sullivan, 2010). Survival from egg to phyllosoma, puerulus and juvenile stages is currently less than 5% (Hall, pers comm), which in part is due to the limited information on larval feeding and nutritional requirements for this species (Johnston, 2006; Johnston et al., 2008). Larval nutrition, and in particular for the contents and composition of highly unsaturated fatty acids, is deemed a critical area to the successful hatchery production of this species (Wu et al., 2012a). Previous studies have shown that optimized fatty acids nutrition in the diet, particularly PUFA, can improve survival, shorten the development time and enhance growth in some other lobster larvae, such as the western rock lobster *Panulirus cygnus* (Liddy et al., 2005), the American lobster *Homarus americanus* (Fiore and Tlusty, 2005) and the southern rock lobster *Jasus edwardsii* (Nelson et al., 2004). This important information in these studies provides an intriguing insight for the larval culture of *P. ornatus* as the improved diet might shorten the larval development time and improve the survival of Palinurid larvae under the culture conditions.

Although there are a large number of publications on the topics of fatty acids nutrition for crustacean species (Liddy et al., 2004a, b; Liddy et al., 2005; Sui et al., 2007; Sui et al., 2011), there are limited informations on the essential fatty acids. The dietary optimal level of essential fatty acids has not been identified for the larval stage of any crustacean species. However, there is are a limited number of reports on the juveniles of some shrimp species, including *Penaeus japonicus* (Kanazawa et al., 1977; Kanazawa et al., 1978; Kanazawa et al., 1979b; Kanazawa et al., 1985b), *Penaeus monodon* (Merican and Shim, 1996b; 1997b; Glencross and Smith, 1999b; Glencross and Smith, 2001; Glencross et al., 2001; Glencross et al., 2002), *Macrobrachium rosenbergii* (Reigh and Stickney, 1989b; D'Abramo and Sheen, 1993; D'Abramo, 1998), *Litopenaeus vannamei* (González-Félix et al., 2002a; González-Félix et al., 2002b;

González-Félix et al., 2003). As formulated diets are unlikely to completely replace live foods for most crustacean larvae, it is difficult to investigate fatty acid requirement for crustacean larvae using formulated diets (Jones, 1998). Live foods, commonly rotifers and *Artemia*, have several beneficial characteristics, such as attractiveness to larvae, less water contamination and availability in a range of sizes. Due to these attributes they are widely used in the seed production of commercially important crustaceans (Takeuchi et al., 1999b; Han et al., 2001; Suprayudi et al., 2004a). Currently, *Artemia* has been used as major food source for the larval culture of *P. pelagicus* and early Stage *P. ornatus* phyllosoma (Romano and Zeng, 2008; O'Sullivan, 2010). However, *Artemia* generally lacks highly unsaturated fatty acids, which will affect growth and survival for many crustacean larvae (Sui et al., 2007; Holme et al., 2009c). Many previous studies have shown enrichment is an effective method to improve the HUFA composition and contents of the *Artemia*, which would led to the improvement of the survival and growth for crustacean larvae (Levine and Sulkin, 1984; Suprayudi et al., 2004c; Liddy et al., 2005; Nghia et al., 2007). However, there are no reports of investigations of the fatty acids nutrition on the early life stages of *P. pelagicus* and *P. ornatus*.

A suitable model crustacean species can speed and facilitate the intensive investigation to understand the nutrition requirements and their basic physiological functions. Although the blue swimmer crab is a good candidate model species for the investigation of larval nutrition of marine crabs (Castine et al., 2008), including fast growth, ease of maintainance in indoor tanks, requirement for only small rearing tanks, disease resistance and frequent spawning in one year, the lipid nutrition of early life stage of *P. pelagicus* is still largely unknown. However, the culture technology of all life stage *P. pelagicus* have been established at James Cook University (Romano and Zeng, 2008; Andrés et al., 2010), and therefore offers the opportunity to further study the larval nutrition for this species. In an attempt to improve seed production and larval quality of commercially tropical crustaceans, *P. pelagicus* is considered as a model species to investigate the fatty acids nutrition, especially for 20:4n6 (ARA), 20:5n3 (EPA), 22:6n3 (DHA), during the early life stages. Findings of these baseline studies will be applied and optimized for the larval culture of blue swimmer crab (*P. pelagicus*) and rock lobster (*P. ornatus*) to improve hatchery production and reduce the cost of operation.

1.2 Functions of fatty acids during the early life stages of crustacean

1.2.1 Energy provision

The utilization of lipids and fatty acids for energy during embryogenesis and early larval development in many species of crustacean has some unique and interesting features that require special mention. To appreciate these features, some description of egg lipid composition

and the species differences is required. In terms of lipid content and lipid class composition, crustacean eggs can fall into basically two categories. Eggs with relatively high polar lipid contents (60 to 90% of total lipid on average, mainly phosphatidylcholine (PC) and phosphatidylethanolamine (PE)), and eggs that have higher neutral lipids (> 50% of total lipid, mainly triacylglycerol (TG)), often in the form of oil globules or droplets, in addition to the phospholipid-rich yolk lipid (Yao et al., 2006; Wu et al., 2007b). In general, eggs from typical marine crustaceans, e.g. *Litopenaeus vannamei* (Wouters et al., 2001), swimming crab, *Portunus trituberculatus* (Chen et al., 2007), tend to be lower neutral lipid contents, but higher phospholipid and n-3 HUFA levels compared to the eggs from freshwater species, such as Chinese mitten crab *Eriocheir sinensis* (Wu et al., 2007b), *Macrobrachium rosenbergii* (Cavalli et al., 1999). In contrast, the eggs of most freshwater fish contain higher levels of n-6 poly-unsaturated fatty acids (PUFA), particularly 18:2n-6 and 20:4n-6, than marine crustacean eggs (Yao et al., 2006; Wu et al., 2007b). Although formulated diet for captive crustacean generally have high 18:2n6 and 18:1n9, it seems that the eggs of captive broodstock appear to be under selection pressure to maintain levels of n-3 HUFA within a species-specific range (Wouters et al., 2001).

The utilization of lipids and fatty acids during embryonic and early larval development varies considerably between crustacean species. Lipids were utilized as an energy source mainly after the initiation of the heart-beating stage of the embryo for most of crustacean species (Yao et al., 2006; Chen et al., 2007). The specific classes utilized again depends largely on the type of eggs, with neutral lipid–rich eggs primarily utilizing neutral lipids, such as in Chinese mitten crab *E. sinensis* and giant shrimp *M. rosenbergii* (Cheng et al., 1998; Yao et al., 2006), whereas phosphoglyceride-rich eggs such as those from swimming crab, *P. trituberculatus* and Pacific white shrimp *L. vannamei* primarily utilized phospholipids, mainly PC (Wouters et al., 2001; Chen et al., 2007). Therefore, phosphoglyceride-rich eggs tend to utilize phosphoglycerides, particularly PC, whereas neutral lipid-rich eggs utilize primarily triacylglycerols and also steryl and wax esters where present (Wehrtmann and Kattner, 1998; Torres et al., 2008). Irrespective of what lipid class, catabolism of lipids results in the release of free fatty acids which can either be utilized for energy or re-acylated back into lipid pools for the other uses during embryogenesis and early larval development, which can be for the formation of rapidly developing larval tissues (Wehrtmann and Graeve, 1998; Walker et al., 2006). In n-3 HUFA-rich marine eggs, it is perhaps obvious that PUFA and HUFA could be catabolized for energy, particularly in phosphoglyceride-rich eggs. Catabolism of HUFA for energy, such as EPA and DHA, has been reported in the Norway lobster, *Nephrops norvegicus* (Rosa et al., 2003), the European lobster, *Homarus gammarus* (Rosa et al., 2005), shrimp, *Palaemon*

serratus (Narciso and Morais, 2001), and deep sea pandalids *Chlorotocus crassicornis* and *Polybius henslowii* (Rosa et al., 2007). In the neutral lipid-rich eggs, such as freshwater giant shrimp *Macrobrachium rosenbergii* (Clarke et al., 1990), and portunid crab *Macropipus tuberculatus* (Rosa et al., 2007), n-3 HUFA and 20:4n-6 tended to be conserved during embryonic development, whereas saturated and monounsaturated fatty acids were primarily utilized during catabolism of triacylglycerols in those species (Yao et al., 2006). It is worth noting that for some species, egg mono-unsaturated fatty acids (MUFA) (primarily 16:1n7, 18:1n9, and 18:1n7) were utilised at a higher rate than saturated fatty acids (SFA) during embryogenesis (Rosa et al., 2005; Rosa et al., 2007) although the exact reason is not clear. Based on studies on mainly marine decapod crustacean, Auel et al. (2002) suggested that the utilization of HUFA, including 22:6n-3, occurred mainly in the earlier stages of development when yolk was still present, whereas later, in non-feeding larvae, 22:6n-3 and 20:4n-6 were selectively retained in PLs, at the expense of other fatty acids, including 20:5n-3, which were mainly catabolized (Auel et al., 2002). Thus, HUFA, as well as having clearly established roles in membrane structure and function for embryonic and early larval development, can also serve as an important energy source during embryonic development, especially marine species (Rosa et al., 2007).

In general, the newly hatched larva, containing a lower total lipid and FA level, has less independence of lecithotrophic nutrition and largely dependent of external energy sources. However, those with higher total lipid and FA content, are more independence from the external environment while rich-endotrophy of those larvae appears to support the growth and survival of early stages (Wehrtmann and Kattner, 1998).

1.2.2 Membrane structure and functions

Phosphoglycerides and their fatty acid compositions have a major and well-established role in maintaining the structure and function of cellular biomembranes (Harrison, 1990; Coutteau et al., 1997). Although metabolism is a very dynamic situation, the membrane phosphoglyceride and fatty acid compositions are relatively more stable than triacylglycerol compositions (Teshima, 1997). Although there are few data that directly demonstrate a clearly defined role for specific fatty acids in membrane functions in crustacean, the importance of 22:6n-3 in neural tissues of all vertebrates and invertebrates, including crustacean and fish, has recently been the subject of increasing interest (Tocher, 2003b; Lavar ás et al., 2006). Thus, in crustacean, dietary deficiency of 22:6n-3 resulted in low hatchability of eggs for many species (Cahu et al., 1994; Xu et al., 1994b; Wen et al., 2002), and decrease larval survival accompanied with high incidence of ing death syndrome (MDS) (Takeuchi et al., 1999b; Suprayudi et al., 2004a). Recent studies have

demonstrated a critical role for 22:6n-3 in the functioning of neural tissue (especially brain and eye) and the importance of dietary 22:6n-3 in marine fish (Masuda et al., 1998; Tocher, 2003b). Unfortunately, there is no research in this area for crustaceans.

Although essential for membrane function, PUFA are also very susceptible to attack by oxygen (oxygen radicals) and other organic radicals. The resultant oxidative damage to PUFA in membrane phosphoglycerides can have serious consequences for cell membrane structure and fluidity, with potential pathological effects on cells and tissues. Crustacean cellular membranes with their uniquely high levels of n-3 HUFA that are constituently vital are at particularly risk from oxidative attack and damage (Chapelle, 1977; 1978; Kong et al., 2006). Although the mechanisms, consequences, and nutritional implications of lipid and fatty acid peroxidation and antioxidant protection in fish have been intensively studies for a long period (Sargent et al., 2002), only a couple of recent publications have reported on and elucidated the possible roles that endogenous enzyme mechanisms may have in protecting membrane HUFA from oxidative damage in crustacean species (Kong et al., 2007; Kong et al., 2008).

The protective enzyme system comprises a series of enzyme scavengers of oxyradicals and other free radicals, such as Superoxide dismutase (SOD), peroxisomes and Glutathione-*S*-transferases (GST) (Kong et al., 2008; Lavarías et al., 2011). Studies in which these enzyme activities have been measured in fish have often focused on their role in pollutant detoxification (Martínez-Lara et al., 1996; Lavarías et al., 2006). Feeding high HUFA diets resulted in signs of increased peroxidative stress in juvenile marine fish and the Chinese mitten crab, *E. sinensis* as evidenced by increased levels of tissue lipid peroxidation products, and some moderate effects on liver antioxidant defense enzyme activities have been reported (Tocher, 2003a; Liu, 2005). In a recent study using diets containing very high levels of n-3 HUFA and preoxidized oil, the activity of the enzyme defense system was clearly increased by feeding oxidized oil and reduced by supplementing the diets with vitamin E in sea bream (Mourente et al., 2002) as well as in the black tiger prawn *Penaeus monodon* (Laohabanjong et al., 2009).

1.2.3 Eicosanoids production

It is well-known that PUFA can be metabolized to produce highly bioactive molecules, which is central to a major functional role of HUFA by dioxygenase enzyme-catalyzed oxidation. As these highly bioactive molecules derivatives are derived primarily from the C-20 PUFA, such as 20:3n-6, 20:4n-6, and 20:5n-3, they are termed eicosanoids. The two main enzymes involved in this process are cyclooxygenase, that produces cyclic oxygenated

derivatives or prostanoids, including prostaglandins (PG), prostacyclins (PGI) and thromboxanes (TX), and lipoxygenases, that produce linear oxygenated derivatives, including hydroperoxy- and hydroxy fatty acids, leukotrienes (LT), and lipoxins (LX) (Yang et al., 2002). The eicosanoids are hormone–like compounds produced by cells to act in their immediate vicinity with a short half-life. Every tissue in the body produces eicosanoids and have a wide range of physiological actions, for example, in blood clotting, the immune response, the inflammatory response, cardiovascular tone, renal function, neural function, and reproduction (Muskiet et al., 2006). Recently, specific eicosanoids, including LTB4 and 15-deoxy $\Delta^{12,14}$-PGJ2, have been postulated to be the natural ligands for a group of nuclear hormone receptors termed peroxisome proliferator-activated receptors (PPAR) that have established functions in the regulation of lipid metabolism in mammals (Forman et al., 1995; Kliewer et al., 1997). The pathway from extracellular stimulus to the production of eicosanoids forms a cascade termed the "arachidonic acid cascade" as arachidonic acid (20:4n-6) is the primary precursor fatty acid in mammals. Briefly, activation of cell surface receptors results in the production of free precursor acid either *via* phospholipase A2 activity or *via* the sequential action of phospholipase C and diacylglycerol lipase. The activation of phospholipase A2 may occur through elevation of intracellular Ca^{2+} (it is activated *in vitro* by high Ca^{2+} levels) or it may be regulated by a G protein, as phospholipase C appears to be in the phosphoinositide cycle (Burgoyne et al., 1987).

The subsequent pathways for the synthesis of individual eicosanoids from fatty acid precursors are complex with many different steps (Smith, 1989). Due to the obvious differences in the C20 PUFA composition of the phosphoglycerides between mammals and crustacean, there has been considerable interest in the general production of eicosanoids in crustacean. Although there are many valuable information on the pathways for the synthesis of individual eicosanoids in fish (Tocher, 2003b), there is only very limited information on the pathways for the synthesis of individual eicosanoids and their functions for crustaceans (Reddy et al., 2004; Meunpol et al., 2010). In mammals, 20:4n-6 is the chief precursor of the eicosanoids, generating 2-series prostanoids and 4-series leukotrienes. However, 20:5n-3 competes with 20:4n-6 in eicosanoid production, and is itself converted to 3-series prostanoids and 5-series leukotrienes, which are generally less biologically active than the corresponding 2-series prostanoids and 4-series leukotrienes produced from 20:4n-6 (Abayasekara, 1999). Thus, eicosanoid actions are determined by the ratio of 20:4n-6:20:5n-3 in cellular membranes, this in turn being determined by the dietary intake of n-6 and n-3 PUFA (Figure 1.1). In fish and mammals, eicosanoid production is influenced by the cellular ratio of 20:4n-6:20:5n-3, an imbalanced ratio of 20:4n-6:20:5n-3 appears to be as damaging in fish as in mammals (Abayasekara, 1999). Although for the vast majority of fish, their membrane phosphoglycerides contain a large excess

of 20:5n-3 relative to 20:4n-6, the chief source of eicosanoids in fish is 20:4n-6 where it is concentrated in phosphoinositides, and thus it is possible to conclude that the phosphoinositides are the origin of the 20:4n-6 used to produce eicosanoids in fish (Tocher, 2003). Dietary supplementation with 20:5n-3, as fish or fish oil, can be beneficial by damping down excess eicosanoid production from 20:4n-6 for mammals (Yang et al., 2002). It has also been established in fish that 20:5n-3 and dihomo-γ-linolenic acid, 20:3n-6, which can also serve as a substrate for fish cyclooxygenase enzymes, competitively depress the production of eicosanoids from 20:4n-6, as does 20:4n-3 (Bell et al., 1994; Tocher et al., 1997). Other than reproduction, in which the roles of eicosanoids in fish are well known and have been comprehensively reviewed, but there are few studies on the functions of eicosanoids in crustacean (Tahara and Yano, 2003; Reddy et al., 2004; Meunpol et al., 2010).

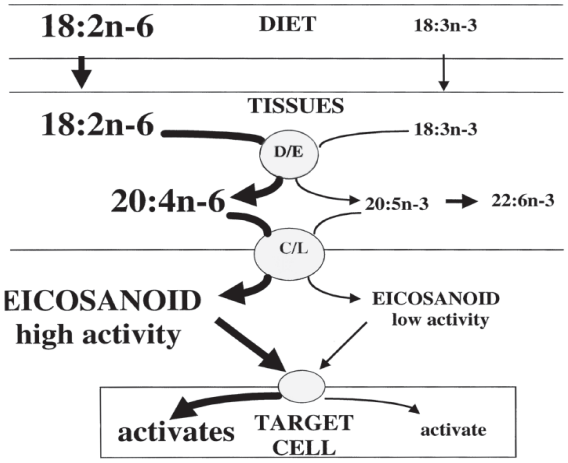

Figure 1.1 Links between dietary PUFA, tissue HUFA and eicosanoid production. Arachidonic acid, 20:4n-6, and eicosapentaenoic acid, 20:5n-3, obtained from the diet or be produced by desaturation and elongation of dietary 18:2n-6 and 18:3n-3, compete for the same cyclo-oxygenase and lipoxygenase enzymes (C/L) to produce, 2-series prostanoids and 4-series leukotrienes, and 3-series prostanoids and 5-series leukotrienes, respectively. Therefore, the ratio of 20:4n-6:20:5n-3 determines the ratio of high activity: low activity eicosanoids (From Tocher, 2003).

1.3 Larval digestion, absorption, storage and transport of lipids

The digestive process in crustaceans is complicated (Ceccaldi, 1998). The processes involve a mechanical digestion, the secretion of digestive enzyme, chemical digestion, physiological variations of enzyme, hormonal control of enzyme synthesis and bacterial flora. The adult decapod crustacean digestive tract is divided into three main parts: fore, mid, and hindgut. Generally, the foregut is composed of the small esophagus and a large part of the stomach, where the masticating parts are present. The hepatopancreatic mass of the midgut

consists primarily of hundreds of tubes. It is the secretory site of digestive enzymes and nutrient absorption (Dall and Moriarty, 1983). The midgut is not lined with chitin while the hindgut is nearly straight and chitin is present. It is enlarged posteriorly into the rectum and terminates at the anus (Ceccaldi, 1997). Here, we attempt to clarify the functions of larvae digestive system, ontogenetic development of the digestive tract, lipid digestion, absorption, storage and transport.

1.3.1 Digestion and absorption

Studies on organogenesis of the digestive tract of crustacean are scarce (Zhu et al., 2000). In the majority of the Crustacea, the foregut (stomadaeum) and hindgut (proctodaeum) are differentiated very early in embryogenesis (Wu et al., 2009). After hatching, the simple digestive tract becomes more complex. The newly hatched nauplii of shrimp have a nonfunctional digestive tract with a mouth but no anus. The anus opens after 2 or 3 moults. Initially straight, the digestive tract progressively acquires movement and enzymatic activities. The fore part widens into a sack that differentiates in two side pockets. The tract is progressively transformed, including the section that gives rise to the gastric mill and hepatopancreas, until it resembles the tract of the adult. Among the nauplius forms, complete differentiation of the internal organs is not complete until nauplius 6. At the zoea and mysis stages, four lobes of digestive tubules appear laterally to the future stomach. The midgut caecum appears also during these particular larval stages (Ceccaldi, 1997). For the swimming crab *Portunus trituberculatus* larvae, the midgut gland shows further differentiation during the larval metamorphosis (Nakamura, 1990). This general scheme of organogenesis has been described in several crustacean species, including *Palaemonetes varians* (Le Roux, 1971), *Homarus americanus* (Factor, 1981), *Penaeus. japonicus* (Nakamura and Tsuru, 1987) and *P. trituberculatus* (Nakamura, 1990).

The crustacean mouth is generally associated with specialized buccal prehensile appendages, maxillula, maxilla, mandibula, and maxillipeds (Lemmens and Knott, 1994). In decapod crustaceans, there is the presence of “dents stomacales” (teeth of the stomach), which are the protrusions on the internal surface of the posterior of the cardiac stomach, are coated with a thick layer of chitin. As the function, these protrusions are often calcified and help smash the diet by moving of particular muscles located outside the wall. This is process is controlled by a group of characteristic nerves (Ceccaldi, 1998). In the brachyuran crabs, the anomaurans and macrourans development of the gastric mill occurs during larval metamorphosis (Nakamura, 1990). During the larval development, the number of tubules continues to increase during each step of the growth of the larvae and the juvenile (Zhu et al., 2000). The thin integument and tissues of the larvae allow observation of particle movements into the digestive tract. They

move alternately between the stomach and inside the lumen of each tubule, following the play of constricting waves. If the four first tubules branches are in the form of an X with the stomach in the center, particles pass from one tubule to the opposite one. When the number of tubules is high, it becomes very complex, named midgut gland or hepatopancreas (Factor, 1981).

The hepatopancreas is also a complex system, which produces several types of digestive enzymes. In crustacean, the hepatopancreas generally has four types of cells, including embryonic cell (E cell), blister-like cell (B cell), resorptive cell (R cell) and fibrillar cell (F cell). The later three cells are thought to originate from E cells through its mitosis and amitosis. R cells are responsible for the nutrient reserves of lipid, glycogen, calcium and the others. F cells are regarded as the primary sites for the biosynthesis and secretion of the digestive enzymes, while B cells mainly execute the absorptive and degrading function of nutrients from the hepatopancreas lumen by the process of pinocytosiss (Vogt, 1994). The diverse functions of the midgut gland are reflected in the different types of epithelial cells (Dall and Moriarty, 1983). Digestive enzymes can be produced by F and B cells. It is well known that triacylglycerol lipases and phospholipases are two family lipases for crustaceans, which can hydrolyze triacylglycerol and phospholipid (Ceccaldi, 1997). Then, the fatty acids will be absorbed by R cells or transported to other tissues, such as muscle (Ceccaldi, 1998). During the larval development, the lipase activity generally increase from early stage to late stage (Pan and Wang, 1997).

The digestibility always varies among the species, lipid source and fatty acids. Generally, crustaceans have a high digestibility on neutral lipids and phospholipids (Merican and Shim, 1996b). However, the different digestibilities could be found on different fatty acids. For example, *Penaeus monodon* has a lower digestibility for 16:0 and 18:0 than 20:5n3, 20:4n6 and 22:6n3 (Glencross and Smith, 1999b). A previous study also suggested that digestibility of saturated fatty acids decreased with increasing chain length while the digestibility of mono-unsaturated fatty acids increased with the chain length of those fatty acids (D'Abramo, 1997). Digestibilities of SFA were believed to be more efficient when it is in free fatty acids form rather than bind together with glycerol (Leger, 1985). It is possible that free fatty acids can be directly utilized instead of glycerol compounds which need to be hydrolysis before it can be used by crustaceans. However, this finding is contrast with the results on *P. monodon* which showed that apparent digestibilities of the same species on triacylglycerols and free fatty acids does result in any significant differences, but that the shrimp fed the diets supplemented with triacylglycerols has better growth performance than those fed free fatty acids (Glencross and

Smith, 1997). Furthermore, digestibility of individual fatty acids appears to be influenced by the presence of other fatty acids (Glencross and Smith, 2001).

1.3.2 Storage and transport

The hepatopancreas is not only for the digestion but also a main storage organ for nutrients, especially for lipid (Yao et al., 2008). Most of the lipid is stored in R cells, in the form of lipid droplets. These reserve cells also contain copper granules, zinc, sulfur, and other less-abundant elements for many crustacean species (Vogt, 1994). The concentration of those metals varies with the stage of inter, reaching their maximum level during the period of intermoult to premoult stage (Heath and Barnes, 1970; Teshima et al., 1977). The storage of material and its reuse immediately after exuviation to re-constitute new tissues is a major factor in crustacean physiological equilibrium (Dall, 1981). The quality of the reserves may play an important role in maturation, growth of the ovary, quality of egg and larvae, and the viability of the larvae (Castille and Lawrence, 1989). Before moulting, crustaceans always reserve high level nutrients in the body, especially in the hepatopancreas. As a result, the body water content will decrease form 70-80% at post stage to 60-65% at early pre stage (Freeman et al., 1987). However, after moulting, the hepatopancreas lipid level will decrease dramatically (Teshima et al., 1977; Jeckel et al., 1990). Neutral lipids are main deposited lipid in hepatopancreas while phospholipids are involved in emulsification to transport lipid from the hepatopancreas to the other tissues (Coutteau et al., 1997; Teshima, 1997). After moulting, lipid is transported to the muscle to the hypodermis but prior to the next ing, the hepatopancreas will again accumulate neutral lipid to support the coming moulting (Jeckel et al., 1990).

It is well known that lipid transport depends on lipoproteins in the haemolymph (Ravid et al., 1999). The most important lipoproteins are high-density lipoprotein (HDL) and very high-density lipoprotein (VHDL) for the transport of lipids. Using radio-labelled C14 lipid, O'Connor and Gilbert (1968) found in *Gecarcinus lateralis* that there is an increase in the specific activity of lipid in hemolymph following destalking. Despite increasing specific activity of lipid, the total lipid level in the hemolymph will decrease after eye stalk ablation. The ratio of the specific activity of hepatopancreatic lipid to the specific activity of lipid in hemolymph was found to be a measure of the relative rate of release of the 14C-lipid from the hepatopancreas into the incubation-medium (O'connor and Gilbert, 1968). The fact that this ratio is higher in destalked animals indicates that the hepatopancreas of destalked animals synthesizes lipid at a greater rate than controls. It is possible that the increase in synthesis of lipids in the early stages of pre is not compensated by a more rapid release of lipid into the hemolymph (Chandumpai et al., 1991). In the late premoult stages, there is a decrease in the rate at which lipid is synthesized

and an increase in the rate at which it is released, resulting in a net decline in the content of lipid in the hepatopancreas (Heath and Barnes, 1970).

During ovarian development, lipid transport is more complicated than simple growth. Hall et al. (1995) showed that male HDL/BGBP contains an average of 47.6 % (w/w) lipids, while VHDL (or clotting protein) contains 11.4 % of lipids and 0.2 % of haemocyanin. They proposed that the clotting protein and haemocyanin may play a significant role in lipid transport in crayfish (Hall et al., 1995). The HDL fraction of *P. vannamei* has been reported to carry substantial amounts (52–80 %) of the lipids found in the haemolymph (RuizVerdugo et al., 1997; Yepiz-Plascencia et al., 2000). Previous study have suggest that PLs in the haemolymph are possibly the main source of PLs and TAGs in the ovary (Teshima and Kanazawa, 1983; Shafir et al., 1992), but it is not clear whether TAGs in the haemolymph are the source of TAGs in the ovary for crustacean (Ravid et al., 1999). However, the measurement of a lipid turnover rate is complicated by the occurrence of intra-ovarian lipid synthesis and by the possibility of additional synthesis in the hepatopancreas and oxidation of lipids in all tissues.

1.4 Catabolism and biosynthesis (embryonic stages and larvae)

1.4.1 Fatty acid catabolism

Fatty acid catabolism is the major source of energy by far in many crustacean species (D'Abramo, 1997). The biosynthesis of fatty acids occurs in the cytosol microsome whereas the catabolism of fatty acids occurs in the cellular organelles, including the mitochondria (and peroxisomes) via a completely different set of enzymes (Iraz ú et al., 1992; Lavar ás et al., 2006). The process is termed β-oxidation and involves the sequential cleavage of two-carbon units, released as acetyl-CoA, through a cyclic series of reactions catalyzed by several distinct enzyme activities rather than a multi-enzyme complex. Briefly, activated fatty acids are transported into the mitochondrion in the form of fatty acylcarnitine esters formed through the action of carnitine acyltransferase, converted back into fatty acyl-CoA derivatives, and then undergo a round of dehydrogenation, hydration, second hydrogenation, and cleavage steps to produce acetyl-CoA and NADH (Kallapur et al., 1983a; Kallapur et al., 1983b). The acetyl-CoA can then be metabolized via the tricarboxylic cycle to produce more NADH. The NADH produced from the oxidation of fatty acids can then provide metabolic energy in the form of ATP through the process of oxidative phosphorylation. Under certain conditions such as fasting, acetyl-CoA can be exported from the liver in the form of ketone bodies, acetoacetate and 3-hydroxybutyrate, which are used by peripheral tissues as fuel through oxidation back to acetyl-CoA (Kondrup and Lazarow, 1985). During the last two decades, the key processes of mitochondrial β-oxidation and ketone body formation have been established in crustacean, based on the results from the

shrimp *Macrobrachium borellii* (González-Baro et al., 1990; González-Baró and Pollero, 1993; González-Baró and Pollero, 1998; González-Baró et al., 2000; Lavarías et al., 2006; Lavarías et al., 2007). Although hepatopancreas is a centre of lipid metabolism, fatty acid oxidation could be found in several crustacean tissues, including heart, gill, hepatopancreas and muscles (González-Baro et al., 1990; Irazú et al., 1992). Recent studies have demonstrated peroxisomes are also another site of β-oxidation for crustaceans (González-Baró et al., 2000) as well as mammals (Lazo et al., 1988).

β-oxidation of PUFA is variable depend on the structure of different PUFAs, which is be more complicated that of SFA and MUFA (Tocher, 2003). In mammals, 20:5n-3 is readily β-oxidized by mitochondria and can actually, induce the formation of mitochondria in rats (Madsen et al., 1999). However, catabolism of 22:6n-3 requires peroxisomal β-oxidation. This is because, as insertion of the Δ4 ethylenic bond in 22:6n-3 requires a special mechanism (described above), so does its removal. Thus, the initial 2, 3 (α, β) dehydrogenation of 22:6n-3, the first step in the β-oxidation of all fatty acids, requires that it be followed in the case of 22:6n-3 by an NADPH–dependent 2, 4-dienoyl CoA reductase and then by a 3*cis*–2*trans* isomerase so as to enable the completion of oxidation of its β-carbon (Madsen et al., 1999; Tocher, 2003). These processes, which are well evolved in peroxisomes, have yet to be studied in crustacean.

The key enzymes involved in fatty acid catabolism, such as microsomal palmitoyl-CoA synthetase and mitochondrial palmitoyl-CoA synthetase, are affected by environmental temperature and the interaction of other fatty acids for crustaceans (Irazú et al., 1992; McKenzie et al., 2008). Therefore, it is very important and interesting to investigate the optimal temperature and fatty acid composition of these species to promote growth and reduce energy consumption (Querijero et al., 1997; Lavarías et al., 2006). In addition, different life stages of crustacean have variable ability of fatty acid oxidation as well as different tissue for the same species (González-Baró et al., 2000; Heras et al., 2000; Lavarías et al., 2006). For example, the embryonic pigmentation stage of the shrimp *Macrobrachium borellii* has stronger fatty acid oxidation than the other embryonic stages (González-Baró et al., 2000).

1.4.2 Fatty acids biosynthesis

The ultimate carbon source for the biosynthesis of new fatty acids is acetyl-CoA formed in mitochondria from the oxidative decarboxylation of pyruvate (carbohydrate source) or the oxidative degradation of some amino acids (protein source) (Kucharski and Dasilva, 1991). The key pathway in fatty acids biosynthesis is catalyzed by the cytosolic fatty acid synthetase (FAS)

multi-enzyme complex that occurs and has been characterized in crustacean (Li, 2005). The main products of FAS are the saturated fatty acids 16:0 (palmitic acid) and 18:0 (stearic acid), which can be biosynthesized *de novo* by all known organisms, including crustaceans (Kucharski and Dasilva, 1991; D'Abramo, 1997). Eight two-carbon acetyl units are required for the biosynthesis of 16:0 with one acetyl-CoA unit serving as a primer and the further seven acetyl units being carboxylated by acetyl-CoA carboxylase to malonyl-CoA before being combined via FAS in a series of sequential condensation steps requiring NADPH (Li, 2005).

Although crustacean can biosynthesize SFA *de novo*, most of them still need a certain level of SFA as an energy source and bioconvertion to MUFA (González-Baró and Pollero, 1993; Querijero et al., 1997), especially for marine crustaceans as they naturally consume diets rich in lipid. For example, the swimming crab always restores high lipid content in the hepatopancreas in the summer and autumn, which is related to the rich lipid of their food in this season (Yao et al., 2008). Therefore, marine crabs are not likely to biosynthesize fatty acids *de novo* to any significant extent. It is well known that the hepatopancreas is the principal site of fatty acids biosynthesis for crustaceans (Whitney, 1974). The rate of fatty acids biosynthesis is regulated by a number of dietary/nutritional factors. For example, dietary n-3HUFA themselves reduced FAS activity in hepatopancreas and muscle of the Chinese mitten crab *E. sinensis* (Li, 2005).

Similar to saturated fatty acids, all organisms, including crustacean, are capable of desaturating 16:0 and 18:0 to yield, respectively, 16:1n-7 (palmitoleic acid) and 18:1n-9 (oleic acid). Desaturation of fatty acids in crustaceans, like all animals, takes place in the endoplasmic reticulum of cells of particular tissues via an aerobic process utilizing CoA-linked substrates and requiring NAD(P)H and O_2, catalyzed by multi-component systems comprising NAD(P)H-cytochrome b5 reductase, cytochrome b5, and terminal desaturase enzymes (Brenner, 1974). This reaction is of particular physiological importance in that the monounsaturated products formed (16:1n-7 and 18:1n-9) have markedly lower melting points (phase transition temperatures) than their saturated precursors (16:0 and 18:0) (Querijero et al., 1997). Hence, Δ9 fatty acid desaturase provides a means of regulating the viscosity of cell membranes by altering the phase transition temperatures of the fatty acids in their constituent phosphoglycerides (Tocher, 2003). As MUFA have lower melting points than SFA, the crustacean tend to biosynthesis more MUFA at the lower temperature than those at the higher temperature (Farkas and Nevenzel, 1981).

Although in crustacea the hepatopancreas, muscle and gills are able to biosynthesis SFA and elongating them (from 16:0 to 18:0), only the hepatopancreas can desaturate palmitic and oleic acid to their correspondent MUFA (16:1n7 and 18:1n9, respectively) (Farkas and

Nevenzel, 1981; González-Baró and Pollero, 1993). Similar to the mammal liver, the crustacean hepatopancreas desaturates oleic acid more efficiently than palmitic acid (González-Baró and Pollero, 1993). A previous study has shown amino acids are the preferred carbon source for the biosynthesis of SFA and MUFA *de novo* (Kucharski and Dasilva, 1991). Obviously, biosynthesis of fatty acids at the expense of amino acids is not economic in terms of aquaculture profits. Therefore, supplementation of certain SFA and MUFA seems necessary for crustacean feeds (D'Abramo, 1997).

Similar to the other vertebrates, crustaceans lack Δ12 (ω6) and Δ15 (ω3) desaturases so they cannot form 18:2n-6 and 18:3n-3 from 18:1n-9 (D'Abramo, 1997; Merican and Shim, 1997b). Therefore, 18:2n-6 and 18:3n-3 are essential fatty acids in the diets of crustaceans (D'Abramo, 1997). These dietary essential fatty acids can be de-saturated further and elongated to form the physiologically essential C20 and C22 PUFA, such as 20:4n-6, 20:5n-3, and 22:6n-3 as shown in Figure 1.2 for some animals including freshwater fishes (Tocher, 2003). The degree to which an animal can perform these conversions is dependent on the relative activities of fatty acid elongases and desaturases, such as Δ6 and Δ5, in their tissues, and these activities in turn are dependent on the extent to which the species can or cannot readily obtain the end product 20:4n-6, 20:5n-3, and 22:6n-3 fatty acids preformed from their natural diets (Glencross, 2009). The tissues of both freshwater and marine crustacean are generally very rich in 20:5n-3 and 22:6n-3, and so the origins of these fatty acids are of particular interest. Some crustacean have ability to biosynthesis 20:5n-3 and 22:6n-3 from 18:3n-3, such as spider crab *Libinia emarginata* Leeth (Whitney, 1974), crayfish *Procambarus clarkii* (Farkas and Nevenzel, 1981) and black tiger prawn *P. monodon* (Merican and Shim, 1996) ; and the conversion of 20:4n6 from 18:2n6, Chinese mitten crab, *E. sinensis* (Wu et al., 2010a) and prawn *Penaeus setiferus* (Lilly and Bottino, 1981). However, the pathway of those biosynthesis and conversion is not clear for crustacean (González-Baró and Pollero, 1998; Wu et al., 2010a).

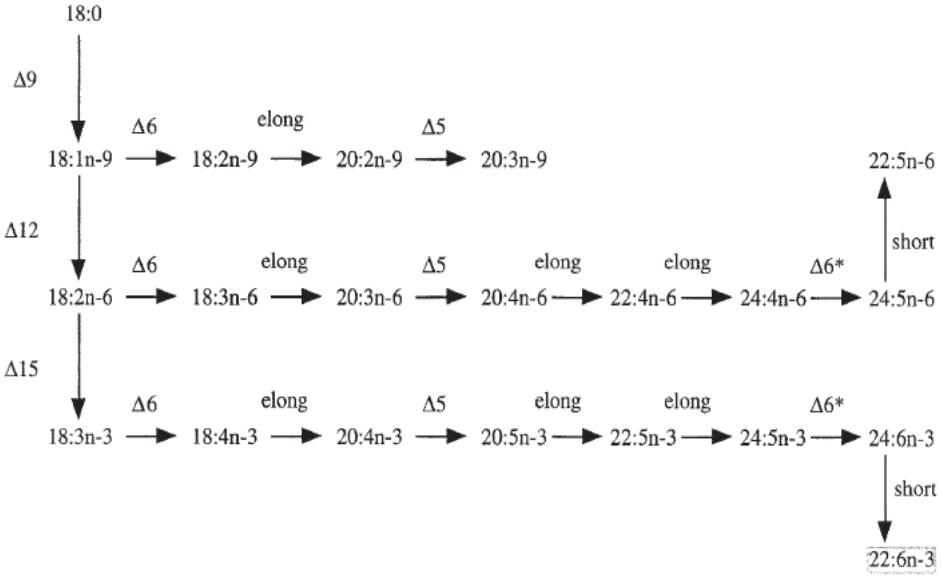

Figure 1.2 General pathways of biosynthesis of C20 and C22 HUFA from n-3, n-6 and n-9 C18 precursors. Δ5, Δ6, Δ6*, Δ9, Δ12, Δ15, Fatty acyl desaturases; Elong, Fatty acyl elongases; Short, chain shortening. Δ9 desaturase is found in all animals and plants whereas Δ12 and Δ15 desaturases are generally only found in plants and so 18:2n-6 and 18:3n-3 are "essential" fatty acids (EFA) for many animals including some crustacean species. Carnivores and many marine crustaceans generally cannot or have only limited ability to carry out the conversions above due to specific deficiencies in desaturases and/or elongases and so 20:5n-3 and 22:6n-3 are their EFA. The Δ6* enzyme acting on C24 fatty acids may or may not be the same enzyme (Δ6) that acts on C18 fatty acids (From Tocher, 2003).

For crustaceans, the reactions of de-saturation and elongation mainly occur in the microsomal and mitochondrial fraction of the hepatopancreas (González-Baró and Pollero, 1998). As the details in Figure 1.2 show there are many fatty acyl desaturases, elongases and shortenases in these biosynthesis pathways, including Δ5, Δ6, Δ9, Δ12, Δ15 series. As Δ12 and Δ15 desaturases are generally only found in plants 18:2n-6 and 18:3n-3 are "essential" fatty

Table 1.1 Summary of biosynthesis and conversion of PUFA in crustacean species

	Life stage	LNA→EPA	LOA→ARA	LNA→DHA	EPA→DHA	DHA→EPA	Reference
Freshwater species							
Macrobrachium borellii	Adult 1.0–1.2 g	No	No	—	—	—	González-Baró and Pollero (1998)
Macrobrachium rosenbergii	Juvenile 0.06-0.5g	No/very low	No/very low	No/very low	Yes	—	D'Abramo and Sheen (1993)
Macrobrachium rosenbergii	Pre-adult 6-16g	Yes	Yes	Yes	—	—	Reigh and Stickney (1989)
Procambaruss clarkii	Adult 50-70g	Yes	Yes	Yes	—	—	Farkas (1981)
Astacus astacus	Adult or pre-adult	Yes	Yes	Yes	—	—	Zandee (1966)
Eriocheir sinensis	Precocity 15-17g	—	Yes	—	—	—	Wu et al (2010a)
Marine species							
Penaeus monodon	Juvenile 0.1-0.4g	Very limited	Yes	No	Yes	Yes	Merican and Shim (1996, 1997)
Penaeus kerathurus	Adult 12.9 + 2.9g	Low ability	Yes	No	Yes Strong	—	Mourente (1996)
Penaeus setiferus	—	Very limited	—	Very limited	—	—	Lilly and Bottino (1981)
Penaeus japonicus	Larvae Z1-Z3	Yes	Yes	Yes	—	—	Jones et al (1979)
Penaeus japonicus	Juveniles —	Moderate ability	Moderate ability	Moderate ability	—	Yes/strong	Kanazawa et al (1979)
Mysid Gnathophausia	—	Yes	Yes	Yes	—	—	Morris and Sagent(1973)
Acanthepyra purpurea	—	Yes	Yes	Yes	—	—	Morris and Sagent(1973)
Nematobrachion sexspinosus	—	Yes	Yes	Yes	—	—	Morris and Sagent(1973)
Scylla serrata	Juvenile 0.06-0.7	Very limited	No	No	—	Yes	Sheen and Wu (2003)
Scylla serrata	Larvae Z1-Megalopa	Very low	Very low	Very low	—	—	Suprayudi et al (2004b)

Notes: "—" means not studied or no available information in the reference. LNA: 18:3n3; LOA: 18:2n6; ARA: 20:4n6; EPA: 20:5n3; DHA: 22:6n3.

acids (EFA) for many crustaceans. The pathways of 18:3n-3 to 22:6n-3 and 18:2n6 to 20:4n6 has been established for several fishes (see Figure 1.2) (Tocher, 2003). Unfortunately, no detailed research of PUFA biosynthesis has been elucidated for any crustacean. However, there is an extensive body of evidence based on direct feeding studies as well as on the conversion of radioisotopes administered *in vivo* that the conversion, including 18:3n-3 to 20:5n-3 and then to 22:6n-3, 18:2n6 to 20:4n6, do occurs in many freshwater crustacean species whereas the ability of this conversion is very poor for most marine decapod species (see Table 1.1). This difference is fundamental and very important to determine the dietary essential fatty acid requirements of crustacean (Mourente, 1996; Merican and Shim, 1997). It reflects the fact that 20:5n-3 and 22:6n-3 are very abundant in the marine environment, therefore, marine decapod crustacean have an ample supply of 20:5n-3 and 22:6n-3 in their natural diets (Lilly and Bottino, 1981; Saunders et al., 2012). In contrast, the natural prey of freshwater crustacean, particularly their invertebrate prey, is not rich in 22:6n-3, being rich instead in 18:2n-6, 18:3n-3 and to a lesser extent 20:5n-3 (Glencross, 2009). Thus, conversion of 18:3n-3 and 20:5n-3 to 22:6n-3 is necessary for freshwater crustacean (Farkas and Nevenzel, 1981; Reigh and Stickney, 1989b;

Wu et al., 2010a). Overall, most of marine decapod crustacean generally have only limited ability to carry out the above conversions due to the specific deficiencies of desaturases and/or elongases, and then 20:5n-3 and 22:6n-3 are their EFAs (Kanazawa et al., 1979a; Merican and Shim, 1996; Suprayudi et al., 2004b).

Gonzalez-Baro and Porello (1998) suggested the inability of some decapods to elongate PUFA to HUFA is because of an inactive enzymatic system of Δ6 and Δ5 desaturate activity. However, previous results on crustacean showed that their situation is a complicated complex varying with different life stage and different species, particularly in terms of their expressing some of the enzyme activities necessary to convert C18 PUFA precursors to C22 PUFA products. For example, although preadult *Macrobrachium rosenbergii* (6-16g) have ability to convert C-18 PUFA precursors to C-22 PUFA, juvenile shrimps do not have or only have very limited ability of these pathway (Reigh and Stickney, 1989b; D'Abramo and Sheen, 1993). The Δ5 fatty acid desaturase maybe the limiting desaturase for the conversion of C-18 PUFA to HUFA of *P. kerathurus* (Mourente, 1996) while the Δ6 is the first limiting factor for *M. borellii* (González-Baró and Pollero, 1998). Table 1.1 summarized the biosynthesis and conversion of C-18 PUFA to HUFA for some common crustacean species.

Recent studies have shown that the Δ5 and Δ6 fatty acid desaturase gene and C18-C20 and C20-C22 elongase gene(s) are well established in almost all animals (Hastings et al., 2001; Monroig et al., 2013). The apparent inability of marine crustacean to convert 18:3n-3 to 20:5n-3 and 22:6n-3 is not due to the complete absence of the required genes in a particular species but to one or more of the required genes not being sufficiently well expressed (Tocher, 2003). Therefore, the problem may be how to switch on the recalcitrant genes rather than to introduce them by genetic engineering. Very recently, fatty acid elongase and desaturase genes have been cloned from Chinese mitten crab, *E. sinensis* (Guo et al., 2013; Yang et al., 2013), but it is unclear for the function of these genes and their enzymes in the fatty acid pathway.

It is very interesting that crustaceans and fishes, having the conversion ability of C18 to C20, always contained lower n-6 HUFA (ARA) than n-3 HUFA (DHA and EPA), which suggest that the fatty acid elongases and desaturase enzymes have stronger effect on n-3 HUFA substrates than n-6 HUFA substrates/precursors (Agaba et al., 2005; Glencross, 2009; Wu et al., 2010a). Clearly, with the development of new molecular technologies, more intensive investigation in greater depth should be conducted to understand those pathway and molecular mechanism.

1.5 Fatty acids requirements and their interactions

1.5.1 Study history and their limitations of larvae and juvenile crustacean

Although the study of crustacean nutrition started in the 1960s, the studies of fatty acids requirements were not conducted until the late 1980s. With the development of formulated diets, especially microbound diets, most of diets defined lipid composition was initially used to investigate fatty acids requirements for juvenile *Penaeus japonicus* (Kanazawa et al., 1978; Kanazawa et al., 1979b; Kanazawa et al., 1985b). However, leaching loss of dietary nutrients into the water was an obstacle to the development of robust conclusions on nutrient requirements of shrimps, particularly the larvae and early juvenile (Teshima et al., 1993; Jones, 1998; Teshima, 1998). Therefore, after 1980s, microencapsulated diets were used to investigate the nutrition requirement or as a replacement for live food for larval/early juvenile crustacean (Jones et al., 1987; Le Vay et al., 1993).

Although total replacement of live feeds is routinely accomplished in the laboratory for penaeids, it has been less successful in large commercial scale for many crustacean larvae (Jones, 1998; Holme et al., 2009a). With the consideration of practical application for aquaculture hatcheries, most of fatty acids researches were still conducted to compare the fatty acid composition of different live food or different enrichment emulsions as well as their effects on gross performance for larvae or early juvenile (Rees et al., 1994; Nghia et al., 2007; Sui et al., 2007). Although the micro-particle diets is not very successful in completely replacing live foods on commercial scales, the development of microparticles (MBD and MED) as feed for crustaceans has facilitated research into the identification of fatty acid requirements (Jones, 1998; Holme et al., 2009a).

As purified fatty acids (in the type of free fatty acid and triacylglycerol) are very expensive, most of research has been conducted only based on different lipid sources (vegetable oil and animal oil) or different ratios of them (González-Félix et al., 2002a; González-Félix et al., 2002b; Holme et al., 2007b; Holme et al., 2009c). It is well known that there are strong interactions of dietary different fatty acids; therefore, it is difficult to draw the precise conclusion of fatty acids requirements from those publications (D'Abramo and Sheen, 1993; D'Abramo, 1998; Glencross et al., 2002). Therefore, future experimentation leading to the precise determination of fatty acid requirements should be directed toward the provision of graded levels of pure fatty acids in a triglyceride or methyl ester form, rather than through the provision of oils rich in the fatty acids under investigation (D'Abramo, 1998).

1.5.2 Requirement criteria

The criteria used to measure the requirement for fatty acids has generally been only used gross performance indicators, such as survival, growth and inter period (Reigh and Stickney, 1989b; D'Abramo and Sheen, 1993; Glencross et al., 2002; Wu et al., 2007c). Thus, in most studies, the larval/juvenile diets supplemented with appropriate level of EFA, especially HUFA, has been reported to not only increase growth and survival, but also reduce inter period and "moulting death syndrome" (MDS) (Holme et al., 2007b; Sui et al., 2007). Except for the analysis of the dietary fatty acids, it is really important and necessary to analyse the lipid composition of crustacean tissue when complement feeding trial and prior to the initiation of an experiment (D'Abramo, 1997). Comparing tissue and dietary fatty acid composition, particularly for potential EFAs, should assist the identification of EFAs, biosynthetic pathways of fatty acids and possible indicator of an EFA deficiency (Glencross and Smith, 2001; Sheen and Wu, 2002). Until 1990s, a limited number of studies have also looked at stress resistance, such as osmotic and temperature shock and those results had shown that appropriate diet EFA levels could increase the stress resistance by optimization of membrane fatty acid composition (Rees et al., 1994; Smith et al., 2004; Sui et al., 2007). Very recently, physiological, metabolic, biochemical or molecular parameters have been investigated to the studies of crustacean nutrition (Guo et al., 2013). Although feed conversion efficiency (weight gain/apparent feed intake, FCR) are important to evaluate animal nutritional requirements, it is very difficult to apply this criteria for larval and juvenile crustacean (Jones, 1998). This is because it is difficult to collect uneaten diet and exclude feces from the uneaten feed and therefore, it is not possible to obtain robust FCR data parts of the larvae and juveniles (Teshima, 1998).

1.5.3 Saturated and Monounsaturated Fatty Acids

Although a high proportion of SFA and MUFA could be found on crustacean tissues including muscle, hepatopancreas and gonads, only little attention has been given to the requirements of SFA and MUFA for crustaceans (D'Abramo, 1998; Teshima, 1998). This is partially because crustaceans have the ability to synthesize saturated fatty acids and possess desaturase and elongatase enzymes that can convert SFA to MUFA (D'Abramo, 1997). In fact, the percentage of SFA and MUFA in the total fatty acids and the compositions are important to the growth and feed conversion efficiency (weight gain/apparent feed intake, FCR) for crustacean (Teshima, 1998). In generally, compared to PUFA, SFA and MUFA are considered as preferred energy sources for crustacean (Querijero et al., 1997) while PUFA was preferentially conserved as they play more roles in cell structure. However, the preferred utilization and digestibility of those SFA and MUFA as energy source maybe vary from species

to species (Glencross and Smith, 2001). A previous study has confirmed that dietary 16:0 and 18:0 are energy source responsible for good growth of *M. rosenbergii* (D'Abramo, 1998). The melting point is one of the factors affecting the nutritive value of dietary lipids as energy sources (Teshima, 1998). Studies on *P. monodon* illustrated that SFA digestibilities in various oils decreased with increased chain length ranging from 95% for 14:0 to 66% for 18:0, while the digestibilities of MUFA increased with increasing chain length (Merican and Shim, 1994). However, excessive lipid content (beyond the optimal total lipid content) will depress the digestibilities of fatty acids (Glencross and Smith, 2001).

Recently, the replacement of fish oil with plant oils has been a priority in aquaculture nutrition, but less attention have been given to the understanding of the balance of dietary SFA: MUFA: PUFA ratios (Holme et al., 2007a). However, lowering or replacing the percentage of fish oil affects the balance of dietary fatty acids, particularly the ratio of SFA: MUFA: PUFA (Teshima, 1998). Our recent research on juvenile Chinese mitten crab, *E. sinensis* has shown that when diet is lacking in 18:1n9, the juvenile *E. sinensis* will absorb/biosynthesis and accumulate high 16:0 in the hepatopancreas for energy provision, especially during ing (Wu et al., 2011). Moreover, the use of short-chain fatty acids (SCFA, i.e. 2:0, 4:0), medium-chain triacylglycerols (MCT) containing 6:0, 8:0, 10:0, and 12:0 as alternative sources of energy have been studied in fish (Tocher, 2003). Some benefits, such as improved growth, high digestibility and low muscle lipid deposition could be found for some fish species (Fontagné et al., 2000). However, no similar studies have been carried out in this area for crustaceans. In addition, providing sufficient amounts of SFA and MUFA in the diets is particularly important in reducing oxidation of the diets (Watanabe, 2002). Based on this review, there is little information available on the SFA and MUFA requirement and their functions for crustacean, it is clearly desirable that more attention should be paid on the importance in the nutrition of the SFA and MUFA, in future studies.

1.5.4 C18-Polyunsaturated Fatty Acids

Compared to SFA and MUFA, PUFA in crustaceans has been studied intensively during the past 40 years. PUFA can be divided into groups according to the number of carbons and double bonds, C18-PUFA (with or more than two double bonds, with 18 carbons) and HUFA (with or more than three double bonds, with or more than 20 carbons). C18-PUFA that consist of linolenic acid (18:3n3, LNA) and linoleic (18:2n6, LOA) acid has been recognized as an important nutrient for crustaceans (Sheen and Wu, 2003). Deficiencies of PUFA in the diets will lead to detrimental effects in crustacean growth (Read, 1981; Merican and Shim, 1996b). Generally, freshwater and estuary crustacean may need more C18-PUFAs (LNA and LOA)

compared to marine species as their natural food typically contains high level of those fatty acids (Read, 1981; D'Abramo and Sheen, 1993; Wu et al., 2007b).

The dietary essentiality of LNA and LOA for crustaceans is variable among the different species and the importance of providing sufficient amounts of these fatty acids is debated (D'Abramo and Sheen, 1993; Merican and Shim, 1997b; Glencross and Smith, 2001). For most penaeids and marine crabs, many experiments suggested that LNA has greater nutritional value

Table 1.2 Summary of requirements of dietary LNA and LOA levels for crustacean larvae and juveniles

Species	Stage	Fatty acid form and contents	LNA	LOA	Value	Criteria	Feeding period	Reference
Penaeus aztecus	Juvenile	Purified FA	1%	—	—	G	--	Shewbart and Mies (1973)
Marsupeaeus japonicus	Juvenile	Purified FA	1%	1%	LNA>LOA	G, S	--	Kanazawa et al (1979)
Penaeus indicus	Juvenile (0.35-2.23g)	Purified FA, 0- 2%DW	0.5%	0.5%	LOA>LNA	G, S, BFA	4 weeks	Read (1981)
Penaeus chinensis	Juvenile (0.37-1.62g)	Purified FA, 0 and 1%DW	1%	1%	LNA>LOA	G, S, MF, BFA	32 days	Xu et al. (1993)
Penaeus chinensis	Juvenile (0.37-1.62g)	Purified FA LOA: 0-1%DW; LNA: 0-1%DW	0.7-1.0%	0.5%	LNA>LOA	G, S, MF, BFA	60 days	Xu et al. (1994)
Macrobrachium rosenbergii	Juvenile (0.06-0.5g)	Purified FA LOA: 0-0.6%DW; LNA: 0-1.2%DW	0.45-0.6%	0.6%	LNA=LOA	G, S, MF, BFA	150 days	D'Abramo and Sheen (1993)
Litopenaeus vannamei	Juvenile (0.37-3.11g)	Purified fatty acids LOA: 0-1%DW; LNA: 0-1%DW	0.5-1%	0.5-1%	LNA>LOA	G, S, BFA	6 weeks	Gonz ález-F élix et 2003a
Penaeus monodon	Juvenile (0.07-0.37g)	Purified FA LNA: 0-3%DW, without HUFA	2.5%	—	—	G, S, BFA	4 weeks	Merican and Shim (1997)
Penaeus monodon	Juvenile (1.87-5.85g)	Vegetable oil and Purified FA LOA:0.29-1.47%DW;LNA: 0-1.16%DW	0.88%DW	0.58%	LNA>LOA	G, S, BFA	50 days	Glencross and Smith (1999)
Eurypanopeus depressus	Larvae to megalopa	Rotifer and *Artermia* enriched with purified FA; 0, 1% of LNA and LOA, respectively.	1%	1%	LNA=LOA	S, DP	22days	Levine and Sulkin (1984)
Scylla serrata	Juvenile (0.06-0.78g)	Purified fatty acids LOA: 0-0.2%DW; LNA: 0-0.2%DW	0.2%	0.2%	LNA>LOA	G, S, BFA, MF	10 weeks	Sheen and Wu (2003)
Scylla serrata	Larvae to megalopa	Rotifer enriched with purified FA LNA: 1.35-2.85%DW; LNA:0.48-1.49%DW respectively	1.49%	2.85%	LNA=LOA	S,DP, CW, BFA	20 days	Suprayudi et al (2004)

Notes: %DW: % of diet dry weight; %TFA: % of total fatty acids; S: survival; G: growth; BFA: body/tissue fatty acid analysis; SR: stress resistance; MF: ing frequency; FC: Feed conversion; DP: development period; CW: carapace width.

compare to LOA (Read, 1981; Xu et al., 1993; Xu et al., 1994b; Lim et al., 1997; González-Félix et al 2003a). In *P. chinensis* comparison of 1% LNA and 1% LOA included in the diets illustrated significant superior growth rate, moulting frequency and survival in shrimps fed diet containing LNA treatment (Xu et al., 1993). However, the experiments with *M. rosenbergii* gave contrast results: LOA have better nutritional values than LNA for this freshwater species (Reigh and Stickney, 1989; D'Abramo and Sheen, 1993). Dietary supplementation of LNA at 1.5-3% in the diet significantly increased survival and growth of juvenile *P. monodon* (Merican and Shim, 1997). For the other species, the nutritional values of LNA and LOA are similar and both of them can enhance growth (Read, 1981; Glencross and Smith, 1999). The LOA and LNA requirements and their importance are summarized in Table 1.2 for the crustaceans which have been studied.

Although there is no concrete experimental explanation for the biochemical aspects of LNA and LOA supremacy, it is noted that in freshwater crustaceans, e.g *M. rosenbergii* and *E. sinensis*, need predominantly n-6 fatty acids compared to marine species which have a preference for n-3 fatty acids (D'Abramo, 1997). Previous studies have also shown that freshwater crustaceans contain relatively higher levels of n-6 fatty acids than marine species (D'Abramo, 1998; Wu et al., 2010a). Marine crustaceans on the other hand, such as *P. chinensis*, show high utilization of LNA compare to LOA (Xu et al., 1993) and it has been shown that marine crustaceans utilized fish oil rich in n-3 fatty acids more efficiently than plant sources that are generally high in n-6 fatty acids (Lim et al., 1997). Overall, it is possible that the difference of PUFA requirement among this species is related to their feeding habits and physiological adaptation to their surroundings.

Comparisons between the HUFA and C18-PUFA studies indicated the importance of dietary HUFA. Sheen and Wu (2002) pointed out that the mud crab *S. serrata* fed diets containing HUFA (DHA, ARA and DHA + ARA) gave better performance, up to 249% weight gain, compared to mud crabs fed diets containing LNA or LOA. Similar results were obtained in a previous study on *P. monodon* (Merican and Shim, 1997). Although HUFA appears to be superior to PUFA, juvenile *P. monodon* fed a diet containing pure free fatty acid of 22:6n-3 gave inferior weight gain compared to shrimps fed diets with cod liver oils that rich in PUFA and HUFA (Merican and Shim, 1996). It suggests that the optimal balance of fatty acids in the diets is more important compared to addressing the fatty acids individually. Interactions between fatty acids may play very important roles rather than the function of each of them (Glencross et al., 2002; Sheen and Wu, 2002).

1.5.5 Highly Unsaturated Fatty Acids

Generally, almost of all decapod crustacean larvae need a certain dietary HUFA, but the HUFA requirement of freshwater crustaceans appears to be approximately one tenth of the level suggested for marine crustaceans (D'Abramo, 1997; D'Abramo, 1998). In addition, cold-water species may need higher dietary HUFA level than warm-water species (D'Abramo, 1997). Common HUFAs include 20:5n3, 20:4n6 and22:6n3, which are considered to have higher nutritional value than those C18-PUFAs (Xu et al., 1993; Xu et al., 1994a; Merican and Shim, 1996b; Suprayudi et al., 2004c). The total HUFA requirements of crustacean larvae and juveniles are summarized in Table 1.3. However, some crustacean species have the ability to convert C18-PUFA to HUFA (see section 1.4.2) and therefore the nutritional value of those two group fatty acids are variable among the different species (D'Abramo and Sheen, 1993; Merican and Shim, 1996; Wu et al., 2011b).

As n-3 HUFA, especially EPA and DHA, play many important physiological roles for crustaceans, such as the components of membrane phospholipids, precursors of biologically active eicosanoids and enhancement of neural development (Wouters et al., 2001; Sui et al., 2007; Sui et al., 2011), most of studies were focused on the n-3 HUFA requirement and their effect on larval or early juvenile crustaceans (Xu et al., 1993; Xu et al., 1994a; Nghia et al., 2007). Most of previous researches have shown that a diet deficient n-3 HUFA usually resulted in slower growth rate and high mortality, and prolonged intermoult periods for the larvae of many marine crustaceans species (Xu et al., 1994a; Suprayudi et al., 2004b). However, excessive amount of dietary n-3 HUFA also severely reduced growth and survival (Sui et al., 2007; Dan and Hamasaki, 2011). Kanazawa et al. (1985) observed that survival of *P. japonicus* increased with increasing dietary n-3 HUFA levels from 0-1% but decreased at 2% (Kanazawa et al., 1985). Depressed growth rate at high levels of n-3 HUFA may be related to metabolic response of shrimps to excessive HUFA levels (Yu and Sinnhuber, 1976). As HUFA is prone to oxidation, then overdose of HUFA or contamination by oxidation products of HUFA is harmful to crustacean (Kanazawa et al., 1985).

Table 1.3 Optimal HUFA requirements of crustacean larvae and juveniles

Species	Stage	FA form and contents	HUFA (%DW)	Total lipids (%DW)	Criteria	Feeding period	Reference
Macrobrachium rosenbergii	Juvenile 0.06-0.5g	Refined cod liver oil HUFA: 0-0.6%DW	0.15%	6.0%	G, S, MF, BFA	150 days	D'Abramo and Sheen (1993)
Litopenaeus vannamei	Juvenile 0.37-1.42g	Refined fish oil HUFA: 0-0.5% DW	0.25%	7.63%	G, S, BFA	6 weeks	Gonz âlez-F élix et al (2002a)
Penaeus monodon	Juvenile 0.05-0.40g	Purified DHA, ARA and EPA; HUFA: 0-1.1% DW	0.73%	7.43%	G, S, BFA	4 weeks	Merican and Shim (1996)
Penaeus monodon	Juvenile 1.87-5.85g	Vegetable/animal oil and Purified FA; HUFA: 0.3-1.95%	1.0%	7.5%	G, S, BFA	6 weeks	Glencross and Smith (2002)
Portunus tribuberculatus	Larvae to megalopa	Rotifer and *Artemia* enriched refined EPA and DHA oil	3.6-4.9%	25.1 - 26.8%	S, DP, CW, BFA	25 days	Takeuchi et al (1999b)
Scylla serrata	Juvenile 0.06-0.78g	Purified DHA and ARA	0.2%	3.8%	G, S, BFA, MF	10 weeks	Sheen and Wu (2003)
Scylla serrata	Larvae to megalopa	*Artemia* enriched with refined HUFA oil	1.96-2.91%	21.0 - 22.5%	S, DP, CW, BFA	20 days	Suprayudi et al (2004a)
Eriocheir sinensis	Larvae to megalopa	Rotifer and *Artemia* enriched refined HUFA	2.0%	15 - 20%	S, DP, CW, BFA, SR	25 days	Sui et al (2007)

Notes: %DW: % of diet dry weight; HUFA: highly unsaturated fatty acids; S: survival; G: growth; BFA: body/tissue fatty acid analysis; SR: stress resistance; MF: ing frequency; FC: Feed conversion; DP: development period; CW: carapace width.

EPA and DHA may have different physiological functions in larvae. For *P. monodon* (Merican and Shim, 1997), *P. trituberculatus* (Takeuchi et al., 1999a, b) and *Eurypanopeus depressus* (Levine and Sulkin, 1984), DHAs are more influential for larval ing and growth while EPAs are related to the improvement of survival. However, for larval *S. serrata*, dietary DHA can also affect survival (Suprayudi et al., 2004). Surprisingly, among the numerous studies on n-3 HUFA, only a limited amount of attention has been given to addressing quantitative requirements of EPA and DHA as individual components (Read, 1981; Merican and Shim, 1996; Gonz âlez-F élix et al., 2003). Among these publications, most of them focus on DHA (Merican and Shim, 1997; Mourente and Rodriguez, 1997; Sheen and Wu, 2003).

Although DHA is superior to EPA for many marine crustaceans, it is likely that critical ratios of DHA and EPA are vital for larval survival and growth (Takeuchi et al., 1999b; Sui et al., 2007). In addition, DHA also may be able to retro-convert itself to EPA e.g. *M. rosenbergii* (D'Abramo and Sheen, 1993) and *P. monodon* (Merican and Shim, 1996). It is likely that DHA is processed through the β-oxidation cycle and produces EPA (Kanazawa et al., 1979a). Therefore, dietary DHA/EPA ratio and their individual levels are likely important to crustaceans (Glencross et al., 2002; Sheen and Wu, 2002b; Nghia et al., 2007). A disproportion ratio of DHA/EPA in the diets will affect body physiological process and thus affect the growth of *P. monodon* as well as the experienced adverse effects of weight gain when fatty acids ratios were imbalanced (Glencross et al., 2002). It is likely to be important to supply appropriate levels of EPA and DHA for crustacean according to their physiological need (Sui et al., 2007). For juvenile *P. monodon*, the optimal ratio of DHA/EPA seems to be around 1 (Glencross et al., 2002).

The importance of n-3 HUFA in crustacean diets has been a focus for many years but has also resulted in neglecting the role of arachidonic acid (ARA; 20:4n-6) as an EFA. As a HUFA, ARA has a greater nutritive value compared to LNA (D'Abramo, 1997). Although there are few studies on the nutritional value and requirement of ARA in crustaceans, it is well known that ARA does have many important functions, being involved in moulting hormone dynamics and immunity (Bell and Sargent, 2003; Suprayudi et al., 2004a, b). The moulting process in crustaceans is regulated by eicosanoids from ARA (González-Félix et al., 2003b). Eicosanoids are hormones that are generated *in situ* when needed with prostaglandin as a product after ARA biosynthesis (Gurr and Harwood, 1991). In *Penaeus esculentus*, when injected with prostaglandin E_2, a type of eicosanoids, there is a shorter moult cycle and better growth compared to controls (Koskela et al., 1992). However, ARA is generally considered to have a lower nutritional value compared to DHA and EPA. For juvenile *P. chinensis*, survival and final weight gain fed a diet containing 1% of ARA, was significantly lower than those from 1% of DHA supplemented treatments (Xu et al., 1994). Deleting ARA from *P. monodon* diet containing other EFA illustrated no noteworthy response in growth rate (Merican and Shim, 1996). It is likely that these species does not require ARA when other HUFA are available. However, for the larval mud crab *Scylla paramamosain*, ARA is more important than EPA (Nghia et al., 2007). For juveniles of the mud crab, *S. serrata*, the diets inclusion of DHA and ARA depressed growth (Sheen and Wu, 2002). This difference may indicate differences in HUFA requirements (Merican and Shim, 1996). Clearly, quantitative values of ARA are necessary for many commercially important crustaceans and the interaction among ARA and other important fatty acids should be taken into consideration in future experiments. Table 1.4 showed the summary of dietary optimum EPA, ARA and DHA levels for some crustacean larvae and juveniles.

1.5.6 The interactions of different fatty acids

Many of the previous results discussed above are contradictory, even within the same species (Merican and Shim, 1996b; Glencross et al., 2001). This may be explained by the interactions of different fatty acids (González-Félix et al., 2003; Wu et al., 2010a), such as n-3PUFA/n-6PUFA, DHA/EPA, ARA/EPA, C18-PUFA/HUFA and SFA: MUFA: PUFA. Unfortunately, few efforts have focussed on this area possibly due to the expensive and complicated experimental design (González-Félix et al., 2002a; González-Félix et al., 2003a,b). Most of our understanding comes indirectly from the researches of fish oil replacement and EFA requirements, but those results have given some indication on fatty acid interactions (Holme et al., 2007a). For example, when juvenile Chinese mitten crab *E. sinensis* are fed HUFA free diets, they accumulate higher LOA in their muscle due to the lack of HUFA (Wu et

al., 2010a). For juvenile mud crab *S. serrata*, dietary DHA will decrease the ARA content in the polar lipid in the hepatopancreas and body muscle (Sheen and Wu, 2002). This may reveal the competition of DHA and ARA in the fatty acid composition of biological membranes. Moreover, the fatty acids interactions and competition may increase as the chain length and level of unsaturation increases (Glencross et al., 2002; Glencross, 2009). Some potential interactions are summarized below.

Firstly, the balance of dietary ratio of SFA: MUFA: PUFA is very important (Teshima, 1998). Recent research on juvenile the Chinese mitten crab has shown that when the diet is lacking in 18:1n9, juveniles will absorb/biosynthesis and accumulate high concentrations of 16:0 in the hepatopancreas, presumably for energy provision, especially during the moulting (Wu et al., 2011a). Furthermore, the replacing of the percentage of fish oil in formulated diets was affected not only by a minimum PUFA and HUFA requirement, but also by a balanced fatty acid composition (Holme et al., 2007a).

Table 1.4 Summary of dietary optimum EPA, ARA and DHA levels for crustacean larvae/juveniles (% dry weight)

Species	Stage	FA form	EPA	ARA	DHA	Value	Criteria	Feeding period	Reference
Peaeus japonicus	Juvenile	Purified FA	1%	—	1%	EPA=DHA	G, S	--	Kanazawa et al (1979)
Penaeus chinensis	Juvenile (0.37-1.62g)	Purified FA	—	1%	1%	DHA>ARA	G, S, MF, BFA	32 days	Xu et al. (1993)
Macrobrachium rosenbergii	Juvenile (0.06-0.5g)	Purified FA	—	0.075%	0.075%	DHA>ARA	G, S, MF, BFA	150 days	D'Abramo and Sheen (1993)
Penaeus monodon	Juvenile (0.05-0.40g)	Purified FA	0.5-1%	0-0.2%	0.5-1%	DHA>EPA>ARA	G, S, BFA	4 weeks	Merican and Shim (1996)
Penaeus monodon	Juvenile (0.07-0.37g)	Purified DHA, without other PUFAs	—	—	1.5%	—	G, S, BFA	4 weeks	Merican and Shim (1997)
Penaeus monodon	Juvenile (1.78-5.10g)	Vegetable/animal oil and Purified FA	1.0%	—	1.0%	EPA=DHA	G, S, BFA	50 days	Glencross and Smith (2001a)
Penaeus monodon	Juvenile (2.34-10g)	Vegetable/animal oil and Purified ARA; with optimized DHA and EPA	—	0	—	—	G, S, BFA	50 days	Glencross and Smith (2001b)
Litopenaeus vannamei	Juvenile (0.39-4.71g)	Purified EPA, ARA and DHA of 0-0.5%DW	**0.5%**	**0.5%**	**0.5%**	DHA=EPA=ARA	G, S, BFA	6 weeks	González-Félix et 2003a
Scylla serrata	Juvenile (0.06-0.78g)	Purified ARA and DHA of 0-0.2% DW	—	0.2%	0.2%	DHA =ARA	G, S, BFA, MF	10 weeks	Sheen and Wu (2003)
Scylla serrata	Larvae to megalopa	*Artemia* enriched purified EPA and DHA	0.7-0.9%	—	0.5-0.7%	DHA >EPA	S,DP, CW, BFA	20 days	Suprayudi et al (2004b)
Scylla paramamosain	Larvae to megalopa	*Artemia* enriched purified EPA and DHA	1.3-2.5%	—	0.5%	DHA >EPA	S,DP, CW, BFA	20 days	Kobayashi et al (2000)
Eriocheir sinensis	Larvae to megalopa	Rotifer and *Artemia* enriched with different DHA/EPA ratios	0.7-1.1%	--	0.6-1.0%	DHA >EPA	S,DP, CW, BFA, SR	25 days	Sui et al (2007)

Notes: S: survival; G: growth; BFA: body/tissue fatty acid analysis; SR: stress resistance; MF: moulting frequency; FC: Feed conversion; DP: development period; CW: carapace width.

Secondly, the requirements of n-3 HUFA and n-6 HUFA could be partly replaced by LNA and LOA (Merican and Shim, 1997b; Gonz âlez-F élix et al., 2002c; Gonz âlez-F élix et al., 2003). Therefore, plant oils rich of LOA and LNA could be used to replace partial marine fish oil for commercial crustacean diets, such as shrimps and crabs. For juvenile *L. vannamei*, the studies have shown that the best growth was obtained when the same amounts of EPA and DHA were supplied (1.5g kg^{-1}) together with 6.5 g LOA kg^{-1} and 7.5 g LNA kg^{-1}.A (Gonz âlez-F élix et al., 2002b). Previous studies have suggested that fatty acid composition, particularly for EFA composition, is more important than dietary absolute EFA contents for juvenile *P. monodon* (Glencross et al., 2002).

Thirdly, the proportion of HUFAs is responsible to the fatty acids interaction as discussed in section 1.5.5. It is well known that DHA, EPA and ARA have different physiological role for crustaceans, and there may exist the bio-conversion between DHA and EPA for some species (Reigh and Stickney, 1989; Merican and Shim, 1996). Therefore, the optimization of their composition may reduce the dietary cost and promote growth and survival (D'Abramo, 1997). Although 20:4n-6 is the chief precursor of the eicosanoids, generating 2-series prostanoids and 4-series leukotrienes, 20:5n3 competes with 20:4n6 in eicosanoid production, which itself is converted to 3-series prostanoids and 5-series leukotrienes, which are generally less biologically active than the corresponding 2-series prostanoids and 4-series leukotrienes produced from 20:4n-6 (Abayasekara, 1999). As eicosanoid production is influenced by the dietary and cellular ratio of 20:4n6:20:5n3, and imbalanced ratio of 20:4n6/20:5n3, it appears to be as damaging in fish as in crustaceans and therefore the ratio of 20:4n6/20:5n3 in the diet seems more important (Bell and Sargent, 2003; Nghia et al., 2007).

1.6 Conclusions

During the past 40 years, significant progress has been made in metabolism and nutrition of fatty acids in crustacean, particularly for larval and juvenile stage shrimps. Although *P. pelagicus* and *P. ornatus* are two commercially important fishery species and target aquaculture candidates in the tropical and sub-tropical regions of the Indo-Pacific (Smith et al., 2009a; Wu et al., 2010d), our knowledge of lipid nutrition of early life stage *P. pelagicus* and *P. ornatus* is largely unknown. This is one of the major obstacles for the improvement of hatchery technology for both species. The further understanding of lipid nutrition for early life stages *P. pelagicus* and *P. ornatus* will facilitate the formulation of cost-effective and nutritionally balanced hatchery foods and the improvement of quality and quantity of artificial seeds for them. This present PhD study aimed to fill the gap of lipid nutrition for early life stage *P. pelagicus* and *P.*

ornatus, which would be a major step towards to cost-effective and reliable hatchery production of blue swimmer crab and ornate rock lobster.

1.7 Aims of this study

The overall objective of this PhD project is to expand the understanding of lipid nutrition for early life stage *P. pelagicus* and *P. ornatus*, with special emphasis on highly unsaturated fatty acids. A series of experiments were designed and carried out using a quantitative approach and using enriched *Artemia* in order to maximize implementation potentials into hatchery practice. The specific aims of this study were as the following:

1. Review information available on metabolism and nutrition of fatty acids in the early life stage of crustaceans, with particular focus on embryonic and larval stages to provide knowledge basis for subsequent lipid nutrition studies on larval *P. pelagicus* and *P. ornatus* (Chapter 1).
2. To assess the ontogenetic changes of lipid class and fatty acid composition during the embryonic development of *P. pelagicus* (Chapter 2).
3. To assess the ontogenetic changes of lipid class and fatty acid composition during the embryonic development of *P. ornatus* (Chapter 3).
4. To determine the ontogenetic patterns of growth and lipid composition during the larval development of *P. pelagicus* (Chapter 4).
5. To determine the ontogenetic patterns of growth and lipid composition during early to mid-stages of development in *P. ornatus* phyllosoma (Chapter 5).
6. To assess the effects of starvation on survival, biomass and lipid composition of newly hatched *P. pelagicus* larvae (Chapter 6).
7. To assess the effects of starvation on survival, biomass and lipid composition of newly hatched *P. ornatus* phyllosoma larvae (Chapter 7).
8. To determine the effects of the dietary arachidonic acid levels on survival, growth and occurrence of moulting death syndrome for *P. pelagicus* larvae (Chapter 8).
9. To establish dietary arachidonic acid requirement for early stage phyllosoma of *P. ornatus* (Chapter 9).
10. To identify the dietary optimal DHA/EPA ratio for *P. pelagicus* larvae (Chapter 10).
11. To determine the optimal ratio of dietary DHA/EPA for early stage phyllosoma of *P. ornatus* (Chapter 11).

Chapter 2

Changes in volume, biomass and lipid composition during the embryonic development of the blue swimmer crab, *Portunus pelagicus* (Linnaeus, 1758)

2.1 Introduction

Females of many decapod crustaceans carry their eggs under their abdomen after egg extrusion and brood them until hatching (Melville-Smith et al., 2007a; Figueiredo and Narciso, 2008). The embryonic development is an important stage in the life history of such crustaceans since nutrient and energy reserves utilized during the development could significantly affect early ontogeny of the larvae (Clarke, 1993; Chen et al., 2007). While egg size might reflect the energy and nutrient contents and indicate maternal investment (Yao et al., 2006; Rosa et al., 2007; Torres et al., 2008), quantifying biochemical dynamics during embryonic development is important for the evaluation of nutritional requirements for the broodstock as well as early larvae, helping improve broodstock management and larval culture success of commercially important crustaceans (Rosa et al., 2003; Rosa et al., 2005; Figueiredo et al., 2008).

Of major nutrients, lipids, including phospholipids (PL), cholesterol (CHO), triacylglycerol (TG) and free fatty acids (FFA), play important roles during crustacean embryonic development (Wehrtmann and Graeve, 1998; Wehrtmann and Kattner, 1998; Reppond et al., 2008) because firstly, lipids are most important energy source and generally contribute > 60% of total energy expenditure during crustacean embryonic development (Wehrtmann and Graeve, 1998; Jacobs et al., 2003; Yao et al., 2006; Chen et al., 2007). Secondly, lipids are generally essential as structural components of cellular biomembranes and as precursors of eicosanoids during egg development (Torres et al., 2008; Garcia-Guerrero, 2009). For example, the contents and ratios of important fatty acids, such as 20:5n-3, 20:4n-6 and 22:6n-3, could affect the egg hatchability and quantity and quality of newly hatched larvae (Cavalli et al., 1999; Wouters et al., 2001; Wu et al., 2007b).

The blue swimmer crab, *Portunus pelagicus* (Linnaeus, 1758), is a commercially important crab species that distributes widely in the Indo-Pacific, particularly in the tropical and subtropical waters of the region (Wu et al., 2010d). There is an increasing market demand for *P. pelagicus* worldwide (Soundarapandian and Dominic Arul Raja, 2008). However, wild *P. pelagicus* has been fully exploited (Dixon and Hooper, 2009), hence the future expansion in *P. pelagicus* production can only come from aquaculture or stock enhancement programs, both

rely on successful hatchery seed production (Maheswarudu et al., 2008). Larval nutrition, particular lipid nutrition, is considered as one of major contributors to the successful larval rearing of this species (Maheswarudu et al., 2008; Romano and Zeng, 2008), which is directly linked to the nutrient reserve and utilization during embryonic development. Previous studies have shown that examination of lipid composition changes during the embryonic development can provide valuable information not only on lipid utilization during organogenesis, but also contribute to our understanding of lipid nutrition for both broodstock and early larvae of crustaceans (Rosa et al., 2003; Rosa et al., 2005; Rosa et al., 2007; Torres et al., 2008). However, to date there is no such published information for *P. pelagicus*. Therefore, the current study was carried out to investigate changes in volume, individual wet weight (WW) and dry weight (DW), as well as lipid composition (lipid classes and fatty acids) during the embryonic development of *P. pelagicus*.

2.2 Materials and methods

2.2.1. Broodstock maintenance and egg sampling

Six adult blue swimmer crabs were collected in estuarine areas around Townsville (19°15′S, 146°48′E), north Queensland, Australia, using baited traps during August to September, 2008. The broodstock crabs were disinfected in formalin treated seawater (100µl/L) for 4-6 hours, and then were kept in outdoor 1000-L oval recirculating tanks. The tanks were provided with sand substrate and PVC tube shelter (diameter: 16 cm). The water temperature and salinity were maintained between 26-30 ^{o}C and 30-36 ‰, respectively. The broodstock were fed on an alternation of banana prawns (*Penaeus merguiensis*, Stockland Woolworths, Townsville, Australia), green lipped mussel (*Perna canaliculus*, Germain and Sons Pty. Ltd, Brisbane, Australia) and California squid (*Loligo opalescens*, Del Mar Seafoods. Inc., Watsonville, CA, USA) at a ration of 5-8% of total crab body weight adjustable based on daily observation of residual food in each tank. Prior to daily feeding, feces and uneaten feed were removed by siphoning. The spawning and mortality were checked and recorded every day in the morning. When a berried female was found, it was labeled with an identification number on the carapace to enable tracking the individual embryonic development. Under the above mentioned condition, the embryonic development took ca. 10-12 days. The egg sampling was performed every 2-7 days depending on the embryonic stage, and the embryonic development stage was identified under a stereo microscope (Leica MZ16A, Leica Microsystems, Germany). For the propose of current experiment and based on the staging criteria from Petersen and Anger (1997) and Arshad et al. (2006), the embryonic development of *P. pelagicus* was divided into five major stages. That is, I. Cleavage: eggs in orange colour; cell-borders become visible; II.

Gastrula: a small, transparent yolk-free area becomes visible but eye spots and pigment/chromatophores is not yet formed; III. Pigmentation: eye-pigment and red chromatophores become visible; IV. Early heart beating: heart-beating is visible but less than 80 beats/min, red chromatophores increase in number, and the embryos begin to move inside egg membranes; V. Pre-hatching: the heart-beating generally more than 150 beats/min; only small patches of yolk remain and eggs are grey in colour (Petersen and Anger, 1997; Arshad et al., 2006). For each embryonic developmental stage, approximate 5 g samples (wet weight) were collected and they were stored separately at -80 ℃ for later biochemical analysis. Totally, four berried females were sampled until egg hatching. Because one sampled female had very small egg masses, the lipid analysis was only conducted on the samples from three females.

2.2.2 Egg volume and weight measurement

For each embryonic stage, thirty eggs were detached from the berried female, and their width (W, short diameter) and length (L, long diameter) were measured under a microscope. Then, individual egg volume was calculated using the formula for oblate spheroids, $V = 1/6 \times \pi \times W^2 \times L$ (Wehrtmann and Kattner, 1998). Bulk samples of eggs were also obtained for the estimation of average individual egg weight due to low weight values of individual eggs. Four sub-samples (approximately 50 eggs/sample) were randomly taken from each berried female for the measurement of average individual egg wet weight (WW) and dry weight (DW). Prior to weighting, the egg samples were rinsed 3 times with 0.5 M ammonium bicarbonate to remove traces of salts, and then blotted on filter paper to remove excess moisture. The solution of 0.5 M ammonium bicarbonate is iso-osmotic to the seawater, which facilitated the removal of trace salt from the surface of larvae, but prevents the ionic loss of body fluids (Zhu and Lee, 1997). The total egg number in each sample was then counted and egg WW were subsequently determined using a Cahn C-33 micro-balance (precision=1μg, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA), DW were obtained after oven-drying the samples for 24 h at 60 ℃. Average individual WW and DW were calculated by dividing the WW and DW of the sample by total egg number of the sample.The egg volume and water contents were determined from four berried females.

2.2.3 Lipid analysis

Prior to biochemical analysis, all samples were freeze-dried and homogenized separately. Moisture content of each sample was obtained by drying the tissue in an oven set at 60 ℃ for 24 h. Total lipid (TL) was extracted with chloroform-methanol (2:1, v/v) (Folch et al., 1957). Lipid fractions were separated and quantified using an Iatroscan MK-6s TLC-FID analyzer (Iatron Laboratories Inc., Tokyo, Japan) as described in detail in Wu et al. (2010c). The developing

solvent system was hexane/diethyl ether/formic acid (42/28/0.3, v/v/v). Lipid classes were quantified for total PL (including PC, PE, PI and others PL), triacylglycerol (TG), free fatty acids (FFA) and cholesterol (CHO). The levels of each lipid fraction were expressed as percentage of both dry weight (% dry weight) and percentage of total lipids (% total lipid).

For fatty acid analysis, fatty acid methyl esters (FAME) were prepared by transesterification with boiling 14% borontrifluoride/methanol (w/w) (Morrison and Smith, 1964). FAME was analytically verified by flame ionization detection (FID) after injecting a sample into an Agilent 6890 gas chromatograph fitted with an Omegawax 320 fused silica capillary column (30m × 0.32mm; Supelco, Billefonte, PA, USA). The injector and detector temperature was kept at 260℃. The detailed method was provided in Wu et al. (2010a). Fatty acid compositions are expressed as percentage of each fatty acid to the total fatty acids (% total fatty acids). Fatty acid contents are expressed as mg of each fatty acid per g egg dry weight (mg/g dry weight). The lipid analysis was determined on the eggs from three berried females.

2.2.4 Statistical analysis

Homogeneity of variance of data was tested with Levene's test. When necessary, arcsine-square root or logarithmic transformation was performed prior to analysis. One-way ANOVA was used to determine the differences among different embryonic stages. If any significant difference was detected, Tukey's multiple range test was used as the means separation procedure. When a normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Kruskal-Wallis H nonparametric test followed by the Games-Howell nonparametric multiple comparison test. $P < 0.05$ was regarded as the statistically significant level. All statistics was performed using SPSS package (version 12.0).

2.3 Results

2.3.1 Egg volume, water content and biomass

During the embryonic development, egg volume and water content increased significantly ($P < 0.05$) and by 92.86% and 67.12%, respectively (Figure 2.1). The highest increase in egg volume and water content occurred during development from stage II (gastrula) to III (pigmentation) while the lowest increase happened when developed from stage III to IV. A significantly positive correlation was detected between egg volume and water content (n=20, r=0.893, $P < 0.05$).

Although there was a slight but not significant increase in DW from stage I (cleavage) to II (gastrula), the mean DW of individual egg decreased significantly (2.96 μg/egg) during the embryonic development, which equaled to a reduction of 21.62% initial egg dry weight (Figure 2.2). In opposite to DW, the mean egg WW and individual egg water content showed significant increases over the embryonic development ($P < 0.05$, Figures 2.2 and 2.3), increased by 80.88% and 198.67%, respectively. A significantly negative correlation was found between egg DW and WW (n=20, r = -0.515, $P < 0.05$).

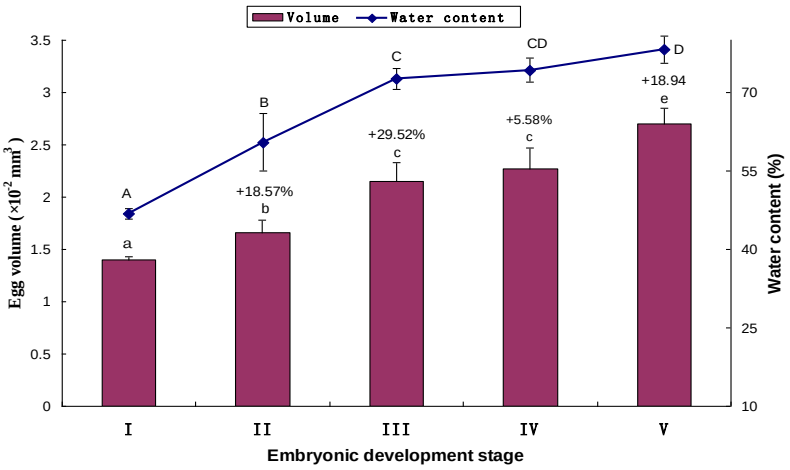

Figure 2.1 The changes of volume and water content during the embryonic development of the blue swimmer crab, *Portunus pelagicus*. Data are presented as mean ±SD (*n*=4). The bars that do not share a same letter indicate significantly different (*P*<0.05). Values on the top of bars indicate the percentage of egg volume increase to the previous stage. Egg developmental stages: I. Cleavage; II. Gastrula; III. Pigmentation; IV. Early heartbeating; V. Pre-hatching.

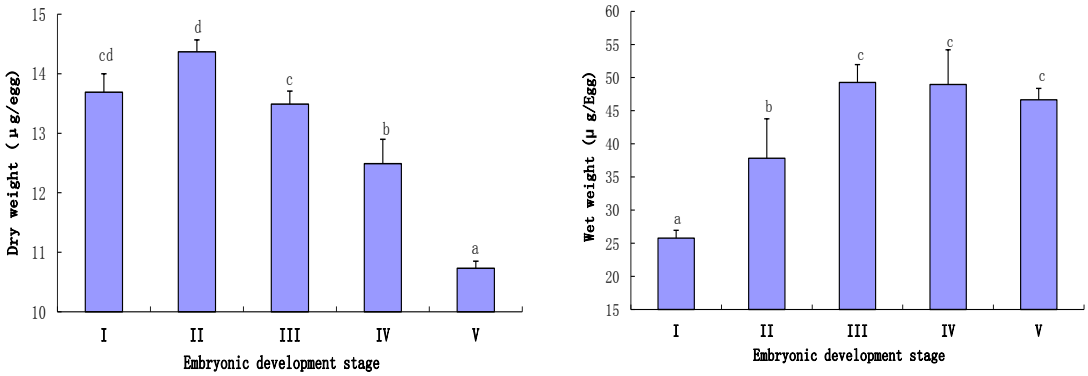

Figure 2.2 Developmental changes in individual egg wet weight (WW) and dry weight (DW) of blue swimmer crab, *Portunus pelagicus* during the embryonic development. Values are presented as means ±SD (n=4). The bars that do not share a same letter indicate significantly different (*P*<0.05).

2.3.2 Total lipids and lipid classes

Both total lipids in individual egg (μg/egg) and as a percentage of egg dry weight (% DW) decreased significantly during the embryonic development ($P < 0.05$) (Figure 2.4). Overall, 1.63

μg of total lipids in an individual egg was utilized, which accounted for 65.14% of the total lipids of a newly extruded egg. The highest decrease for total lipids appeared to occur during the later stage of embryonic development from stage IV to V, for instance, total lipids as a percentage of DW decreased sharply from 17.46% to 11.32% over the period (Figure 2.4).

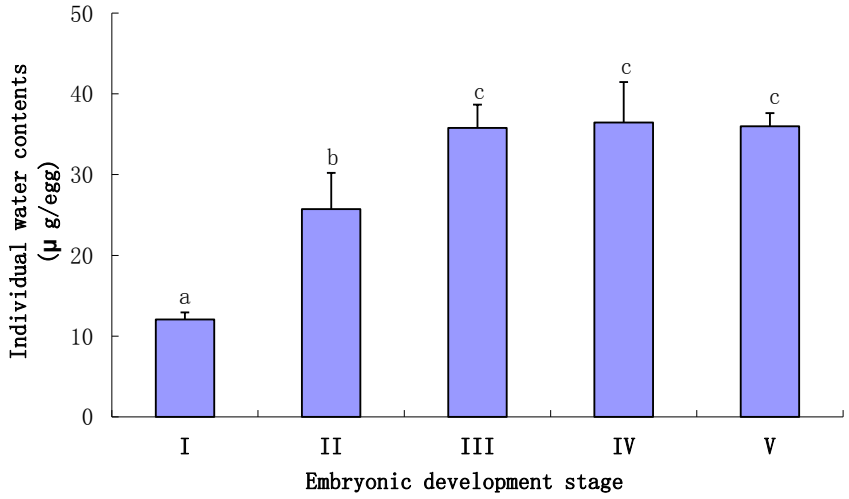

Figure 2.3 The changes of individual egg water content (μg/egg) during the embryonic development of the blue swimmer crab, *Portunus pelagicus*. Data are presented as mean ±SD (*n*=4). The bars that do not share a same letter indicate significantly different (*P*<0.05).

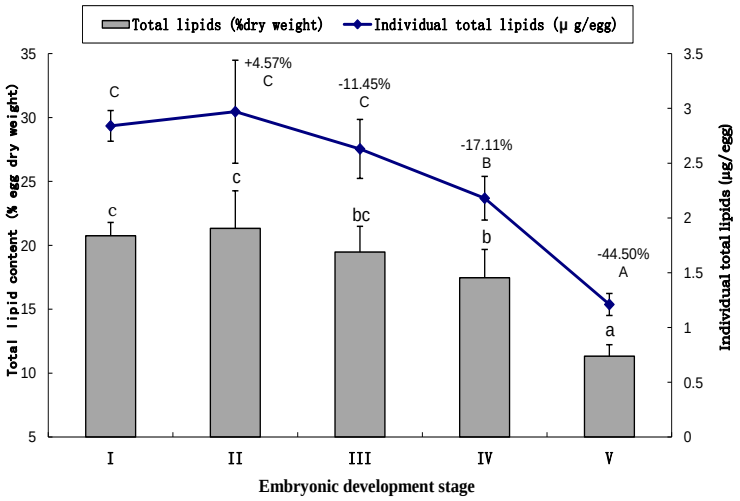

Figure 2.4 The changes of total lipids as % of egg dry weight and in individual egg (μg/egg) during embryonic development of the blue swimmer crab, *Portunus pelagicus*. Data are presented as mean ±SD (*n*=3). The columns that do not share a same letter indicate significantly different (*P*<0.05). Values above the lines indicate total lipids decreases as a percentage of stage I eggs for the subsequent egg development stages.

Of various lipid classes, phospholipids (PL) was the most dominant lipid fraction, ranging from 67.70% to 40.29% of the total lipids, which was followed by triacylglycerol (TG), cholesterol (CHO) and free fatty acids (FFA) (Table 2.1). As a percentage of total lipids, PL and CHO decreased significantly while the FFA increased significantly during the embryonic

development. When measured in the term of decrease in content per egg, 1.43 μg of PL and 0.44 μg of TG were utilized respectively during the egg development, which accounted for more than 55% of their initial contents (Table 2.2).

Table 2.1 Egg lipid class composition as % of total lipids during the embryonic development of the blue swimmer crab, *Portunus pelagicus*

Embryonic stage	Triacylglycerol	Free fatty acids	Cholesterol	Monoacylglycerol	Phospholipids
I (Cleavage)	26.68±2.29	0.45±0.26^{a}	5.22±1.08^{b}	-	67.60±3.03^{b}
II (Gastrula)	26.11±6.47	0.69±0.33^{a}	5.20±1.12^{b}	-	68.01±7.35^{b}
III (Pigmentation)	28.24±1.87	16.30±7.22^{b}	3.69±0.65^{ab}	0.33±0.14	51.20±8.22^{a}
IV (Early heartbeating)	24.59±1.36	21.92±4.57^{b}	3.45±0.27^{a}	-	50.03±4.36^{a}
V (Pre-hatching)	26.45±1.98	29.52±5.37^{c}	3.74±0.37^{ab}	-	40.29±3.76^{a}

Data are presented as mean±SD (n=3). Values within a column having different superscript letters are significantly different (P<0.05). "--": undetectable.

Table 2.2 Egg lipid class contents (μg. Egg^{-1}) of an individual egg during the embryonic development of the blue swimmer crab, *Portunus pelagicus*

Embryonic stage	Triacylglycerol	Free fatty acids	Cholesterol	Monoacylglycerol	Phospholipids	Total lipids
Ⅰ (Cleavage)	0.76±0.04^{c}	0.01±0.005^{a}	0.15±0.02^{c}	--	1.92±0.18^{c}	2.84±0.14^{b}
Ⅱ (Gastrula)	0.79±0.19^{c}	0.02±0.01^{a}	0.16±0.04^{c}	--	1.98±0.42^{c}	2.97±0.47^{b}
Ⅲ(Pigmentation)	0.74±0.12^{c}	0.44±0.18^{b}	0.10±0.01^{b}	0.01±0.00	1.33±0.07^{b}	2.63±0.27^{b}
Ⅳ(Early heartbeating)	0.53±0.02^{b}	0.48±0.13^{b}	0.08±0.01^{ab}	--	1.09±0.13^{b}	2.18±0.20^{ab}
Ⅴ (Pre-hatching)	0.32±0.05^{a}	0.35±0.04^{b}	0.04±0.00^{a}	--	0.49±0.08^{a}	1.21±0.10^{a}
Change (%)	-57.89	+3400.00	-73.33	--	-74.48	-65.14

Data are presented as mean±SD (n=3). Values within a column having different superscript letters are significantly different (P < 0.05). "--": undetectable.

2.3.3 Fatty acids

The changes in fatty acid compositions as % of total fatty acids in *P. pelagicus* eggs during the embryonic development are presented in Table 2.3. A total of 29 fatty acids were identified in samples of all five embryonic developmental stages. Of these fatty acids, 16:0 (16.67-21.46%), 18:1n-9 (8.09-10.48%), 18:1n-7 (6.08-8.02%), 20:5n-3 (8.29-18.02%) and 22:6n-3 (5.13-7.68%) were the five most abundant fatty acids (≥5%) found in all developmental stages, which generally accounted for 53.42-60.16% of egg total fatty acids. Among saturated fatty acids (SFA), 16:0 and 18:0 were the major constituents at all developmental stages. The significant decrease in the percentage of 16:0 only occurred during the late stage of embryonic development from stage IV to V while 18:0 was substantially lower at both stage III and IV (Table 2.3). For mono-unsaturated fatty acids (MUFA), 16:1n-7, 18:1n-9 and 18:1n-7 were the three most dominant fatty acids. During the embryonic development, a decreasing trend was detected for both 16:1n-7 and 18:1n-7 as a percentage of total fatty acids while no clear trend

was found for 18:1n-9, which was at the highest percentage at stage IV. Of poly-unsaturated fatty acids (PUFA), during egg development, 18:2n-6, 18:3n-3 and 22:2n-6 as a percentage of total fatty acids decreased significantly while 18:4n-3, 20:4n-6, 20:3n-6 and 20:5n-3 (EPA) increased significantly. Consequently, the ratio of n-3PUFA/n-6PUFA increased significantly, but DHA/EPA ratio decreased from 0.85 at stage I to 0.43 at stage V (Table 2.3).

Table 2.3 Egg fatty acid composition (% total fatty acids) during the embryonic development of the blue swimmer crab, *Portunus pelagicus*

Fatty acid	I	II	III	IV	V
14:0	3.19±0.24^{b}	2.59±0.21^{ab}	2.54±0.13^{ab}	2.43±0.10^{ab}	1.94±0.60a
15:0	0.67±0.49	0.83±0.01	0.61±0.17	0.68±0.06	0.51±0.05
16:0	21.46±1.39^{b}	20.99±1.57^{b}	21.27±0.98^{b}	19.96±1.28^{b}	16.67±1.25^{a}
18:0	7.67±1.73^{b}	7.98±0.46^{b}	3.89±0.33^{b}	3.56±0.37^{a}	8.68±1.03^{b}
23:0	0.91±0.18^{b}	0.30±0.14^{a}	0.21±0.07^{a}	0.50±0.18^{a}	0.23±0.14^{a}
∑SFA	34.05±0.53^{b}	33.07±1.54^{b}	29.06±1.15^{a}	27.84±1.30^{a}	28.40±1.97^{a}
16:1n-7	8.16±0.44^{c}	7.83±0.71^{bc}	8.48±1.19^{c}	6.25±0.56^{b}	4.54±0.37^{a}
18:1n-9	9.00±0.62^{ab}	8.81±0.47^{ab}	9.79±0.51^{ab}	10.48±0.81^{b}	8.09±1.48^{a}
18:1n-7	8.02±0.21	7.69±0.28	7.34±0.92	7.43±0.68	6.08±3.07
20:1n-9	1.33±0.42	1.67±0.89	1.26±0.56	0.86±0.17	0.97±0.48
20:1n-7	1.24±0.50	1.05±0.20	0.81±0.20	0.88±0.09	0.96±0.04
∑MUFA	27.99±1.76^{b}	27.43±1.97^{ab}	28.16±1.65^{b}	26.19±0.45^{ab}	21.55±4.30^{a}
16:2n-4	0.83±0.13^{b}	0.79±0.02^{b}	0.71±0.10^{ab}	0.71±0.03^{ab}	0.59±0.06^{a}
16:3n-4	1.07±0.04	1.03±0.03	0.72±0.50	0.63±0.66	0.76±0.54
18:2n-6	3.00±0.41^{bc}	3.17±0.76^{bc}	3.93±0.47^{c}	2.13±0.87^{ab}	1.49±0.78^{a}
18:3n-6	0.72±0.09	0.44±0.19	0.60±0.39	0.58±0.07	0.95±1.12
18:3n-4	0.58±0.16	0.37±0.02	0.32±0.10	0.57±0.30	0.36±0.21
18:3n-3	0.51±0.13^{b}	0.39±0.16^{ab}	0.28±0.06^{a}	0.27±0.02^{a}	0.27±0.14^{a}
18:4n-3	0.68±0.16^{bc}	0.36±0.04^{a}	0.47±0.10^{ab}	0.63±0.05^{bc}	0.83±0.13^{c}
20:2n-6	1.07±0.28	0.96±0.37	0.81±0.26	0.98±0.26	0.99±0.06
20:3n-6	0.29±0.05^{a}	0.13±0.11^{a}	0.53±0.19^{ab}	1.15±0.56^{b}	1.05±0.32^{b}
20:4n-6	2.97±0.62^{a}	3.66±0.73^{ab}	3.94±1.18^{ab}	4.37±0.32^{b}	4.91±1.42^{b}
20:4n-3	0.29±0.02^{a}	0.51±0.10^{b}	0.46±0.01^{b}	0.47±0.16a^{b}	0.29±0.11^{a}
20:5n-3	8.29±0.37^{a}	10.07±1.11^{a}	13.76±1.04^{b}	15.25±0.17^{b}	18.02±1.08^{c}
22:2n-6	1.30±0.03^{b}	0.24±0.11^{a}	0.24±0.08^{a}	0.51±0.14^{a}	0.48±0.26^{a}
22:5n-3	2.06±0.70	2.24±0.09	2.18±0.12	2.51±0.60	1.55±0.68
22:6n-3	6.99±0.39	5.87±0.24	5.13±0.42	7.03±1.29	7.68±1.96
∑PUFA(≥ 18:2n)	28.53±1.28^{a}	28.50±0.38^{a}	32.72±1.08^{b}	36.84±1.29^{c}	38.68±2.07^{c}
∑n-3PUFA	18.62±0.97^{a}	19.55±1.35^{ab}	22.35±0.75^{b}	26.25±1.37^{c}	28.77±2.41^{c}
∑n-6PUFA	9.34±0.47	8.59±0.94	10.04±0.43	9.72±0.64	9.86±0.62
n-3/n-6	1.99±0.03^{a}	2.30±0.41^{ab}	2.23±0.04^{ab}	2.75±0.04b^{c}	2.93±0.42^{c}
∑HUFA(≥ 20:3n)	20.87±1.37^{a}	22.59±0.62^{ab}	26.08±1.71^{b}	30.87±1.68^{c}	33.63±3.19^{c}
DHA/EPA	0.85±0.09^{b}	0.59±0.04^{a}	0.38±0.06^{a}	0.46±0.09^{a}	0.43±0.13^{a}

Data are presented as mean±SD (n=3). Values within a line having different superscript letters are significantly different (P<0.05). "--": undetectable.

Table 2.4 presented the changes in absolute contents of principal fatty acids in individual egg (μg/egg) during the embryonic development. All principal fatty acids contents reduced dramatically during the embryonic development. Five fatty acids, i.e. 16:0, 16:1n-7, 18:1n-7, 18:1n-9 and 18:2n-6, in particular reduced by more than 60%. However, the reduction of 22:6n-3 was 50.38% while the reduction of EPA and ARA was relatively mild, being only

11.26% and 23.79%, respectively. Overall, the decrease in total fatty acids content was 59.70% over the period of embryonic development.

Table 2.4 Egg fatty acid contents (μg. Egg^{-1}) of individual egg during the embryonic development of the blue swimmer crab, *Portunus pelagicus*

Fatty acid	I	II	III	IV	V	% change
16:0	0.24 ± 0.03^{c}	0.24 ± 0.01^{c}	0.21 ± 0.02^{c}	0.16 ± 0.01^{b}	0.08 ± 0.01^{a}	- 67.04
18:0	0.08 ± 0.03^{c}	0.09 ± 0.01^{c}	0.04 ± 0.00^{ab}	0.03 ± 0.00^{a}	0.04 ± 0.00^{a}	- 52.69
∑SFA	0.38 ± 0.02^{d}	0.37 ± 0.03^{d}	0.29 ± 0.03^{c}	0.22 ± 0.01^{b}	0.13 ± 0.00^{a}	- 64.80
16:1n-7	0.09 ± 0.01^{c}	0.09 ± 0.00^{c}	0.08 ± 0.01^{c}	0.05 ± 0.00^{b}	0.02 ± 0.00^{a}	- 77.23
18:1n-9	0.10 ± 0.01^{c}	0.10 ± 0.01^{c}	0.10 ± 0.01^{c}	0.08 ± 0.00^{b}	0.03 ± 0.00^{a}	- 65.50
18:1n-7	0.09 ± 0.00^{c}	0.09 ± 0.01^{c}	0.07 ± 0.01^{b}	0.06 ± 0.01^{b}	0.02 ± 0.01^{a}	- 74.11
∑MUFA	0.31 ± 0.03^{d}	0.31 ± 0.01^{d}	0.27 ± 0.02^{c}	0.21 ± 0.02^{b}	0.09 ± 0.01^{a}	- 70.61
18:2n-6	0.03 ± 0.01^{bc}	0.04 ± 0.00^{c}	0.04 ± 0.00^{c}	0.02 ± 0.01^{ab}	0.01 ± 0.00^{a}	- 82.02
20:4n-6	0.03 ± 0.01^{bc}	0.04 ± 0.00^{c}	0.04 ± 0.01^{c}	0.03 ± 0.00^{b}	0.02 ± 0.00^{a}	- 23.79
20:5n-3	0.09 ± 0.00^{a}	0.10 ± 0.03^{b}	0.14 ± 0.02^{b}	0.12 ± 0.01^{b}	0.08 ± 0.01^{a}	- 11.26
22:6n-3	0.08 ± 0.01^{c}	0.07 ± 0.01^{c}	0.05 ± 0.00^{a}	0.06 ± 0.02^{bc}	0.04 ± 0.00^{a}	- 50.38
∑PUFA	0.32 ± 0.00^{b}	0.32 ± 0.05^{b}	0.33 ± 0.03^{b}	0.29 ± 0.04^{b}	0.18 ± 0.02^{a}	- 42.87
∑HUFA	0.23 ± 0.01^{b}	0.25 ± 0.04^{b}	0.25 ± 0.03^{b}	0.25 ± 0.04^{b}	0.16 ± 0.02^{a}	- 31.79
Unknown	0.08 ± 0.01^{ab}	0.11 ± 0.05^{ab}	0.09 ± 0.01^{b}	0.06 ± 0.02^{a}	0.05 ± 0.00^{a}	- 43.13
Total fatty acids	1.00 ± 0.05^{d}	1.01 ± 0.09^{d}	0.89 ± 0.09^{c}	0.72 ± 0.07^{b}	0.40 ± 0.03^{a}	- 59.70

Data are presented as mean±SD (n=3). Values within a line having different superscript letters are significantly different (P<0.05). "--": undetectable.

2.4 Discussion

For the common brachyuran crab species, egg volume increments between 40-100% during the embryonic development have been reported (Jacobs et al., 2003; Chen et al., 2007; Figueiredo et al., 2008; Torres et al., 2008). In this study, the egg volume increase for P. pelagicus was 92.86%, which is similar to Chinese mitten crab, *Eriocheir sinensis* (85.33% increase) (Yu, 2008) and several fiddler crabs, *Uca annulipes*, *U. inversa*, *U. urvillei* and *U. chlorophthalmus* (Torres et al., 2008), but substantially higher than the Spider crab, *Maja brachydactyla* (34.36% increase) (Figueiredo and Narciso, 2008) and the blue crab, *Callinectes sapidus* (37.20% increase) (Jacobs et al., 2003). The egg volume is positively related to water content during the embryonic development of *P. pelagicus*, which is similar to the other crab species (Jacobs et al., 2003; Chen et al., 2007; Yu, 2008).

A dramatic increase in water content occurred during the later stage of embryonic development in many crab species, including *P. trituberculatus* and *C. sapidus* (Jacobs et al., 2003; Chen et al., 2007; Figueiredo and Narciso, 2008). This is because during later stage of

embryonic development, high moisture and osmolality, absorbing water and mineral ions leads to high internal osmotic pressure that helps egg hatching (Pandian, 1970a). The increasing egg water content also leads to a reduction in specific gravity and buoyancy of the newly hatched larvae (Pandian, 1970b). Pandian et al. (1970b) reported that during egg development of the lobster *Homarus gammarus*, approximately 91% of water intake was through the egg membrane while ca. 9% produced by the oxidation of yolk substances, such as protein, lipid, carbohydrate. During the embryonic development, most decapod crustaceans generally display remarkable fat metabolism, but suppressed protein metabolism (Pandian, 1970b; Chen et al., 2007). This is probably because lipids contain higher energy levels (33.0KJ/g) than protein (17.9 KJ/g) (Heras et al., 2000). Therefore, during the embryonic development, lipids generally serve as main energy source while protein more as structural components for organogenesis (Heras et al., 2000; Figueiredo and Narciso, 2008; Reppond et al., 2008).

The total lipids as % of DW in eggs of a crab could be affected by various factors. The previous study has shown that the total lipids of the first brood eggs (31.65%DW) produced by *P. trituberculatus* was significantly higher than that from the second brood (24.54%DW) (Wu et al., 2010b). For blue crab *C. sapidus* of North Carolina population, the embryos from spring spawning contained higher total lipids (26.4%) than those from summer spawning (21.5%DW) (Amsler and George, 1984). The similar condition maybe exists for the blue swimmer crab, *P. pelagicus*. Overall, newly extruded *P. pelagicus* eggs have similar lipid classes profile to the blue crab, *C. sapidus* and the swimming crab, *P. trituberculatus* (Chen et al., 2007; Li et al., 2012). However, egg lipid classes profile of *P. pelagicus* is different to the Chinese mitten crab, *E. sinensis* and the mud crab, *Scylla serrata* (Yu, 2008; Wu et al., Unpublished data), which had the higher PL percentage and lower TG than *P. pelagicus* egg. During the embryonic development of *P. pelagicus*, the TG loss only accounted for 26.99% of total lipids loss. Therefore, TG appeared to be not the major energy source during the embryonic development. In opposite, PL was the dominant lipid fraction (40.29-68.01%) at all embryonic stages and accounted for 87.77% of total lipids loss. During the embryonic development, there was a 73.33% of cholesterol reduction. This may be explained by the fact that cholesterol is essential for the crustacean embryonic development and it is a precursor of ecdysone of steroids hormone (Subramoniam, 2000). The increased percentage of free fatty acids is possibly due to the decompounding/hydrolysis of PL and TG that formed free fatty acids. The lipid classes of pre-hatching *P. pelagicus* eggs are close to those of *C. sapidus* (Li et al., 2012) (Table 2.5), but contained higher TG than that of *S. serrata* (Wu et al., Unpublished data) and lower TG than that of *E. sinensis* (Yu, 2008). Such difference may reflect the embryogenesis adaptation of each species to life-history pattern, feeding ecology and habitat that they live within (Rosa et al.,

2007). For instance, *P. pelagicus* is a typical oceanic species inhabiting 10-30 m depth while *E. sinensis* is a freshwater species that migrates to the estuary for reproduction (Josileen and Menon, 2004; Wu et al., 2007a).

Table 2.5 A comparison of major lipid classes (% total lipids) of newly extruded and pre-hatching eggs of major commercially important crab species.

Egg developmental stage	Crab species	Triacylglycerol	Free fatty acids	Cholesterol	Phospholipid	Source
Newly extruded egg	*Eriocheir sinensis*	53.57	--	0.83	45.59	Yu (2008)
	Scylla serrata	44.68	--	3.00	51.59	Wu et al. unpublished data
	Portunus trituberculatus	36.58	--	2.08	61.34	Chen et al. (2007)
	Callinectes sapidus	33.96	3.74	4.48	57.81	Li et al. (2012)
	Portunus pelagicus	26.68	0.45	5.22	67.60	Present study
Pre-hatching egg	*Eriocheir sinensis*	42.85	10.78	3.54	43.17	Yu (2008)
	Scylla seratta	10.89	9.44	3.93	72.66	Wu et al. unpublished data
	Portunus trituberculatus	37.59	7.27	2.92	51.92	Chen et al. (2007)
	Callinectes sapidus	25.31	35.09	6.94	32.66	Li et al. (2012)
	Portunus pelagicus	26.45	29.52	3.74	40.29	Present study

"--": undetectable.

Overall, the fatty acid profile of newly extruded egg of *P. pelagicus* is similar to that of *S. serrata* (Wu et al., Unpublished data) and *C. sapidus* (Li et al., 2012), but having higher SFA and lower MUFA and PUFA than that of *E. sinensis* (Yu, 2008) and *P. trituberculatus* (Chen et al., 2007) (Table 2.6). Among four commercially important marine Portunidae species, i.e swimming crab *P. trituberculatus*, blue swimmer crab *P. pelagicus*, blue crab *C. sapidus* and mud crab *S. serrata*, newly spawned eggs of *P. pelagicus* have the highest 18:0, 18:1n-7, 18:2n-6 and EPA, but the lowest 18:1n-9, DHA, n-3/n-6 and DHA/EPA ratios (Table 2.6). Compared to freshwater species, newly extruded eggs of *P. pelagicus* have lower MUFA and 18:2n-6, but higher DHA, HUFA and n-3/n-6, DHA/EPA. Generally, embryos of freshwater decapod crustaceans, such as the Chinese mitten crab, *E. sinensis* and the giant freshwater prawn, *Macrobrachium rosenbergii*, have higher MUFA and 18:2n-6, but lower DHA, HUFA and ratios of n-3/n-6 and DHA/EPA (Cavalli et al., 1999; Yu, 2008), which is likely related to differences in nutritional profile of their food.

Table 2.6 A comparison of principal fatty acid profile of newly extruded and pre-hatching eggs among major commercially important crab species.

	Newly extruded egg					Pre-hatching egg				
Fatty acid	*Eriocheir sinensis*	*Scylla serrata*	*Portunus trituberculatus*	*Callinectes sapidus*	*Portunus pelagicus*	*Eriocheir sinensis*	*Scylla serrata*	*Portunus trituberculatus*	*Callinectes sapidus*	*Portunus pelagicus*
16:0	13.09	21.11	15.93	21.42	21.46	14.02	20.86	16.26	22.97	16.67
18:0	3.34	6.47	5.74	5.62	7.67	3.71	6.90	5.39	8.86	8.68
∑SFA	18.02	31.87	24.21	30.57	34.05	19.42	30.90	24.34	34.85	28.40
16:1n-7	10.96	7.24	8.88	10.08	8.16	10.61	4.91	8.28	7.25	4.54
18:1n-9	24.42	19.20	23.61	12.19	9.00	21.85	14.98	22.92	11.74	8.09
18:1n-7	3.97	6.97	3.57	3.44	8.02	5.23	7.45	3.86	3.21	6.08
20:1n-9	1.12	1.19	1.30	2.73	1.33	0.98	1.53	1.42	2.00	0.97
∑MUFA	43.79	36.03	40.56	33.94	27.99	43.02	31.05	39.89	28.77	21.55
18:2n-6	14.16	2.18	0.88	1.49	3.00	12.08	1.58	0.84	1.91	1.49
18:3n-3	3.69	0.14	0.53	--	0.51	2.48	0.44	0.47	0.35	0.27
20:4n-6	4.37	4.28	2.63	2.13	2.97	3.27	5.09	2.70	3.40	4.91
20:5n-3	5.26	6.19	7.64	7.71	8.29	7.27	11.74	8.66	10.14	18.02
22:6n-3	3.73	9.07	15.20	12.19	6.99	5.67	10.25	15.45	10.02	7.68
∑PUFA(≥ 18:2n)	35.14	26.86	30.21	29.89	28.53	34.57	34.46	31.62	30.34	38.68
∑HUFA(≥ 20:3n)	15.05	21.88	27.73	24.17	20.89	17.60	28.68	28.84	25.41	33.63
n-3/n-6	0.74	2.06	6.17	4.29	1.99	0.99	2.62	6.20	3.21	2.93
DHA/EPA	0.69	1.47	1.98	1.60	0.85	0.79	0.87	1.79	0.99	0.43
Source	Yu (2008)	Wu et al. unpublished data	Chen et al. (2007)	Li et al. (2012)	Present study	Yu (2008)	Wu et al. unpublished data	Chen et al. (2007)	Li et al. (2012)	Present study

During the embryonic development of *P. pelagicus*, around 60% of total fatty acids in an individual egg was consumed. Among various fatty acids, the consumption of PUFA and HUFA were lower than that of SFA and MUFA, which suggests egg PUFA and HUFA were relatively conserved during embryonic development. For many crustaceans, SFA and MUFA are the main fuels during the embryonic development while HUFA, especially ARA, EPA and DHA, are comparatively conserved. These HUFAs play important physiological roles, including as the components of membrane, precursors of biologically active eicosanoids and important for neural development during embryonic and larval development (Heras et al., 2000; Narciso and Morais, 2001; Wouters et al., 2001; Morais et al., 2002), hence have been identified as essential fatty acids (EFAs) for various crustacean species (D'Abramo and Sheen, 1993; Rees et al., 1994; Xu et al., 1994d). To date, it is still unknown as to which fatty acids are EFAs for *P. pelagicus*, but it is very likely that HUFAs are important for larval and juvenile survival and development as having been shown in other Portunidae species (Hamasaki et al., 1998; Suprayudi et al., 2004a,b). ARA and EPA generally serve as the important components of membrane phospholipids and also the precursors of prostaglandins (Mourente et al., 1995; Heras et al., 2000; Wouters et al., 2001) while DHA affects crustacean neural development and hatchability (Xu et al., 1994b). Therefore, it is not surprising that these fatty acids are relatively conserved during *P. pelagicus* embryonic development. However, among important HUFAs, the consumption of DHA (50.38% initial total fatty acids) was dramatically higher than EPA (11.26%) and ARA (23.79%). This led to significantly reduced DHA/EPA ratio toward the end of embryonic development. A similar trend was observed for *S. serrata* (Wu et al., Unpublished data) and *C. sapidus* (Li et al., 2012). Finally, it should be noted that SFA are nonessential and can be synthesized *de novo* while MUFA could be produced by the desaturation of SFA, such as from 16:0 to 16:1n-7 and 18:0 to 18:1n-9 during embryonic development of decapod crustaceans (D'Abramo, 1997; Heras et al., 2000). Therefore, the actual consumption of SFA and MUFA during egg development is difficult to estimate since they could be synthesized/produced by the embryos themselves (Rosa et al., 2003; Rosa et al., 2005).

The lipid class and fatty acid profile of pre-hatching *P. pelagicus* eggs can provide the important clues for the development of suitable dietary regime for the newly hatched larvae, which normally is a crucial period during larval culture. The pre-hatching embryos of *P. pelagicus* contained very limited yolk reserves and the lowest total lipids, which suggest newly hatched larvae would need to feed soon after hatching and the availability of a suitable diet is crucial (Morais et al., 2002). The high EPA (18.02%) and DHA (7.68%) and increasing ARA as a percentage (4.91%) of total fatty acids toward the end of egg development indicate that newly hatched larvae need a diet rich

in these HUFAs. Presently, the preys commonly used for *P. pelagicus* larval culture are rotifers and *Artemia* nauplii (Castine et al., 2008). However, rotifer and *Artemia* lack of DHA and have a lower DHA/EPA ratio (Hamasaki et al., 1998; Liddy et al., 2005). It indicates that improving *Artemia* DHA level via enrichment or development artificial diets containing balanced fatty acids could improve *P. pelagicus* larval culture. Previous studies have shown optimization of dietary HUFA composition enhanced larval survival and shorten development duration for *S. serrata* and *P. trituberculatus* (Hamasaki et al., 1998; Suprayudi et al., 2004a,b). Clearly, optimal dietary ARA, EPA, DHA and their ratios should be investigated in the future for *P. pelagicus*.

2.5 Conclusion

In summary, during the embryonic development of *P. pelagicus*, the egg volume, water content and individual WW increased substantially, while individual DW, total lipids content as % of egg DW and in individual egg (μg/egg) decreased significantly. At all embryonic development stages, PL and TG were the two most dominant lipid fractions while PL is the major energy source during embryonic development. Among fatty acids, 16:0, 16:1n-7, 18:1n-9 and 18:1n-7 were preferentially utilized while ARA, EPA and DHA, were relatively conserved during the embryonic development. These results provide useful information for better understanding lipid utilization and dynamics during embryonic development of *P. pelagicus*, which also will have implications for lipid nutrition and formulated diet development for broodstock and early larvae of *P. pelagicus*.

Chapter 3

Changes in volume, biomass and lipid composition during the embryonic development of the ornate rock lobster, *Panulirus ornatus*

3.1 Introduction

Females of some decapods extrude and carry their eggs under the abdomen until hatching (Melville-Smith et al., 2007a; Figueiredo and Narciso, 2008). The developing embryo is lecithotrophic relying on energy reserves packaged into the egg before female ovopositioning (Herring, 1974; Clarke, 1993; Chen et al., 2007). Egg size can not only reflect the energy and nutrient contents but also indicate the maternal investment per embryo (Yao et al., 2006; Rosa et al., 2007; Torres et al., 2008). The understanding of variations in biochemical composition through embryonic development is essential for the estimation of broodstock nutritional requirements during oogenesis. Improvements in crustacean broodstock nutrition are important to improve larval survival and quality of commercially important crustaceans in aquaculture (Rosa et al., 2003; Rosa et al., 2005; Figueiredo et al., 2008).

Although protein is the major component for crustacean eggs, lipids play several central roles in embryonic development and metabolism (Wehrtmann and Graeve, 1998; Wehrtmann and Kattner, 1998; Reppond et al., 2008). Firstly, lipids are generally essential as structural components for cellular biomembranes and precursors of eicosanoids during egg development (Torres et al., 2008; Garcia-Guerrero, 2009). Secondly, lipids are most important energy source and generally contribute more than 60% of total energy expenditure during crustacean embryonic development (Wehrtmann and Graeve, 1998; Jacobs et al., 2003; Yao et al., 2006; Chen et al., 2007). Moreover, the contents and ratios of some important fatty acids, such as 20:5n-3, 20:4n-6 and 22:6n-3 will affect the embryonic development, egg hatchability and suivival and quality of newly hatched larvae (Cavalli et al., 1999; Wouters et al., 2001; Wu et al., 2007b). Therefore, the quality and quantity of lipids in broodstock diets have the potential to affect embryonic development, egg hatchability and survival of newly hatched larva (Xu et al., 1994b; Cavalli et al., 1999; Smith et al., 2004). Generally, examination of lipid composition during the embryonic development can provide valuable information on the lipid nutrition and the understanding of relative importance of specific lipid classes and fatty acids for the broodstock and early larvae (Rosa et al., 2003; Rosa et al., 2005; Rosa et al., 2007; Figueiredo and Narciso, 2008; Torres et al., 2008).

Aquaculture production of seafood is an increasingly important source of supply. The aquaculture production of Penaeid prawns is firmly established with other species being targeted for domestication. The rock spiny lobster, *Panulirus ornatus*, is a high-price and high-demand seafood delicacy. It is a commercially important crustacean species supplied through wild fisheries throughout the Indo-West Pacific region and in particular in the tropical and subtropical waters of the region, e.g. Torres Strait and North eastern Australia (Ye, 2008; Ye and Dennis, 2009). Global demand for *P. ornatus* has exceeded its wild harvest yields (FAO, 2005), and hence there is an increasing interest for the *P. ornatus* aquaculture to meet the increasing market demands (Hung and Tuan, 2009; Irvin et al., 2010). One of current bottleneck in ornate rock lobster aquaculture is the lack of artificial seed. Hatchery technology for larval supply is in its infancy and of insufficient robustness for commercial establishment. Larval survival is variable which may be partially due to poor quality eggs and in particular due to inadequacies in egg biochemical composition as well as inadequate broodstock diets (Wu et al., 2012). Therefore, knowledge of the lipid dynamics and metabolic process during embryonic development may be essential for the understanding and improving of *P. ornatus* larval quality.

This study examined the lipid composition and dynamics during embryonic development of *P. ornatus*. The current study was designed to investigate the changes of individual egg volume, wet weight (WW) and dry weight (DW), as well as ontogenetic changes in the lipid composition (lipid classes and fatty acids) during the embryonic development of *P. ornatus*. Those results will not only provide basic information for lipid utilization and organogenesis during egg development of *P. ornatus*, but also contribute to the understanding of the lipid nutrition for broodstock and early larvae of this species.

3.2 Materials and methods

3.2.1 Broodstock culture and egg sampling

Wild-caught adult *P. ornatus* broodstock were obtained by commercial fishers from M.G. Kallis Pty Ltd collecting in coastal waters of far northern Queensland (latitude: 13-16 °S; 144-145 °E), Australia and transported live to AIMS, Townsville, Queensland. The broodstock were disinfected in formalin treated seawater (100 mg L^{-1}) for 0.5 hours, and then were maintained in indoor 12,000-L circular tanks (diameter: 4.5m) with flow-through seawater with PVC half-pipes to provide shelter (diameter: 30 cm). To induce mating and spawning, broodstock were held under natural or simulated photoperiod, water temperature of 28^{o}C and salinity of 33-36 ‰. All females were labeled with a different colored ring tags (bird leg rings)

attached around the eyestalk to allow visual identification, enabling individual spawns and embryonic development to be tracked.

Females were examined daily for spawning, and if any berried females were found, their ovipositioning dates were recorded. Embryonic development lasted 22-25 days until hatching. The designated sampling date was performed every 2-7 days, and the embryonic development stage was identified under a stereo microscope (Leica MZ16A, Leica Microsystems, Germany). Based on the staging criteria from Petersen and Anger (1997) and Tong et al. (2000), the embryonic development was divided into four stages. I. Cleavage: cell-borders become visible in the amorphous yolk, and the egg colour is orange; II. Gastrula: a small, transparent and yolk-free area becomes visible for all eggs, and no eye spots and pigment/chromatophores could be found on the eggs; III. Differentiation: eye-pigment, heart-beating, appendages and increasing red chromatophores become visible; IV. Pre-hatching: the heart-beating, general more than 150 times/min, and the embryos begin to move inside the eggs. The eggs only contain a small yolk volume and eggs are brown-grey colour (Tong et al., 2000). For each stage, approximate 8 g wet weight samples were collected and stored separately at -80℃ for later biochemical analysis. Totally, three females were sampled during the experiment. Because the egg quality and quantity varied from female to female, only one female had adequate egg masses until the hatching. Therefore, the data presented in the paper was from one female.

3.2.2 Egg volume and weight measurement

For each embryonic stage, thirty eggs were separated from the berried female, and their width (W) and length (L) were measured under a microscope. Then, individual egg volume was calculated using the formula for oblate spheroids $V = 1/6 \times \pi \times W2 \times L$ (Wehrtmann and Kattner, 1998). Egg bulk samples were used to estimate the weight of an individual egg as individual eggs were too small to give accurate measurements. Four sub-samples (approximately 50 eggs/sample) were randomly selected from each berried female for the measurement of individual egg wet weight (WW) and dry weight (DW). Prior to weighing, the egg samples were rinsed three times with 0.5 M Ammonium bicarbonate to remove traces of salts and blotted on filter paper to remove excess moisture. 0.5 M Ammonium bicarbonate is an iso-osmotic solution to the seawater, which facilitates the removal of trace salt from the larvae surface but prevents the ionic loss of internal body fluids (Zhu and Lee, 1997). Egg WW were determined using a Cahn C-33 micro-balance (precision=1μg, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA), DW were obtained after oven-drying for 24 h at 60℃. Individual mean WW and DW were calculated by the division of egg number for each embryonic development stage. The egg

volume and water contents were determined from three replicate samples, but all of them are sampled from one berried female.

3.2.3 Lipid analysis

Lipid analysis was described in Chapter 2 (See section 2.2.3). The lipid samples of each embryonic stage were analyzed in triplicate for lipid classes and fatty acid contents.

3.2.4 Statistical analysis

Statistical analysis was described in Chapter 2 (See section 2.2.4).

3.3 Results

3.3.1 Volume, water contents and biomass

During egg development there was a significant increase in egg volume and water contents (% egg wet weight, $P < 0.05$). The highest increase of egg volume and water contents occurred from stage II (gastrula) to III (differentiation) while the lowest increase happened from stage III to IV (Figure 3.1). A significantly positive correlation exists between egg volume and water content (n=16, r=0.903, $P < 0.01$). Overall, the egg volume and water content increased by 90.9% and 34.6% of their initial values, respectively. Individual egg dry weight (DW) decreased significantly during the embryonic development with a 14.1% loss (i.e. 5.2 µg/egg) during embryonic development (Figure 3.2A). In contrast, there was a significant increase individual egg wet weight (WW) (Figure 3.2B) and individual egg water content ($P < 0.05$, Figure 3.3). Egg WW increased by 61.4% during the embryonic development while individual water content (µg/egg) increased form 51.5µg/egg of Stage I to 112.0 µg/egg of Stage IV.

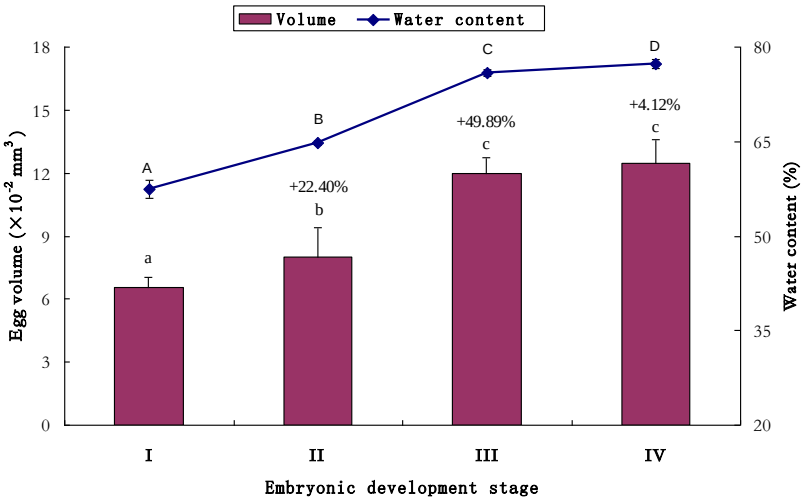

Figure 3.1 Developmental changes in egg volume ($\times 10^{-2}$ mm^3) and water content (%) of *Panulirus ornatus* during embryonic development. Mean ±SD (*n*=4). The bars or diamonds that do not share a same

letter are significantly different (P<0.05). Values in the each bar indicate the percentage of egg volume increase to that of last Stage embryos.

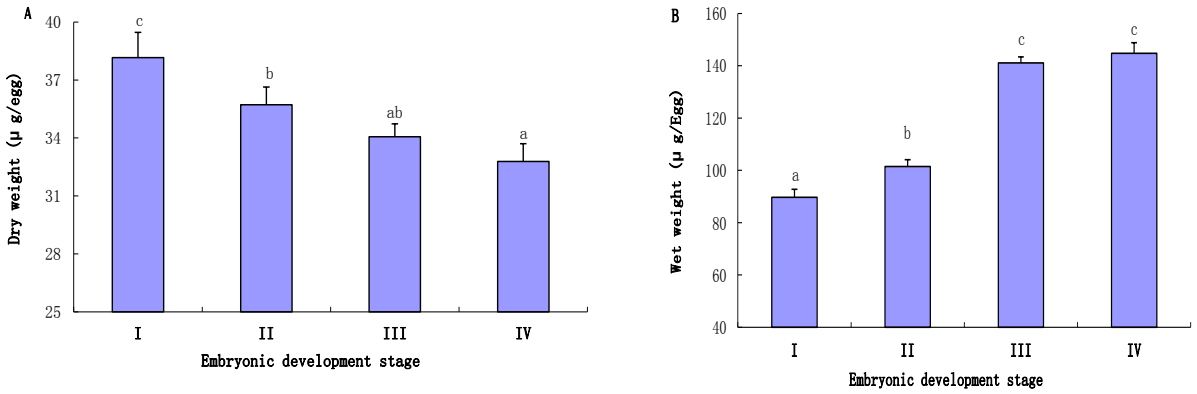

Figure 3.2 Developmental changes in individual egg wet weight (A) and dry weight (B) of *Panulirus ornatus* during embryonic development. Mean ±SD (n=4). The bars that do not share a same letter are significantly different (P<0.05).

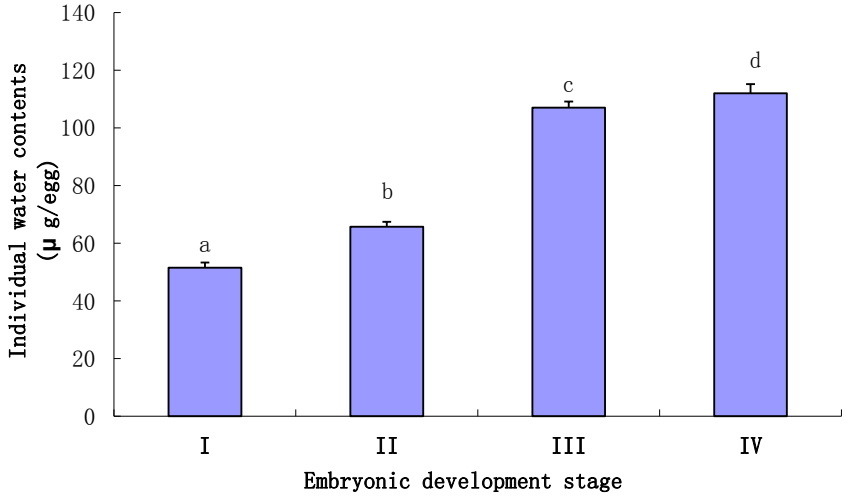

Figure 3.3 Change in individual egg water content (μg/egg) of *Panulirus ornatus* during embryonic development. Mean ±SD (n=4). The bars that do not share a same letter are significantly different (P<0.05).

3.3.2 Total lipids and lipid classes

Total lipids and individual total lipids of eggs progressively decreased through embryonic development (Figure 3.4). There was a significant decrease in egg total lipids (% dry weight) and individual egg total lipids (μg/egg) through embryonic development. For both total lipids and individual egg total lipids, the highest decrease was found on the period of Stage III to IV. For instance, total lipids contents decreased from 20.2% to 12.3% of dry weight during the period of stage III to IV. During the embryonic development, 6.4 μg of total lipids per egg was utilized, which accounted for 61.3% of initially egg total lipids.

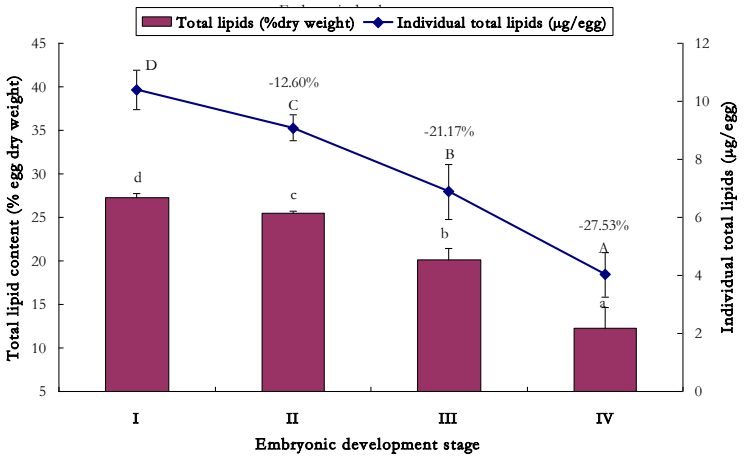

Figure 3.4 Changes in total lipids content (%dry weight) and individual egg total lipids (μg/egg) of *Panulirus ornatus* during embryonic development. Mean ±SD (*n*=3). The bars or diamonds that do not share a same letter are significantly different ($P<0.05$). Values in the each bar indicate the percentage of decreasing lipids to that of last stage embryos.

Of the lipid classes measured, phospholipids were the dominant lipid fraction, ranging from 66.6% to 81.2%, followed by triacylglycerol, cholesterol, monoacylglycerol and free fatty acids, respectively (Table 3.1). Between Stage I to III, phospholipids, cholesterol and monoacylglycerol increased significantly while the percentages of triacylglycerol decreased significantly. Between Stage III and IV, the percentage of triacylglycerol significantly increased to 23.5%. During embryonic development, 4.86 μg of phospholipids and 1.66 μg of triacylglycerol were utilized on a per egg basis, respectively, accounting for more than 60% of initial individual contents (Table 3.2).

Table 3.1 Lipid class composition (% total lipids) of the eggs during embryonic development in the orante rock lobster, *Panulirus ornatus.*

Embryonic stage	Triacylglycerol	Free fatty acids	Cholesterol	Monoacylglycerol	Phospholipids
Stage I (Cleavage)	25.15±2.67^{a}	0.20±0.01^{a}	2.65±0.16^{a}	0.37±0.02^{a}	71.62±2.77^{ab}
Stage II (Gastrula)	16.58±1.83^{b}	0.94±0.09^{b}	3.11±0.37^{a}	0.86±0.20^{a}	78.38±2.11^{bc}
Stage III (Differentiation)	10.17±3.66^{b}	0.09±0.12^{a}	5.33±0.21^{b}	2.81±0.59^{b}	81.21±3.56^{c}
Stage IV (Pre-hatching)	23.53±0.64^{a}	--	7.47±0.11^{c}	2.36±0.27^{b}	66.64±0.74^{a}

Data are presented as mean±SD (n=3). Values within a column having different superscript letter are significantly different ($P<0.05$). "--": undetectable.

3.3.3 Fatty acids

A total of 26 individual fatty acids were identified during embryonic development (Table 3.3). The five most abundant fatty acids (≥5%) in all embryos at different development stages, included 16:0 (18.4-21.8%), 18:0 (8.7-9.2%), 18:1n-9 (9.9-11.9%), 20:5n-3 (9.9-11.8%) and 22:6n-3 (15.9-21.1%). Those five fatty acids accounted for 68.9-70.6% of all total fatty acids in

eggs. Among saturated fatty acids (SFA), 16:0 and 18:0 were the major constituents of SFA for all stages, and their percentages did not change significantly during the embryonic development. Of the mono-unsaturated fatty acids (MUFA), 16:1n-7, 18:1n-9, 18:1n-7 and 20:1n-9 were four dominant. Through embryonic development the percentage of 18:1n-9 and 18:1n-7 increased significantly in comparison to a significant decrease in 20:1n-9. Of the poly-unsaturated fatty acids (PUFA), the percentages of 18:2n-6, 18:3n-3, 20:4n-6 and 20:5n-3 (EPA) increased significantly while 18:4n-3 and 22:6n3 (DHA) decreased significantly, particularly between Stage III to IV. The ratio of n-3PUFA/n-6PUFA and DHA/EPA decreased significantly between Stage III to IV. Table 3.4 presented the changes of principal fatty acid contents (μg/egg) during the embryonic development. All principal fatty acids contents reduced dramatically during the embryonic development. There are two fatty acids with reduction of more than 70%, including 20:1n-9 and DHA. Overall, the loss of total fatty acids was 64.53%.

Table 3.2 Lipid class contents (μg. Egg^{-1}) of the individual egg during embryonic development in the orante rock lobster, *Panulirus ornatus.*

Embryonic stage	Triacylglycerol	Free fatty acids	Cholesterol	Monoacylglycerol	Phospholipids
Stage I (Cleavage)	2.61±0.23c	0.02±0.01a	0.27±0.01a	0.04±0.00c	7.54±0.43c
Stage II (Gastrula)	1.51±0.17b	0.08±0.01b	0.28±0.03a	0.08±0.02c	7.12±0.20c
Stage III (Differentiation)	0.69±0.21a	0.01±0.01a	0.37±0.04b	0.19±0.03b	5.60±0.60b
Stage IV (Pre-hatching)	0.95±0.20ab	--	0.30±0.06a	0.10±0.03a	2.68±0.49a
Change	- 63.68	- 100.00	+ 9.14	+ 153.69	- 64.03

Data are presented as mean±SD (n=3). Values within a column having different superscript letter are significantly different (P<0.05). "--": undetectable.

3.4 Discussion

In Homarid and Palinurid lobster eggs there is between a 60-280% increase in egg volume through embryonic development (Pandian, 1970a,b; Silberbauer, 1971; Herring, 1974; Rosa et al., 2003; Rosa et al., 2005). In this study on *P. ornatus*, egg volume increased by 91.0%; similar to the scalloped spiny rock lobster, *Panulirus homarus* with increases of 120% (Berry, 1971). However, the percentage of egg volume increase for P. ornatus is lower the European lobster, *Homarus gammarus* increased by 177.3% (Rosa et al., 2005), the American lobster, *Homarus americanus* by 270% (Pandian, 1970b) and in the South African rock lobster, *Jasus lalandii* by 200% (Silberbauer, 1971). The individual egg volume is positively related to water contents during the embryonic development of *P. ornatus*. This is similar the other lobster species, including European lobster *Homarus gammarus* (Pandian, 1970a; Rosa et al., 2005), Canadian lobster *Homarus americanus* (Pandian, 1970b), and Norway lobster, *Nephrops norvegicus* (Rosa et al., 2003).

Table 3.3 Fatty acid composition (%total fatty acids) of eggs during embryonic development in the ornate rock lobster, *Panulirus ornatus*.

Fatty acid	Stage I (Cleavage)	Stage II (Gastrula)	Stage III (Differentiation)	Stage IV (Pre-hatching)
14:0	1.13±0.32	1.45±0.00	1.29±0.10	1.28±0.11
15:0	0.56±0.15	0.69±0.04	0.64±0.04	0.63±0.04
16:0	18.45±3.50	21.42±1.00	21.10±0.01	21.82±1.89
18:0	9.22±0.09^{b}	8.81±0.24^{a}	8.74±0.02^{a}	9.09±0.25^{ab}
23:0	0.51±0.03^{c}	0.42±0.02^{b}	0.37±0.00^{a}	0.32±0.05^{a}
∑SFA	29.87±4.05	32.79±0.78	32.13±0.17	33.14±1.84
16:1n-7	3.71±0.79	4.33±0.14	4.16±0.03	4.02±0.19
16:1n-5	0.63±0.04	0.65±0.04	0.66±0.18	0.73±0.04
18:1n-9	9.93±0.07^{a}	10.44±0.44^{ab}	10.85±0.03^{b}	11.99±0.31^{c}
18:1n-7	4.17±0.02^{a}	4.31±0.24^{ab}	4.51±0.02^{b}	4.68±0.27^{b}
20:1n-9	3.64±0.33^{c}	3.33±0.20^{bc}	2.94±0.09^{a}	2.97±0.28^{ab}
20:1n-7	1.72±0.15^{b}	1.63±0.02^{b}	1.38±0.12^{a}	1.35±0.27^{ab}
∑MUFA	23.89±0.36^{a}	24.81±0.65^{ab}	24.66±0.40^{b}	25.93±0.16^{c}
16:2n-4	0.81±0.16^{ab}	0.88±0.04^{b}	0.84±0.03^{ab}	0.78±0.06^{a}
16:3n-4	1.03±0.09^{a}	1.11±0.05^{ab}	1.12±0.01^{b}	1.19±0.05^{c}
18:2n-6	0.84±0.15^{a}	0.80±0.03^{a}	0.84±0.01^{a}	1.26±0.00^{b}
18:3n-6	0.22±0.04^{a}	0.21±0.17^{ab}	0.33±0.00^{b}	0.40±0.02^{c}
18:3n-4	0.41±0.03^{b}	0.23±0.01^{a}	0.23±0.00^{a}	0.25±0.06^{a}
18:3n-3	0.28±0.02^{ab}	0.29±0.01^{b}	0.26±0.00^{a}	0.33±0.06b
18:4n-3	0.95±0.07^{b}	0.95±0.06^{b}	0.93±0.06^{b}	0.74±0.09^{a}
20:2n-6	0.84±0.07	0.82±0.01	0.84±0.02	0.97±0.16
20:4n-6	2.18±0.16^{ab}	2.11±0.02^{a}	2.35±0.03^{b}	2.61±0.06^{c}
20:3n-3	0.61±0.06	0.50±0.09	0.64±0.12	0.65±0.12
20:5n-3	10.14±0.77^{ab}	9.86±0.04^{a}	10.90±0.12^{b}	11.80±0.23^{c}
22:2n-6	0.41±0.22	0.25±0.07	0.28±0.11	0.27±0.06
22:5n-3	0.58±0.08^{a}	0.79±0.03^{b}	0.79±0.16^{ab}	0.88±0.21^{b}
22:6n-3	21.13±2.67^{c}	19.40±0.98^{bc}	18.64±0.25^{b}	15.87±0.90^{a}
∑PUFA(≥ 18:2n)	38.75±3.40	36.36±0.75	37.10±0.96	36.23±1.51
∑n-3PUFA	33.84±3.56	31.94±0.98	32.23±0.38	30.47±1.90
∑n-6PUFA	4.50±0.18^{ab}	4.20±0.23^{a}	4.64±0.05^{b}	5.51±0.25^{c}
n-3/n-6	7.55±1.10^{c}	7.63±0.65^{c}	6.94±0.01^{b}	2.90±0.05^{a}
∑HUFA(≥ 20:3n)	34.79±3.67	32.81±1.01	33.39±0.29	32.02±1.22
DHA/EPA	2.08±0.11^{c}	1.97±0.11^{c}	1.71±0.04^{b}	1.35±0.05^{a}

Data are presented as mean±SD (n=3). Values within a line having a different superscript letter are significantly different (P <0.05).

For many lobster species there is an increase in water content during the late development stages, i.e. *H. gammarus*, *H. americanus*, *N. norvegicus* (Pandian, 1970b; Rosa et al., 2003; Rosa et al., 2005). This increase may be related to osmolality as it could lead to an increased internal osmotic pressure improving successful egg hatching (Pandian, 1970a). It has been suggested that the increased egg water content leads to a reduction in specific gravity resulting in the newly hatched larvae floating (Pandian, 1970b). However, other factors also influences on egg hatching, including circadian control, intra-embryonic moving and some other unknown factors (Davis, 1964; Dorota et al., 2010). Pandian et al. (1970b) further reported for *H. gammarus* egg development, approximately 91% of increasing water is due to water intake

through the egg membrane, and there is ca. 9% of metabolic water, which produced by the oxidation of yolk substances, such as protein, lipid and carbohydrate.

Table 3.4 Fatty acid contents (μg. Egg^{-1}) of the eggs during embryonic development in the orante rock lobster, *Panulirus ornatus*.

Fatty acid	Stage I (Cleavage)	Stage II (Gastrula)	Stage III (Differentiation)	Stage IV (Pre-hatching)	Change (%)
16:0	0.69 ± 0.13^{c}	0.72 ± 0.06^{c}	0.54 ± 0.03^{b}	0.29 ± 0.00^{a}	- 58.20
18:0	0.35 ± 0.00^{c}	0.30 ± 0.02^{c}	0.23 ± 0.01^{b}	0.12 ± 0.01^{a}	- 64.98
∑SFA	1.12 ± 0.15^{c}	1.10 ± 0.07^{c}	0.83 ± 0.04^{b}	0.44 ± 0.01^{a}	- 60.75
16:1n-7	0.14 ± 0.03^{c}	0.15 ± 0.01^{c}	0.11 ± 0.01^{b}	0.05 ± 0.00^{a}	- 61.56
18:1n-9	0.37 ± 0.00^{c}	0.35 ± 0.03^{c}	0.28 ± 0.02^{b}	0.16 ± 0.01^{a}	- 57.22
18:1n-7	0.16 ± 0.00^{d}	0.15 ± 0.01^{c}	0.12 ± 0.01^{b}	0.06 ± 0.00^{a}	- 60.29
20:1n-9	0.14 ± 0.01^{d}	0.11 ± 0.00^{c}	0.08 ± 0.00^{b}	0.04 ± 0.01^{a}	- 70.96
∑MUFA	0.89 ± 0.01^{c}	0.83 ± 0.05^{c}	0.64 ± 0.03^{b}	0.34 ± 0.03^{a}	- 61.52
18:2n-6	0.03 ± 0.01^{b}	0.03 ± 0.00^{b}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	- 47.04
20:4n-6	0.08 ± 0.01^{c}	0.07 ± 0.01^{bc}	0.06 ± 0.00^{b}	0.03 ± 0.00^{a}	- 57.51
20:5n-3	0.38 ± 0.03^{d}	0.33 ± 0.01^{c}	0.28 ± 0.01^{b}	0.16 ± 0.02^{a}	- 58.70
22:6n-3	0.79 ± 0.10^{d}	0.65 ± 0.01^{c}	0.48 ± 0.03^{b}	0.21 ± 0.03^{a}	- 73.29
∑PUFA(≥ 18:2n)	1.45 ± 0.13^{d}	1.22 ± 0.02^{c}	0.96 ± 0.07^{b}	0.48 ± 0.06^{a}	- 66.78
∑HUFA(≥ 20:3n)	1.30 ± 0.14^{d}	1.10 ± 0.01^{c}	0.86 ± 0.06^{b}	0.43 ± 0.05^{a}	- 67.30
Unknown	0.21 ± 0.05^{d}	0.14 ± 0.02^{c}	0.11 ± 0.01^{b}	0.04 ± 0.01^{a}	- 82.65
Total fatty acids	3.74 ± 0.05^{d}	3.36 ± 0.13^{c}	2.58 ± 0.15^{b}	1.33 ± 0.11^{a}	- 64.53

Data are presented as mean ±SD (n=3). Values within a line having different superscript letter are significantly different ($P<0.05$).

Through embryonic development most of decapod crustacean exhibit an enhancement of fat metabolism with a suppression in protein metabolism (Pandian, 1970a; Chen et al., 2007). As lipids are of a higher energy content (33.0KJ/g) than proteins (17.9 KJ/g), it would be a high energy density per weight basis (Heras et al., 2000). Lipids generally serve as main energy source while the major functions of proteins are for structural components during organogenesis (Pandian, 1970b; Wehrtmann and Graeve, 1998; Heras et al., 2000; Figueiredo and Narciso, 2008; Reppond et al., 2008). Total lipids (27.2% DW) of newly spawned *P. ornatus* eggs is similar to that of European lobster, *H. gammarus* (Rosa et al., 2005), but lower than southern rock lobster, *Jasus edwardsii* (ca. 40%) (Smith et al., personal communication). Generally, decapod crustaceans from polar and temperate regions, or deep-sea environment have larger egg size and higher egg lipid levels than those similar species in tropic and subtropical sea, or shallow water (Thatje et al., 2005; Morley et al., 2006)..

Table 3.5 The comparison of major lipid classes (% total lipids) of newly spawned eggs and pre-hatching eggs among the different lobster species.

		Triacylglycerol	Free fatty acids	Cholesterol	Phospholipids	**Source**
Newly spawned egg	*Nephrops norvegicus*	24.32	14.89	21.03	29.80	Rosa et al. (2003)
	Hormarus gammaurs	52.75	4.81	11.87	16.83	Rosa et al. (2005)
	Panulirus ornatus	25.15	0.20	2.65	71.62	Present study
Pre-hatching egg	*Nephrops norvegicus*	6.87	22.31	19.28	51.51	Rosa et al. (2003)
	Hormarus gammaurs	12.35	22.19	2.91	57.13	Rosa et al. (2005)
	Panulirus cygnus	4.20	0.80	8.40	85.50	Melville-smith et al. (2007)
	Panulirus ornatus	23.53	--	7.47	66.64	Present study

"--": undetectable.

During embryonic development in *P. ornatus*, 61.3% of initial total lipids per egg were utilized while the percentage of total lipids (%DW) decreased by 54.9%. Similar patterns have been reported for other lobster species, including the European lobster, *H. gammarus* (Rosa et al., 2005), the American lobster, *H. americanus* (Pandian, 1970b) and the Norway lobster, *Nephrops norvegicus* (Rosa et al., 2003). Compared to European lobster, *Homarus gammarus* (Rosa et al., 2005) and Norway lobster, *Nephrops norvegicus* (Rosa et al., 2003), newly spawned *P. ornatus* eggs contained a higher percentage of PL, but lower free fatty acids and cholesterol. Although TG is a common energy source and the major form of energy storage for the crustacean egg (Rosa et al., 2003; Rosa et al., 2005; Yao et al., 2006), the newly spawned P. ornatus eggs only contained 25.2% of TG in the total lipids. During embryonic development, the TG loss only accounted for 25.1% of the decrease in total lipids. TG is unlikely to be a major energy source during the embryonic development of *P. ornatus*. PL is the dominant lipid fraction (71.6-66.6%) for all embryonic stages and accounts for 76.30% of total lipids loss and may be the major energy source in contrast to TG. Although the percentage of cholesterol increased significantly during embryonic development, the absolute content of cholesterol per egg did not increased significantly. Cholesterol is essential for crustacean embryonic development as is a precursor of ecdysone, steroids hormone and cell membrane constituent (Subramoniam, 2000). The increasing percentage of cholesterol is possibly due to the decompound/hydrolysis of sterol-ester to free fatty acids and cholesterol. As for the lipid classes of pre-hatching *P. ornatus* eggs, except for TG, the lipid composition of *P. ornatus* closely resembles that in the western rock lobster, *P. cygnus* (Melville-Smith et al., 2007) (Table 3.5).

The fatty acid profile of pre-hatching *P. ornatus* eggs can provide important insight into the formulation of an appropriate dietary regime for the newly hatched *P. ornatus* larvae. The high percentages of EPA and DHA, indicates that phyllosoma may need a diet rich in these

PUFAs, as the pre-hatching egg contained higher percentage of EPA and DHA (11.80% and 15.87%, respectively). In the hatchery environment, the diet for *P. ornatus* larval culture is represented by *Artemia* nauplii, on-growing/on-grown *Artemia*, adult *Artemia* and blue mussel gonad (Wu et al., 2012). However, *Artemia* is deficient in DHA, which would inhibit/suppress growth for phyllosoma from hatching to Stage IV, as found in western rock lobster, *P. cygnus* (Liddy et al., 2005). A similar condition may exist for newly hatched *P. ornatus* phyllosomas (O'Sullivan, 2010). It is likely that DHA levels of *Artemia*, via enrichment or through the development of an artificial diet would be necessary for the fatty acids requirements for *P. ornatus* phyllosomas.

During embryonic development of *P. ornatus*, around 64.5% of total fatty acids was consumed. Interesting, the consumption of PUFA and HUFA were higher than that of SFA and MUFA. In crustaceans SFAs are nonessential and can be synthesized *de novo* while MUFA can be produced by the desaturation of SFA, such as from 16:0 to 16:1n-7 and 18:0 to 18:1n-9 (D'Abramo, 1997; Heras et al., 2000). Due to the possibility of metabolic transformation it is not possible to accurately estimate how much SFA and MUFA is consumed during embryonic development (Rosa et al., 2003; Rosa et al., 2005). In crustaceans, HUFA, especially for ARA, EPA and DHA, play many important physiological roles and are components of membrane phospholipids, precursors of biologically active eicosanoids and involved in neural development (Heras et al., 2000; Narciso and Morais, 2001; Wouters et al., 2001; Morais et al., 2002). These fatty acids have been identified as essential fatty acids (EFAs) for some crustacean species (D'Abramo and Sheen, 1993; Rees et al., 1994; Xu et al., 1994a). Although to date, it is not clear for a number of EFA of spiny lobster species, HUFAs apprear to be important for phyllosoma/juvenile survival and development (Smith et al., 2003a; Liddy et al., 2004a; Liddy et al., 2005; Limbourn and Nichols, 2009). ARA and EPA are important components of membrane phospholipids as well as being precursors of prostaglandins (Mourente et al., 1995; Heras et al., 2000; Wouters et al., 2001). It is possible that 20:5n-3 and 20:4n-6 are relatively conserved during embryonic development of *P. ornatus* because of their importance in key developmental processes. Although DHAs are essential for crustacean embryonic development and hatchability (i.e. neural development) it is unclear why 22:6n-3 was preferentially utilized compared to ARA and EPA during embryonic development of *P. ornatus*. This utilization led to a significantly decrease in the percentage of DHA (%total fatty acids) and DHA/EPA ratio. Similar patterns have been described in the Norway lobster, *N. norvegicus* (Rosa et al., 2003) and the freshwater giant prawn *M. rosenbergii* (Yao et al., 2006).

3.5 Conclusion

In summary, during embryonic development of *P. ornatus*, egg volume, water contents and egg wet weight increased, while the individual egg dry weight (DW), total lipids contents (%DW) and individual egg total lipids (μg/egg) decreased significantly. For all stages of eggs, phospholipids (PL) represent the dominant lipid fraction (66.6-71.6% total lipids). PL is probably a major energy source during embryonic development of *P. ornatus*. Fatty acids dynamics were characterized by preferential utilization of 18:0, 20:1n-9 and 22:6n-3 while the other fatty acids, in particular ARA and EPA, were relatively conserved. These results offer valuable insights for the understanding of lipid metabolism in *P. ornatus* during embryonic development, which may contribute to the formulation of balanced diets for broodstock and newly hatched larvae.

Chapter 4

Ontogenetic patterns of growth and lipid composition changes of blue swimmer crab larvae: insights into larval biology and lipid nutrition

4.1 Introduction

The blue swimmer crab, *Portunus pelagicus* (Linnaeus, 1758), is a commercially important crab species that is distributed widely throughout the Indo-Pacific, particularly in the tropical and subtropical waters of the region (Stephenson and Campbell, 1959; Wu et al., 2010d). This species supports an important fishery resource (Dineshbabu et al., 2008; Campbell and Sumpton, 2009), and plays an important role in the coastal and estuarine ecosystems of the region via affecting benthic and planktonic food web (Wu and Shin, 1998; Hall et al., 2006; Dineshbabu et al., 2008). Although there is an increasing market demand for *P. pelagicus* supply worldwide, wild *P. pelagicus* population has been fully exploited or over-exploited (Dineshbabu et al., 2008; FAO, 2012). Therefore, in the future, the expansion of *P. pelagicus* output can only depend on aquaculture and stock enhancement programs (Maheswarudu et al., 2008; Dixon and Hooper, 2009). Pond-culture of *P. pelagicus* has been started in small scale in Australia and South-Asian countries (O'Sullivan and Savage, 2008; Oniam et al., 2010). Hatchery seeds are important for the future aquaculture of *P. pelagicus* (Maheswarudu et al., 2008). Although larval culture of *P. pelagicus* generally has better survival than that of other commercially important portunid crabs, such as the mud crab *Scylla* spp. and the blue crab *Callinectes sapidus* (Zmora et al., 2005; Nghia et al., 2007), variable survival during the larval development is still one of the obstacles for the *P. pelagicus* hatchery production (Castine et al., 2008; Soundarapandian and Dominic Arul Raja, 2008). In contrast to our knowledge of adult and juvenile stages, only very limited information is available on larval biology of this species, which include the description of larval morphology and development (Shinkarenko, 1979; Josileen and Menon, 2004; Arshad et al., 2006) as well as effects of temperature and photoperiod on larval development (Bryars and Havenhand, 2006; Andrés et al., 2010). The larval phase of *P. pelagicus* consists of four zoeal instars and one megalopal stage before settling as the first stage crab (Josileen and Menon, 2004a; Andrés et al., 2010). The duration of *P. pelagicus* megalopa is longer than that of any zoeal stage (Andrés et al., 2010), and megalopa prefers to settle in the shallow sea grass meadows of estuarine areas (Sumpton et al., 2003).

For crustacean larvae, lipids, including phospholipids (PL), cholesterol (CHO), triacylglycerol (TG) and free fatty acids (FFA), play an important role in supporting their survival, development and growth, by providing metabolic energy (D'Abramo, 1997; Anger, 1998; D'Abramo, 1998), maintenance of structure and function of cellular biomembranes and precursors of eicosanoids (Teshima and Kanazawa, 1971; Coutteau et al., 1997). Therefore, the quality and quantity of lipids supplied to the larvae via their diets could significantly affect survival, development and growth as well as general well-being of the larvae (Sui et al., 2007; Wu et al., 2007c). Examination of the lipid compositions of wild crustacean larvae collected from plankton sampling can provide valuable information on lipid requirements and an understanding of the relative importance of specific fatty acids during larval development (Phleger et al., 2001; Limbourn and Nichols, 2009). However, collection and identification of marine larvae from plankton samples is technically difficult, tedious and costly, particularly for species with a number of larval development stages and extended larval duration (Danaher, 2003; Smith et al., 2009). For example, *P. pelagicus* undergoes four pelagic zoeal stages and a postlarval megalopal stage before settling as benthic juvenile, which are assumed to disperse and live in different parts of ocean (Josileen and Menon, 2004). Clearly, collection of sufficient numbers of wild larvae of representative stages and at similar stage of moulting cycle for lipid analysis would be impractical or technically difficult for *P. pelagicus* larvae. Use of laboratory cultured larvae has become an important tool in understanding aspects of larval nutrition of various crustaceans, including the penaeid shrimp, *Penaeus kerathurus* (Mourente et al., 1995), the giant freshwater prawn, *Macrobrachium rosenbergii* (Roustaian et al., 1999), the rock spiny lobster, *Jasus edwardsii* (Ritar et al., 2003; Limbourn and Nichols, 2009), the spider crab, *Maja brachydactyla* (Andrés et al., 2010), the mud crab, *Scylla serrata* (Holme et al., 2009b) and the red king crab, *Paralithodes camtschaticus* (Copeman et al., 2012).

The aims of this study were to investigate the survival, growth patterns in body sizes and weights, as well as ontogenetic changes in lipid composition (lipid classes and fatty acid compositions) during larval development of *P. pelagicus*. Such information would not only help better understanding larval biology and ecology as well as lipid nutrition of *P. pelagicus* larvae, but also allow inter-specific comparison and facilitate better fishery management.

4.2 Materials and methods

4.2.1 Broodstock source and maintenance

Broodstock source and maintenance was described in Chapter 2 (See section 2.2.1). Broodstock were checked and recorded every day in the morning and when a berried female

was found, it was labeled on the carapace with an identification number stick to enable subsequent tracking. A few days prior to hatching, berried females were disinfected again using formalin treated seawater (50μL/L) for 6 h and then transferred to an indoor 300-L hatching tank with salinity of 34 ± 1 g/kg and water temperature of 27 ± 2 ℃. The berried females were hatched separately to acquire the larvae from a single female, i.e. one berried female was kept in one indoor 300-L hatching tank.

4.2.2 Culture of larvae and natural food

Within 8 h of hatching, healthy and actively swimming zoea-I were collected by attracting them to a light source. They were then transferred to several 300 L flat-bottom, shallow rearing tanks with batch exchange water and where they were stocked at a density of 60-100 larvae L^{-1}. Larval culture protocols were modified from Castine *et al.* (2008), and feeding and water exchange regime was summarized in Table 1. Throughout larval culture, water temperature was maintained at 28±1 ℃ while salinity was initially set at 22 g kg^{-1}, but was gradually increased to 33-35 g kg^{-1} as larval development progressed. Light was provided by overhead fluorescent ceiling lights with photoperiod set at 14 h L: 10 h D and light intensity ranged from 350 to 450 lux. Larval culture tanks were siphoned each morning to remove dead larvae, feces and other debris.

Microalgae, *Nannochloropsis* sp. were cultured in 2500 L tanks and aquatic plant fertilizer (AQUASOL, Yates Ltd, New South Wales, Australia) was added at 0.5 mg L^{-1} upon inoculation. When culture density reached approximately 1-2 × 10^7 cells mL^{-1}, the microalgae were pumped to the rotifer culture tanks to feed the ss type rotifers, *Brachionus rotundiformis*, which were then used to feed zoea I and II of *P. pelagicus*. To obtain newly hatched *Artemia* nauplii, *Artemia* cysts (EG grade, INVE Ltd) were hatched daily at 28 ℃ in 1-μm filtered seawater with a salinity of 25 g kg^{-1}. After 24 h, nauplii (size: 430-520 μm) were harvested and either fed directly to the larvae, or held for further enrichment. The latter *Artemia* were stocked at a density of approximately 300 ind. mL^{-1} for 24 h at 28 ℃ with the enrichment emulsion (DHA Selco) dosed at 0.3 g emulsion L^{-1} (DC DHA Selco: HUFA=27.5%, DHA=17.8%, EPA=5.2%; INVE Aquaculture NV, Belgium) at beginning and at 12 h, respectively. After enrichment, the *Artemia* were rinsed thoroughly with filtered seawater to remove residual emulsion before being fed to the larvae. Any enriched *Artemia* meta-nauplii and newly-hatched nauplii that were not used immediately for feeding larvae were kept at 4 ℃ in a fridge to minimize fatty acid catabolism (Merchie, 1996).

4.2.3 Larval sampling and measurement

To determine the biochemical compositions of natural foods used, samples (5-10 g wet weight sample^{-1}) of *Nannochloropsis* sp, rotifers, newly hatched and enriched *Artemia* meta-nauplii were sampled and stored at -70 ℃ prior to biochemical analysis. Meanwhile, various stages of *P. pelagicus*, i.e. newly hatched zoea I, zoea IV, megalopae and the newly settled first stage crabs, were also sampled for analysis of the lipid components. The samplings of zoea IV, megalopae and the first stage crabs from 300 L culture tanks were carried out when 70-80% of population had just moulted to the desired developmental stage. This was done to ensure that larvae sampled were at a similar stage, early in the moulting cycle. Samples were immediately rinsed with filtered seawater and then stored at -70 ℃ for later analysis.

Table 4.1 Protocol developed at James Cook University for mass rearing of *Portunus pelagicus* larvae in 300 L tanks

Larval stage	Feeding regime	Daily batched water exchange	Average larval duration
Zoea I	Rotifers 20-40 mL^{-1} Microalgae 5-10 ×10^{4} mL^{-1}	No water exchange	2 - 3 days
Zoea II	Rotifers 20-40 mL-1 *Artemia* nauplii 0.5 -1 ind. mL^{-1} Microalgae 5-10 ×10^{4} mL^{-1}	10-20%	2 - 3 days
Zoea III	Enriched *Artemia* 2 - 3 ind. mL^{-1}	15–30%	3 - 4 days
Zoea IV	Enriched *Artemia* 2 - 4 ind. mL^{-1}	30-50%	3 - 4 days
Megalopa	Enriched *Artemia* 3–5 ind. mL^{-1}	30-50%	4 - 6 days

Close to each moult, 50-250 individuals (depending on stage) were randomly collected from a 300 L culture tank and transferred into five replicate 500 mL glass beakers. These larvae were cultured on identical feeding regime and conditions as shown in Table 4.1. These larvae were checked every 12 h for newly moulted larvae, which were recorded and then removed from the beakers. These larvae were briefly rinsed with 0.5 M ammonium bicarbonate to remove trace of salt and blotted dry on a filter paper before their wet weights were measured. The use of 0.5 M ammonium bicarbonate for washing larvae was based on the fact that this solution is iso-osmotic to the seawater used, therefore, it would wash off salt from the surfaces of the larvae but not lead to ion losses from larval tissues (Zhu and Lee, 1997). After wet weights were determined, larval samples were oven-dried at 60 ℃ for 24 h and their dry weights (DW) were determined using a Cahn C-33 micro-balance (precision=1μg, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). The number of larvae in each sample was also counted to enable the calculation of mean wet weight (WW) and mean dry weight (DW) per larvae for each stage. For the present study, the number of larvae in each replicate was 40-50 for zoea I and II, 20 - 30 for zoea III and IV, 8-15 for megalopa and 2-4 for the first stage crab. To determine the

mean carapace length (CL), 30 newly hatched zoea I or newly moulted larvae of subsequent stages were randomly sampled from the mass culture tanks. CL was measured under a Leica S8APO dissecting microscope equipped with a computer-controlled display system (Leica Microsystems Inc., Wetzlar, Germany). CL was defined from the base of the rostral spine to the midpoint of the posterior lateral margin of the carapace (Josileen and Menon, 2004a).

To obtain larval survival and mortality data for each stage, 150 newly hatched zoea I larvae were collected randomly and transferred into five 500 mL glass beakers (30 larvae/beaker). Feeding protocol, water temperature and salinity matched those of mass cultured larvae in 300-L tanks (section 2.2) while 100% water exchange was performed daily. Mortality and moulting of larvae were checked and recorded every 12 h, and any newly moulted larvae found during the checking procedure were transferred to a set of new beakers with fresh live food. By doing this, the average intermoult period of each larval instar could be calculated accurately. Cannibalism typically occurs after larvae metamorphose to the megalopae stage when two chelipeds develop (Castine et al., 2008). To avoid cannibalism, megalopae were reared individually in 500 mL flatted bottom vessels as soon as any megalopae were found in the beakers. The experiment was terminated when all larvae either moulted to the first stage crabs or died.

4.2.4 Biochemical analysis

The moisture content of each sample was determined by drying the sample at 60 ℃ for 24 h while Lipid analysis was described in Chapter 2 (See section 2.2.3). The crude protein content (Kjeldahl method, using a 6.25 N to protein conversion factor) of live food were analyzed according to AOAC procedures (AOAC, 1995). For lipid class analysis, both live food and larvae were quantified for total phospholipids (PL; including phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol and other polar fractions), triacylglycerol (TG), free fatty acids (FFA) and cholesterol (CHO). The level of each lipid fraction was expressed as percentage of dry weight (% dry weight) and/or percentage of total lipid (% total lipid). Fatty acid composition was defined as percentage of a particularly fatty acid to the total fatty acids while fatty acid content was expressed as mg fatty acid per g dry weight. Between 2 to 4 replicates were analyzed for each sample.

4.2.5 Data analysis and statistics

The larval mortality, cumulative survival and specific growth rate (SGR, % day^{-1}) for each replicate beaker were calculated using following formulae:

Mortality at a particular larval stage (%) =100%×Total number of larval mortality found during this larval stage/initial number of larvae moulted to this larval stage

Cumulative survival (%) = 100%×Total number of larvae moulted successfully to the next larval stage/30

Specific growth rate (SGR, % day^{-1}) = 100 × (lnWt- lnWo)/D

where Wo is the mean initial larval body weight of a particular stage while Wt is the mean body weight of the larvae that newly moulted to the next stage, D is the duration of the larval stage in days.

Homogeneity of variance of the data was first tested with Levene's test. Where necessary, arcsine-square root or logarithmic transformation was performed prior to analysis. One-way ANOVA was used to determine any significant differences of growth parameters and lipid composition among different live food sources or larval stages. If any significant difference was detected, Tukey's multiple range test was used as the means separation procedure. When a normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Kruskal-Wallis H nonparametric test followed by the Games-Howell nonparametric multiple comparison test. $P < 0.05$ was regarded as the statistically significant level (Sokal and Rohlf, 1995). All statistics was performed using SPSS package (version 12.0).

4.3 Results

4.3.1 Larval survival and development

Figure 4.1 presents larval mortality of each stage and cumulative survival of newly hatched larvae to each subsequent larval stage. The highest mortality of 33.1% occurred at zoea IV stage before larvae metamorphosed to megalopae and the second highest mortality of 29.2% occurred at megalopae to the first crab stage. Overall, the cumulative survival of larvae decreased more gradually after zoea I with the mortality at each subsequent stage ranging from 10% to 18%. Under present rearing conditions (Temperature=28 ℃, Salinity=22-33 g/kg), the newly hatched zoea I of *P. pelagicus* took 14-18 days to develop to the first crab stage with a mean overall cumulative development time (OCDT) at 16.1 ± 0.7 days. The mean development time for each stage are presented in Figure 4.2. Zoea I and zoea II had the shortest periods (2.2-2.3 days) among five larval stages, accounting for only 13-14% of OCDT, while the duration of megalopae was the longest (4.7 days), accounting for 29.28% of OCDT. From zoea II to megalopal stage, a trend of increasing development duration was observed (Figure 4.2). Finally, after natural logarithmic transformation of overall cumulative development time (OCDT) to a particular larval stage and the instar number (I), a significant non-linear relationship was

detected between OCDT and I. The equation was ln(OCDT) = 1.2020×ln(I) + 0.7673 for such a relationship (Table 4.2, P=0.001, n=5).

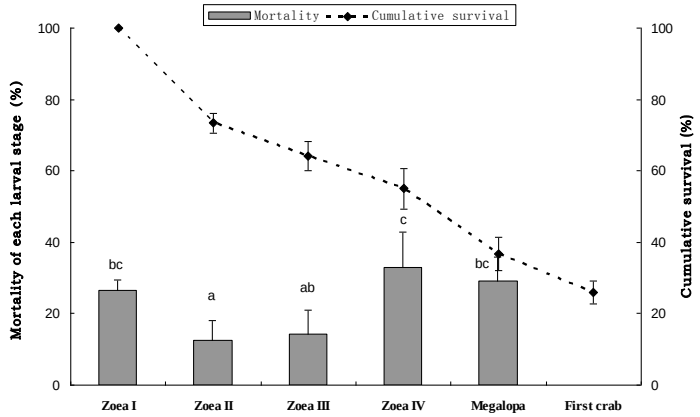

Figure 4.1. Mortality of each larval stage (%) and cumulative survival (% newly hatched larvae moulted successfully to the each subsequent stage) of *Portunus pelagicus* larvae. Values are presented as means±SD (n=5). Bars that do not share a same letter are significantly different (P<0.05).

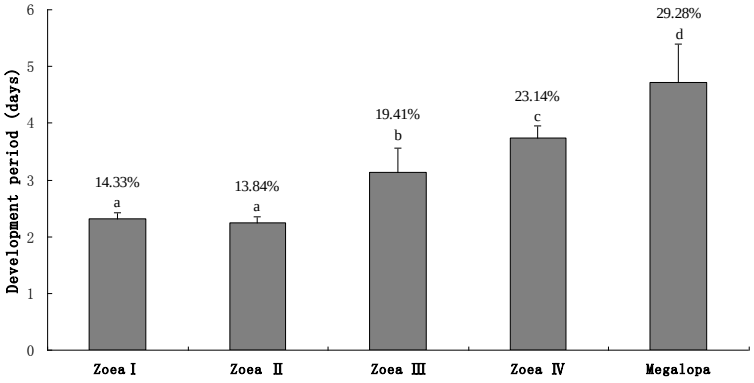

Figure 4.2. Mean development time (days) of each stage of *Portunus pelagicus* larvae. Values are presented as means±SD (n=5). Bars that do not share a same letter are significantly different (P<0.05). Value on the top of a bar is the percentage of the particular stage duration to the overall larval developmental time.

4.3.2 Larval growth

The growth in terms of both wet weight (WW) and dry weight (DW) increases during the course of larval development of *P. pelagicus* is shown in Figure 4.3. Overall, the body WW and DW increased exponentially over larval ontogenetic development and from zoea-I to the first stage crab, a positive correlation between larval WW and DW increases was found (P < 0.001, n = 30), which can be described as DW = 0.1565×WW + 16.118 (Table 4.2). However, when each larval stage was further examined, the percentage increases of WW was similar to the

Table 4.2 Equations of linear regression for various biometric parameters of *Portunus pelagicus* larvae

	Equation	r	P	n	n'
OCDT/I	ln(OCDT) = 1.2020×ln(I) + 0.7673	0.995	0.001	5	150
DW/WW	DW = 0.1565×WW+16.118	0.995	< 0.001	30	1321
WW/CL	ln(WW) = 3.0037×ln(CL) + 5.7927	0.977	0.001	6	180
DW/CL	ln(DW) = 2.6867×ln(CL) + 4.243	0.979	< 0.001	6	180
DW/I	ln(DW) = 0.8293×I + 1.7367	0.996	< 0.001	6	665
WW/I	ln(WW) = 0.9294×I + 2.9828	0.996	< 0.001	6	665
CL/I	ln(CL) = 0.3008×I - 0.9053	0.991	< 0.001	6	180

Notes: OCDT=overall cumulative development time to each larval stage; I = Stage/instar number, zoea I=1, zoea II=2, zoea III=3, zoea IV=4, megalopa=4 and the first crab=5; DW = dry weight; WW = wet weight; CL = carapace length; r = correlation coefficient; *P* = level of significance; n = number of replicates; n' = total number of larvae.

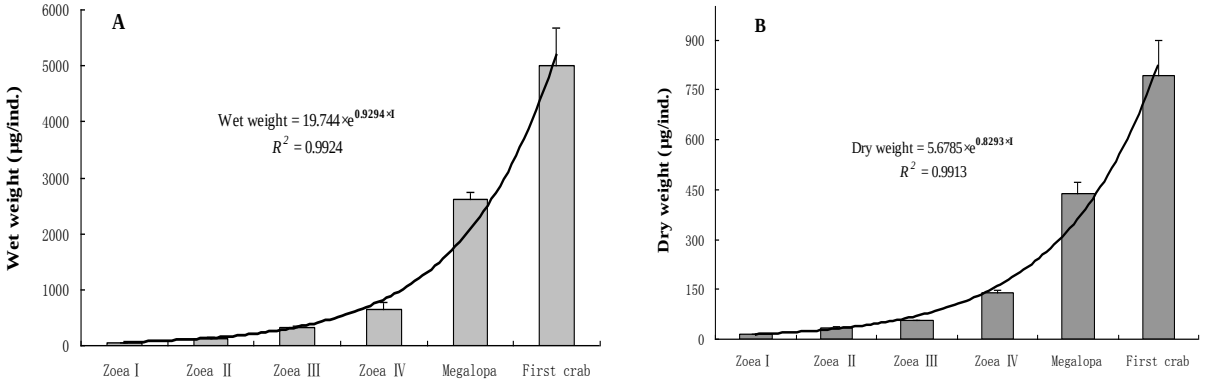

Figure 4.3. Ontogenetic changes in larval wet weight (A) and dry weight (B) through successive stages in *Portunus pelagicus*. Values are presented as means ±SD (n=5). "I": instar number, i.e. zoea I=1, zoea II=2, zoea III=3, zoea IV=4, megalopa=4 and the first crab=5.

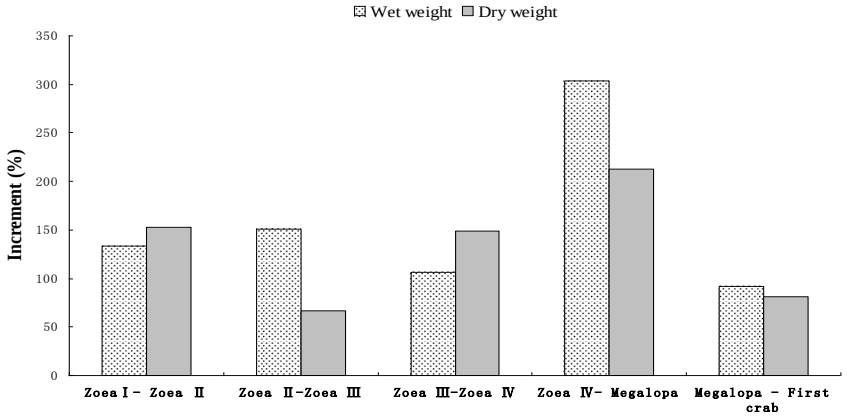

Figure 4.4. Increment (%) in wet weight and dry weight of *Portunus pelagicus* larvae through successive stages.

increases in DW during larval development from zoea I to zoea II, zoea III to zoea IV and from megalopa to the first stage crab (Figure 4.4). Among all five larval stages, the highest percentage increase in both WW (303.9%) and DW (212.3%) occurred during final zoeal stage

as zoea IV larvae developed to megalopal stage. Interestingly, a substantial difference between percentage increase in WW (150.5%) and DW (66.9%) was detected when larvae developed from zoea II to zoea III. The specific growth rate (SGR, % day^{-1}) at each larval stage and over the whole of larval development are summarized in Figure 4.5. The SGR of WW was significantly higher than the SGR of DW when larvae developed from zoea II to zoea III and from zoea IV to megalopa as well as for the OCDT (i.e. zoea I to the first crab stage) ($P < 0.05$) while the differences were not significant for other larval stages. From newly hatched zoea I to newly settled first stage crab, the mean individual WW increased 92.4 times, which is substantially higher than that of DW (58.6 times). Among four zoeal stages, the highest SGR of WW was during larval development from zoea II to zoea III while the highest SGR of DW was from zoea I to zoea II. Interesting, the lowest SGR of both WW and DW among all larval stages was observed during larval development from megalopa to the first stage crab; statistical analysis confirmed that both SGR of WW and DW for megalopal stage were significantly lower than any zoeal stages (P<0.05) (Figure 4.5).

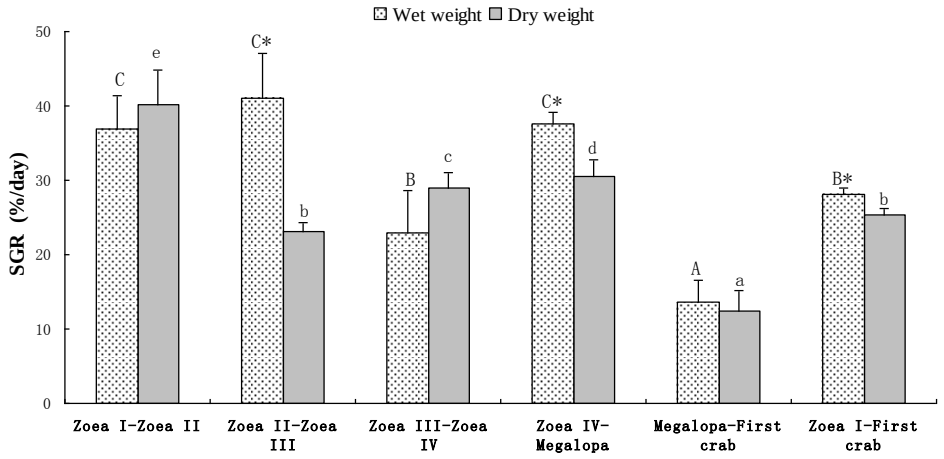

Figure 4.5. The specific growth rate (SGR, % day^{-1}) for each larval stage and overall larval development duration of *Portunus pelagicus*. Values are presented as means ±SD (n=5). The bars that do not share a same letter are significantly different (P<0.05). "*" indicates significant differences between the SGR of wet weight and SGR of dry weight (P< 0.05).

In terms of growth in size over larval development, newly hatched zoea I had an mean carapace length (CL) of 0.50 ± 0.02 mm (Figure 4.6). After 16.12 ± 0.67 days of rearing, the CL had increased to a mean of 2.49 ± 0.16 mm (4.98 fold) when they reached the first juvenile crab stage. An exponential equation could be used to describe the relationship between carapace length and the number of zoeal instars (I) (Figure 4.6). Among four zoeal stages, the highest increases in CL was recorded when larvae developed from zoea I to zoea II (+58%) (Fig. 6). After logarithmic transformation of body weight and carapace length, non-linear relationships were detected between CL and WW as well as CL and DW (Table 4.2).

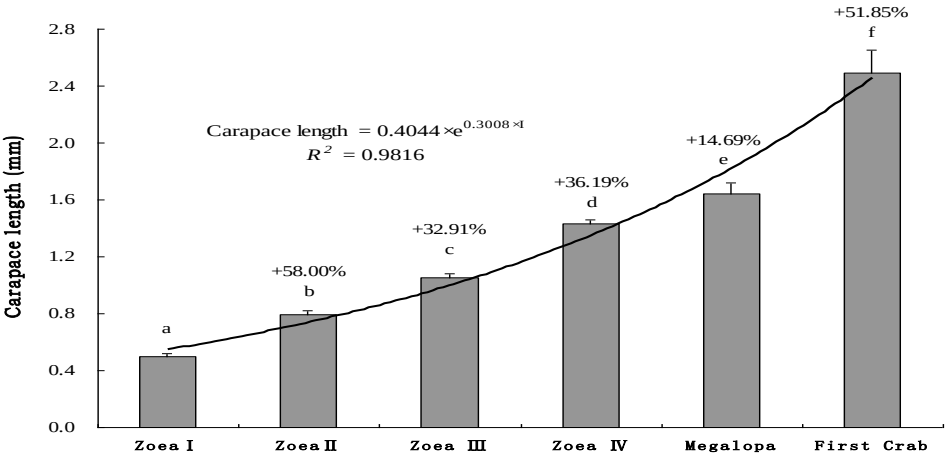

Figure 4.6. Ontogenetic changes in carapace length of *Portunus pelagicus* at each larval stage. Values are presented as means±SD (n=30). The bars that do not share a same letter are significantly different (P<0.05). "I": instar number, i.e. zoea I=1, zoea II=2, zoea III=3, zoea IV=4, megalopa=4 and the first crab=5.

Table 4.3 Gross composition and lipid class compositions (% dry weight) of microalgae *Nannochloropsis* sp., rotifers, newly hatched and enriched *Artemia* used in *Portunus pelagicus* larval culture

	Microalgae *Nannochloropsis*	**Rotifers**	**Newly hatched *Artemia* nauplii**	**Enriched *Artemia* metanauplii**
Gross composition				
Moisture (% wet weight)	85.83±1.21^{a}	92.98±0.43^{c}	86.84±0.14^{a}	90.98±0.03^{b}
Crude protein (% dry weight)	40.48±0.38^{a}	54.96±0.48^{c}	56.68±0.66^{d}	49.27±0.45^{b}
Total lipid (% dry weight)	13.98±0.02^{b}	6.80±0.19^{a}	18.26±0.43^{c}	30.34±0.74^{d}
HUFA (% dry weight)	1.97±0.17^{b}	0.56±0.07^{a}	0.51±0.03^{a}	2.79±0.21^{c}
Lipid classes (% dry weight)				
Phospholipids	11.28±0.33^{d}	5.30±0.22^{a}	7.25±0.18^{b}	9.00±0.10^{c}
Monoacylglycerol	ND	0.03±0.02^{a}	0.13±0.02^{b}	0.22±0.05^{c}
Cholesterol	ND	0.31±0.05^{a}	0.66±0.01^{b}	1.21±0.02^{c}
Pigments	0.42±0.00	ND	ND	ND
Diacylglycerol	0.32±0.04^{b}	ND	0.26±0.02^{a}	0.70±0.05^{c}
Free fatty acids	1.27±0.22^{b}	0.50±0.03^{a}	3.00±0.50^{c}	4.68±0.18^{c}
Triacylglycerol	0.65±0.09^{a}	0.66±0.26^{a}	6.96±0.06^{b}	14.51±0.38^{c}

Values are presented as means±SD (n=3). Values in a same row that do not share a same superscript are significantly different (P<0.05). "ND": values was not detectable or < 0.01%.

4.3.3 Chemical composition of live food

Both proximate compositions and lipid classes of live prey and microalgae used for larval rearing in this study are shown in Table 4.3. Among them, i.e. rotifers, newly hatched *Artemia*

Table 4.4 Fatty acid compositions (% total fatty acids) of microalgae *Nannochloropsis* sp., rotifers, newly hatched and enriched *Artemia* used in *Portunus pelagicus* larval culture

Fatty acids	Microalgae *Nannochloropsis*	Rotifers	Newly hatched *Artemia* nauplii	Enriched *Artemia* metanauplii
12:0	0.14±0.02	0.14±0.00	ND	ND
14:0	3.36±0.07^{d}	2.79±0.04^{c}	0.66±0.03^{a}	1.49±0.03^{b}
15:0	0.24±0.00^{b}	0.50±0.01^{d}	0.15±0.01^{a}	0.28±0.01^{c}
16:0	18.71±0.22^{c}	21.80±0.19^{d}	10.29±0.15^{a}	10.88±0.11^{b}
18:0	0.75±0.01^{a}	6.83±0.03^{c}	6.06±2.38^{c}	4.32±0.05^{b}
20:0	0.24±0.33^{a}	0.77±0.50^{b}	0.42±0.38^{ab}	ND
23:0	0.37±0.26^{b}	1.04±0.08^{c}	0.11±0.02^{a}	0.53±0.06^{b}
∑SFA	**24.01±0.49**^{b}	**34.99±0.46**^{c}	**17.65±2.71**^{a}	**17.62±0.04**^{a}
14:1n-7	0.45±0.00^{a}	0.53±0.02^{b}	1.31±0.03^{d}	0.71±0.02^{c}
16:1n-7	22.65±0.15^{d}	7.28±0.57^{c}	1.98±0.04^{a}	3.49±0.04^{b}
16:1n-5	0.92±0.00^{c}	ND	0.27±0.01^{b}	0.12±0.00^{a}
18:1n-9	5.63±0.06^{a}	5.43±0.33^{a}	15.70±2.08^{b}	21.27±0.02^{c}
18:1n-7	0.73±0.02^{a}	7.15±0.21^{d}	5.11±0.73^{c}	4.43±0.01^{b}
20:1n	ND	3.10±1.82^{b}	0.63±0.04^{a}	1.62±0.01^{b}
∑MUFA	**30.38±0.03b**	**23.55±2.97**^{a}	**25.00±1.37**^{a}	**31.64±0.07**^{c}
16:2n-4	0.24±0.01^{a}	0.45±0.09^{bc}	0.44±0.00^{c}	0.43±0.00^{b}
16:3n-4	0.34±0.00^{c}	1.02±0.03^{d}	0.24±0.00^{a}	0.30±0.01^{b}
18:2n-6 (LA)	4.98±0.03^{b}	4.27±0.13^{a}	5.60±0.03^{c}	8.69±0.02^{d}
18:3n-6	0.72±0.01^{c}	6.21±1.02^{d}	0.68±0.00^{b}	0.30±0.11^{a}
18:3n-3 (LNA)	0.40±0.17^{a}	2.00±0.09^{b}	31.07±0.15^{d}	18.25±0.08^{c}
18:4n-3	0.29±0.06^{a}	0.32±0.06^{a}	6.64±0.07^{c}	3.67±0.12^{b}
20:2n-6	0.15±0.11^{a}	2.12±1.38^{c}	0.22±0.01a	0.35±0.01^{b}
20:3n-6	0.14±0.20^{a}	1.19±0.18^{b}	0.16±0.07a	0.13±0.10^{a}
20:4n-6 (ARA)	3.23±0.06^{b}	3.62±0.11^{c}	1.07±0.11a	1.01±0.02^{a}
20:3n-3	ND	0.52±0.19^{a}	1.00±0.00^{b}	0.64±0.00^{a}
20:4n-3	0.10±0.09^{a}	0.52±0.19^{b}	ND	0.96±0.01^{c}
20:5n-3 (EPA)	24.31±0.51^{d}	7.67±0.01^{c}	1.33±0.04^{a}	4.43±0.09^{b}
22:2n-6	0.14±0.20^{ab}	0.70±0.31^{c}	0.20±0.01^{a}	0.26±0.03^{b}
22:5n-3	ND	1.95±0.77^{c}	0.11±0.07^{a}	0.75±0.02^{b}
22:6n-3 (DHA)	0.28±0.03^{a}	0.96±0.31^{b}	0.23±0.02^{a}	5.22±0.13^{c}
∑PUFA(≥ 18:2n)	**34.80±0.01**^{b}	**32.22±2.59**^{a}	**48.32±0.11**^{d}	**44.65±0.12**^{c}
n-3PUFA	25.48±0.44^{b}	14.11±1.98^{a}	40.38±0.27^{d}	33.91±0.05^{c}
n-6PUFA	9.32±0.46^{b}	18.11±0.61^{d}	7.94±0.16^{a}	10.75±0.17^{c}
n-3/n-6	2.74±0.18^{b}	0.78±0.08^{a}	5.09±0.14^{d}	3.16±0.06^{c}
∑HUFA(≥ 20:3n)	28.15±0.53^{d}	16.61±1.90^{c}	3.90±0.13^{a}	13.14±0.17^{b}
DHA/EPA	0.01±0.00^{a}	0.13±0.04^{b}	0.17±0.02^{c}	1.18±0.00^{d}

Values are presented as means±SD (n=3). Values in a same row that do not share a same superscript are significantly different (P<0.05). "ND": values was not detectable or < 0.1%.

nauplii, enriched *Artemia* metanauplii and microalgae *Nannochloropsis* sp., rotifers contained the highest level of moisture (92.98%) and the lowest total lipid (6.80%). As expected, enriched *Artemia* metanauplii contained much higher levels of total lipid (30.34% dry weight) and highly unsaturated fatty acids (HUFA) (2.79% dry weight) than the newly hatched, un-enriched nauplii (total lipid: 18.26% dry weight; HUFA: 0.51% dry weight). As for the lipid class contents, the

microalgae *Nannochloropsis* sp. contained the highest level of phospholipids (PL) while the enriched *Artemia* metanauplii had the highest cholesterol (CHO) and triacylglycerol (TG) contents (Table 4.3). As rotifers contained the lowest level of total lipid, this was reflected in all lipid classes since they were all found at the lowest levels when compared to other live foods tested.

The fatty acid compositions (% total fatty acids) of the live foods used for the larval culture are shown in Table 4.4. Among saturated fatty acids (SFA), 16:0 was the dominant fatty acid for all of them while rotifers contained the highest percentage of SFA (34.99%) ($P<0.05$) (Table 4.4). For mono-unsaturated fatty acids (MUFA), *Nannochloropsis* sp. contained the highest level of 16:1n-7 fatty acid while enriched *Artemia* metanauplii had the highest percentage of 18:1n-9 fatty acid ($P<0.05$). Of the poly-unsaturated fatty acids (PUFA), newly hatched *Artemia* nauplii had a significantly higher level of 18:3n-3 fatty acid (31.07%) than the other live foods, while microalgae *Nannochloropsis* sp. contained the highest percentage of 20:5n-3 fatty acid (24.31%). Meanwhile, enriched *Artemia* metanauplii had significantly higher levels of 18:2n-6, 20:5n-3 (EPA), 22:6n-3 (DHA) and a higher DHA/EPA ratio than that of newly hatched *Artemia* nauplii ($P<0.05$, Table 4.4).

Table 4.5 Moisture (% wet weight), total lipid and lipid class contents (% dry weight) of newly hatched zoea I larvae, and newly moulted zoea VI, megalopae and first stage crabs of *Portunus pelagicus*

	Zoea I (n=4)	**Zoea IV (n=3)**	**Megalopa (n=2)**	**First stage crab (n=3)**
Moisture (% wet weight)	79.32 ± 2.01^{a}	79.21 ± 2.48^{a}	83.26 ± 1.26^{b}	84.11 ± 1.09^{b}
Total lipid (% dry weight)	6.95 ± 1.68^{a}	11.39 ± 0.41^{c}	8.19 ± 0.41^{a}	10.43 ± 0.26^{b}
Lipid classes (% dry weight)				
Phospholipids	6.05 ± 1.06^{ab}	5.90 ± 0.09^{a}	5.30 ± 0.10^{b}	5.17 ± 0.03^{b}
Monoacylglycerol	0.02 ± 0.02^{a}	ND	ND	0.06 ± 0.01^{b}
Cholesterol	0.67 ± 0.13^{b}	0.66 ± 0.05^{b}	0.48 ± 0.05^{a}	0.42 ± 0.01^{a}
Diacylglycerol	ND	ND	ND	0.08 ± 0.01
Triacylglycerol+Free fatty acids	0.21 ± 0.08^{a}	4.84 ± 0.45^{c}	2.40 ± 0.55^{b}	4.69 ± 0.24^{c}

Values are presented as means ±SD. "n": the number of replicates (2-3g larvae was pooled as one replicate). Values in a same row that do not share a same superscript are significantly different ($P<0.05$). "ND": values was not detectable or < 0.01%.

4.3.4 Total lipid, lipid classes and fatty acid profile of crab larvae

The moisture, total lipid and various lipid classes (% dry weight) of newly hatched zoea I, newly moulted zoea IV, megalopa and the first stage crab of *P. pelagicus* are shown in Table 4.5. Zoea I and zoea IV larvae contained significantly lower moisture contents than megalopae

and the first stage crabs (P <0.05). The highest total lipid and TG+FFA contents were detected in zoea IV larvae and zoea IV, together with zoea I larvae, also contained significantly higher CHO levels (0.66-0.67% dry weight) than megalopae and the first stage crabs. As for the percentage compositions of lipid classes (%total lipid, Table 4.6), zoea I had the highest percentage of PL and CHO, but dramatically lowest percentage of TG+FFA among the four early life stages of *P. pelagicus* analyzed (P <0.05). The first stage crab contained the highest level of TG+FFA (45%) among the four stages.

Table 4.6 Lipid class compositions (% total lipid) of newly hatched zoea I larvae, newly moulted zoea IV, megalopae and first stage crabs of *Portunus pelagicus*

	Zoea I (n=4)	**Zoea IV (n=3)**	**Megalopae (n=2)**	**First stage crab (n=3)**
Phospholipids	87.03 ± 0.82^{c}	51.81 ± 2.64^{a}	64.85 ± 4.39^{b}	49.60 ± 1.22^{a}
Monoacylglycerol	0.30 ± 0.27^{a}	ND	ND	0.62 ± 0.05^{b}
Cholesterol	9.64 ± 0.32^{c}	5.76 ± 0.22^{b}	5.91 ± 0.84^{b}	4.03 ± 0.01^{a}
Diacylglycerol	ND	ND	ND	0.74 ± 0.03
Triacylglycerol + Free fatty acids	3.09 ± 1.01^{a}	42.44 ± 2.42^{c}	29.24 ± 5.23^{b}	45.00 ± 1.13^{c}

Values are presented as means ±SD. "n": the number of replicates (2-3g larvae was pooled as one replicate). Values in a same row that do not share a same superscript are significantly different (P<0.05). "ND": values was not detectable or < 0.1%.

The fatty acid compositions of the four early life stages of *P. pelagicus* were calculated as percentage of total fatty acids (Table 4.7). While the newly hatched zoea I contained the highest percentage of SFA (30.12%), which was significantly higher than all other stages (P <0.05), 16:0 and 18:0 fatty acids were the major constituents of SFA for all stages analyzed. The percentage of mono-unsaturated fatty acids (MUFA) increased with larval development with zoea I had the lowest level (18.94%), followed by zoea IV (28.23%), megalopa (30.21%) and the first stage crab (32.59%), the differences among four stages were all statistically significant (P<0.05). However, for all four stages, 18:1n-9 and 18:1n-7 fatty acids were the two most dominant MUFA. Of carbon 18 poly-unsaturated fatty acids (C18-PUFA), zoea I contained significantly lower levels of 18:2n-6, 18:3n-3 and 18:4n-3 fatty acids than other stages while zoea IV contained significantly higher percentages of 18:3n-3 and 18:4n-3 fatty acids among the four stages (P<0.05). As to highly unsaturated fatty acids (HUFA), zoea I contained the significantly higher 20:4n-6 (ARA), EPA and DHA than other stages (P<0.05). Meanwhile, from zoea IV to the first crab stage, DHA level and DHA/EPA ratio increased gradually while EPA levels remained relative stable at 6.09-7.39% (Table 4.7).

The contents of major fatty acids were also converted as mg per g of larval dry weight for the four stages (mg/g dry weight, Table 4.8). It was shown that the newly hatched zoea I generally contained the lowest levels of MUFA and C18-PUFA among the four stages with the

Table 4.7 Fatty acid compositions (% total fatty acids) of newly hatched zoea I larvae, newly moulted zoea IV, megalopae and first stage crabs of *Portunus pelagicus*

Fatty acids	Zoea I (n=4)	Zoea IV (n=3)	Megalopae (n=2)	First stage crab (n=3)
13:0	0.18 ±0.07^{ab}	0.12 ±0.01^{a}	0.26 ±0.05^{b}	ND
14:0	1.33 ±0.29^{c}	0.65 ±0.13^{ab}	0.86 ±0.01^{b}	0.70 ±0.02^{a}
16:0	16.41 ±2.16^{d}	8.39 ±0.13^{b}	6.57 ±0.67^{a}	9.65 ±0.39^{c}
18:0	11.18 ±0.53^{d}	5.63 ±0.32^{b}	4.21 ±0.31^{a}	6.26 ±0.29^{c}
20:0	0.21 ±0.08^{a}	0.60 ±0.43^{a}	1.37 ±1.28^{ab}	2.05 ±0.02^{b}
23:0	0.24 ±0.13^{ab}	0.13 ±0.04^{a}	0.28 ±0.23^{ab}	0.39 ±0.04^{b}
∑SFA	**30.12 ±2.21**^{c}	**15.71 ±0.69**^{a}	**13.98 ±1.92**^{a}	**19.36 ±0.71**^{b}
14:1n-7	0.19 ±0.08^{a}	0.50 ±0.01^{c}	0.42 ±0.02^{b}	0.69 ±0.00^{d}
16:1n-7	3.04 ±0.77^{b}	1.88 ±0.03^{a}	1.69 ±0.05^{a}	1.83 ±0.03^{a}
16:1n-5	0.17 ±0.21	0.07 ±0.09	0.10 ±0.14	0.17 ±0.04
18:1n-9	8.37 ±0.86^{a}	18.06 ±0.17^{b}	19.42 ±0.05^{c}	21.19 ±0.23^{d}
18:1n-7	5.01 ±1.82	6.50 ±0.02	7.20 ±0.11	7.25 ±0.00
20:1n	2.15 ±0.66^{b}	1.22 ±0.05^{a}	1.38 ±0.11^{a}	1.45 ±0.03^{a}
∑MUFA	**18.94 ±2.69**^{a}	**28.23 ±0.00**^{b}	**30.21 ±0.01**^{c}	**32.59 ±0.18**^{d}
16:2n-4	0.62 ±0.02	0.53 ±0.43	0.67 ±0.65	0.63 ±0.09
16:3n-4	1.03 ±0.13^{b}	0.48 ±0.57^{ab}	0.15 ±0.04^{a}	0.18 ±0.01^{a}
18:2n-6 (LA)	0.54 ±0.47^{a}	7.02 ±0.12^{c}	7.26 ±0.05^{c}	6.74 ±0.04^{b}
18:3n-6	0.39 ±0.31^{ab}	0.42 ±0.17^{ab}	0.41 ±0.01^{b}	0.26 ±0.01^{a}
18:3n-3 (LNA)	0.71 ±0.41^{a}	23.56 ±0.05^{d}	20.19 ±1.24^{c}	16.88 ±0.22^{b}
18:4n-3	1.16 ±0.17^{a}	2.99 ±0.04^{c}	2.57 ±0.14^{b}	2.09 ±0.25^{b}
20:2n-6	1.46 ±0.67^{ab}	0.31 ±0.06^{b}	0.66 ±0.52^{ab}	0.17 ±0.00^{a}
20:3n-6	1.02 ±0.92^{ab}	0.51 ±0.55^{ab}	0.23 ±0.18^{a}	0.91 ±0.00^{b}
20:4n-6 (ARA)	6.89 ±1.02^{c}	1.81 ±0.01a	2.16 ±0.15^{b}	2.16 ±0.02^{b}
20:3n-3	0.11 ±0.08^{a}	2.64 ±0.02^{b}	2.25 ±1.44^{b}	0.02 ±0.02^{a}
20:4n-3	0.21 ±0.26^{a}	1.27 ±0.02^{c}	1.44 ±0.02^{d}	0.99 ±0.01^{b}
20:5n-3 (EPA)	18.39 ±2.41^{d}	6.09 ±0.05^{a}	7.39 ±0.01^{c}	6.58 ±0.03^{b}
22:2n-6	0.69 ±0.19^{b}	0.33 ±0.00^{a}	0.45 ±0.08^{ab}	0.53 ±0.04^{b}
22:5n-3	1.65 ±0.10^{c}	1.11 ±0.02^{b}	1.00 ±0.16^{b}	0.61 ±0.02^{a}
22:6n-3 (DHA)	9.74 ±1.00^{d}	1.73 ±0.04^{a}	3.05 ±0.05^{b}	4.24 ±0.02^{c}
∑PUFA(≥ 18:2n)	**42.43 ±3.71**^{ab}	**49.65 ±1.03**^{c}	**49.06 ±2.18**^{bc}	**42.18 ±0.47**^{a}
n-3PUFA	31.68 ±2.67^{ab}	39.41 ±0.12^{bc}	37.89 ±2.76^{b}	31.40 ±0.47^{a}
n-6PUFA	10.63 ±0.94	10.40 ±0.69	11.16 ±0.58	10.78 ±0.00
n-3/n-6	2.98 ±0.03^{a}	3.80 ±0.24^{b}	3.40 ±0.42^{b}	2.91 ±0.04^{a}
∑HUFA(≥ 20:3n)	**38.00 ±3.62**^{c}	**15.17 ±0.58**^{ab}	**17.52 ±1.62**^{b}	**15.50 ±0.01**^{a}
DHA/EPA	0.54 ±0.11^{c}	0.28 ±0.00^{a}	0.41 ±0.01^{b}	0.64 ±0.00^{c}

Values are presented as means ±SD. "n": the number of replicates (2-3g larvae was pooled as one replicate). Values in a same row that do not share a same superscript are significantly different (P<0.05). "ND": values was not detectable or < 0.1%.

exception of 16:1n-7 fatty acid. Although zoea IV had significant higher PUFA content (33.47 mg/g dry weight) than that of other stages, it had the lowest DHA content (1.16 mg/g dry

weight), which was significantly lower than all other stages (P<0.05). Significantly higher levels of SFA and MUFA contents were found in the first stage crab as compared to the other stages (*P*<0.05) (Table 4.8).

Table 4.8 Major fatty acid contents (mg g^{-1} dry weight) of newly hatched zoea I larvae, newly moulted zoea IV, megalopae and first crabs of *Portunus pelagicus*

Fatty acids	Zoea I (n=4)	Zoea IV (n=3)	Megalopae (n=2)	First stage crab (n=3)
16:0	5.36 ±0.71^{bc}	5.64 ±0.09^{b}	3.23 ±0.33^{a}	6.04 ±0.25^{c}
18:0	3.65 ±0.17^{a}	3.78 ±0.21^{b}	2.07 ±0.15^{a}	3.92 ±0.18^{b}
∑SFA	**9.84 ±0.72**^{b}	**10.56 ±0.46**^{b}	**6.87 ±0.94**^{a}	**12.12 ±0.45**^{c}
16:1n-7	0.99 ±0.25^{ab}	1.26 ±0.02^{c}	0.83 ±0.03^{a}	1.15 ±0.02^{b}
18:1n-9	2.73 ±0.28^{a}	12.14 ±0.11^{c}	9.54 ±0.02^{b}	13.26 ±0.15^{d}
18:1n-7	1.85 ±0.51^{a}	4.37 ±0.01^{c}	3.54 ±0.06^{b}	4.54 ±0.00^{d}
∑MUFA	**6.19 ±0.88**^{a}	**18.87 ±0.13**^{c}	**14.84 ±0.03**^{b}	**20.39 ±0.12**^{d}
18:2n-6	0.18 ±0.15^{a}	4.71 ±0.01^{d}	3.57 ±0.02^{b}	4.22 ±0.02^{c}
18:3n-3	0.23 ±0.13^{a}	15.84 ±0.04^{c}	9.92 ±0.61^{b}	10.56 ±0.14^{b}
18:4n-3	0.28 ±0.19^{a}	2.01 ±0.03^{c}	1.26 ±0.07^{b}	1.31 ±0.16^{b}
20:4n-6	2.25 ±0.33^{d}	1.22 ±0.00^{b}	1.06 ±0.07^{a}	1.35 ±0.00^{c}
20:5n-3	6.01 ±0.79^{c}	4.09 ±0.03^{b}	3.63 ±0.00^{a}	4.12 ±0.02^{b}
22:6n-3	3.18 ±0.33^{d}	1.16 ±0.03^{a}	1.50 ±0.02^{b}	2.65 ±0.01^{c}
∑PUFA(≥ 18:2n)	**13.86 ±1.21**^{a}	**33.47 ±0.54**^{d}	**24.11 ±1.07**^{b}	**26.40 ±0.30**^{c}
∑HUFA(≥ 20:3n)	**12.41 ±1.18**^{d}	**10.20 ±0.39**^{c}	**8.61 ±0.80**^{a}	**9.70 ±0.01**^{b}
Unknown	2.24 ±0.91	3.53 ±0.18	2.92 ±0.43	3.17 ±0.02
Total fatty acids	30.42 ±0.91^{a}	63.67 ±0.18^{d}	46.22 ±0.43^{b}	59.41 ±0.22^{c}

Values are presented as means ±SD. "n": the number of replicates (2-3g larvae was pooled as one replicate). Values in a same row that do not share a same superscript are significantly different (*P*<0.05).

4.4 Discussion

This study described the patterns of larval growth for the blue swimmer crab, *P. pelagicus*, reared in the laboratory. The developmental patterns and equations established allow conversion and estimation of one growth parameter from another, for example, estimating larval dry weight based on wet weight. The basic information obtained on larval development and lipid composition of cultured larvae can be used to compare with that of wild larvae, resulting in the improvement of hatchery diets for the future *P. pelagicus* seed production (Copeman et al., 2012). Therefore, these data have both ecological and aquaculture relevance.

As in many brachyuran crab species, the increase in biomass during larval development of *P. pelagicus* could be described as exponential to successive larval stages. The slopes of the linear regressions were 0.8293 for larval dry weight and 0.9294 for larval wet weight of *P. pelagicus*. These data are similar to those of larval swimming crab, *Portunus trituberculatus*

(slope for dry weight = 0.907; slope for wet weight = 0.905) (Lim and Hirayama, 1991), but higher than that reported for the other several crabs, e.g. the spider crab, *Maja brachydactyla* (slope for dry weight = 0.63) (Andrés et al., 2008), the flying crab, *Liocarcinus holsatus* (slope for dry weight= 0.62) (Harms, 1990) and the salt marsh crab, *Neohelice* (formerly *Chasmagnathus*) *granulata* (slope for dry weight = 0.69) (Anger and Ismael, 1997).

However, since there are substantial inter-specific and intra-specific differences in developmental durations of larval instars, the growth equations mentioned earlier are inappropriate for the comparison of growth. The specific growth rate (SGR) is a common used parameter for evaluating growth rate under different conditions and can be used both intra-species (Guillaume, 2001; Romano and Zeng, 2006) and inter-species (Mcandrew and Majumdar, 1989). Therefore, SGRs were calculated in the present study, which provide a mean for comparing growth rate between species. For instance, over larval development from newly hatched zoea I to the first crab stage, dry weight increase of *P. trituberculatus* (9058%) was substantially higher than that of *P. pelagicus* (5862%), while the SGR calculated for *P. pelagicus* (25.36) is higher than that of *P. trituberculatus* (22.58) (Lim and Hirayama, 1991) (Table 4.9). Hence, *P. pelagicus* larvae had higher growth rate than that of *P. trituberculatus* under the experimental conditions reported. It is evident that *P. pelagicus* larvae grow faster than all other species. Furthermore, the previous study reported a dramatically lower SGR of 8-9 during the development from the 4th to the 9th crab stage for *P. pelagicus* cultured under similar conditions of temperature and salinity (Romano and Zeng, 2006). This is considerably lower than that of larval *P. pelagicus* (25.36) and illustrates the slowing in the rate of relative growth with increasing age of an animal (Southgate and Lucas, 2003). Therefore, SGR can also be used to assess growth at different life stages within the same species (Folkvord, 2005).

For most brachyuran crabs, the megalopal stage is the longest larval stage, that normally accounts for 20-50% of total larval developmental time. The megalopal stage of *P. pelagicus* accounts for 29.28% of total larval development time, which is similar to other Portunid crabs, such as the swimming crab, *P. trituberculatus* (Lim and Hirayama, 1991), the mud crab, *Scylla serrata* (Wang et al., 1995b), the blue crab, *Callinectes sapidus* (Zmora et al., 2005) and the shore crab, *Carcinus maenas* (Dawirs, 1985) and some non-portunid crabs, such as the Chinese mitten crab, *Eriocheir sinensis* (Zhang and Li, 2002) and the salt marsh crab, *N. granulata* (Anger and Ismael, 1997). However, when compared to the spider crab, *M. araneus* (Anger and Dawirs, 1982) and the bristly crab, *Pilumnus hirtellus* (Hartnoll and Mohamedeen, 1987), it is shorter since megalopal stage of these two crabs ranges from 47 to 50% of total larval duration. In the field, an extended duration for megalopa may be an adaption for *P. pelagicus*, to search

and reach suitable habitat to settle (Bryars and Havenhand, 2006). This speculation is supported by the SGR results of the present study showing that both SGR of dry weight and wet weight of *P. pelagicus* megalopae were significantly lower than other larval stages.

Table 4.9 Dry weight of zoea I and the first stage crabs (C1), percentage increase in dry weight, overall cumulative development time (OCDT) and SGR in dry weight relative to the larval development of various crab species

	DW of Z I (μg)	DW of C1(μg)	Percentage increase	OCDT (days)	SGR (% day^{-1})	Water temperature	Source
Hyas araneus	63	420	567%	48	3.95	12 ℃	Anger et al. (1983)
Maja brachydactyla	94.0	627	567%	18	10.54	18 ℃	Adrés et al. (2008)
Carcinus maenas	10.9	188.6	1,085%	32	8.92	18 ℃	Dawirs et al. (1986)
Chasmagnathus granulata	8.45	276	3,160%	32	10.89	18 ℃	Anger and Ismael (1997)
Callinectes sapidus	8.0	726	8,975%	35	12.88	22 ℃	Zmora et al., 2005
Portunus trituberculatus	18.0	1649	9,058%	20	22.57	23-24 ℃	Lim and Hirayama, 1991
Eriocheir sinensis	14.5	1203	8,197%	23	19.21	23-25 ℃	Zhang and Li (2002)
Scylla serrata	10.7	1234	11,433%	28	16.96	26-28 ℃	Wang et al. (1995)
Portunus pelagicus	13.3	793	5,862%	16.1	25.36	28 ℃	This study
Ranina ranina	90.0	14350	15,844%	34	14.92	29 ℃	Minagawa et al. (1993)

DW of Z I: Dry weight of zoea I; DW of C1: Dry weight of the first stage crabs; SGR: Specific growth rate.

It is worth noting that the duration of a larval stage and its proportion to the total larval development time might change depending on quality and quantity of larval diets. For example, when zoea I and II larvae of *P. pelagicus* were fed rotifers only, duration of zoea II stage was always longer than zoea I (Bryars and Havenhand, 2006). However, when zoea I and II larvae were fed solely on newly hatched *Artemia* nauplii (Josileen and Zeng, unpublished data) or a mixture of rotifers and *Artemia* nauplii (current study), the duration of zoea II was shorter/similar than that of zoea I. There are two possible explanations: (1) the newly hatched *Artemia* nauplii contained significantly higher levels of lipids and HUFA than rotifers and it has been reported that high lipid and HUFA contents could accelerate larval development in *E. sinensis* (Sui et al., 2007) and *P. trituberculatus* (Takeuchi et al., 1999a); (2) compared to newly hatched *Artemia* nauplii, rotifers are smaller and contain substantially less energy per individual. As the consequence, energy budget suggest it is highly inefficient for the crab larvae to feed on

rotifers as they would need to capture and ingest many more rotifers to gain the same energy level provided by *Artemia* nauplii (Zeng and Li, 1999). Duration of the larval stage may therefore be extended to accumulate sufficient nutrition and energy reserves to enable a successful moult.

Table 4.10 Number of zoeal stages, megalopal duration, overall cumulative larval development time (OCDT) and percentage of megalopal duration to OCDT of various crab species

	Number of zoeal stages	Megalopal duration (days)	OCDT (days)	Megalopal duration/OCDT (%)	Water temperature	Source
Maja brachydactyla	2	4	18	22.22	18 ℃	Adrés *et al*. (2008)
Hyas araneus	2	24	48	50.00	12 ℃	Anger and Dawirs (1982)
Pilumnus hirtellus	4	14.3	30.1	47.51	20 ℃	Hartnoll and Mohamedeen (1987)
Chasmagnathus granulata	4	12	32	37.50	18 ℃	Anger and Ismael (1997)
Carcinus maenas	4	12	32	37.50	18 ℃	Dawirs (1985)
Portunus trituberculatus	4	6	20	30.30	23-24 ℃	Lim and Hirayama (1991)
Portunus pelagicus	4	4.7	16.1	29.28	28 ℃	This study
Eriocheir sinensis	5	7	23	30.43	23-25 ℃	Zhang and Li (2002)
Menippe mercenaria	5	8	28	28.57	30 ℃	Brown *et al*. (1992)
Scylla serrata	5	8	28	28.57	26-28 ℃	Wang *et al*. (1995)
Charybdis feriatus	6	5-6	25	20.00-24.00	27.5-29.5 ℃	Wang *et al*. (2008)
Ranina ranina	7-8	19	53	35.85	26.1-29.9 ℃	Minagawa *et al*. (1990)
Callinectes sapidus	8	8	35	22.85	22 ℃	Zmora *et al*. 2005
Paralithodes camtschaticus	4	21	35	40.00	11 ℃	Swingle *et al*. (2013)

The mass mortality, occurred during metamorphosis moulting to megalopae and megalopae to the first crab, are common phenomenon for brachyuran crabs, including *S. serrata* (Holme et al., 2007b), *P. trituberculatus* (Lim and Hirayama, 1991) and *E. sinensis* (Wu et al., 2007c). The previous studies have reported this mass mortality is due to moulting death syndrome (MDS), when crab larvae do not have the ability to completely shed the old carapace

or integument of the chelipeds or walking legs (Hamasaki et al., 2002a). It is thought that a major cause of MDS is the insufficient energy and nutrient intakes during the final zoeal stage (Minagawa et al., 1993), and related particularly to HUFA, CHO, PL and total lipids stored in the hepatopancreas (Holme et al., 2007b). Hence, the optimization of larval nutrition and culture physical-chemical conditions, may reduce mortality over this critical period (Hamasaki et al., 2002b; Suprayudi et al., 2004a,b).

During larval development of *P. pelagicus*, total lipid and lipid class contents underwent significant changes. The final zoeal stage, zoea IV were found to have the highest total lipid content among all larval stages and there are two possible explanations for this: firstly, it is likely related to the high energy and nutritional reserves required to facilitate crucial process of metamorphosis to megalopae (Minagawa et al., 1993). Secondly, lipids can contribute to larval buoyancy, and reduce energy consumption associated with maintaining position in water column (Roustaian et al., 2001). As the final holoplanktonic stage, the highest lipid content of zoea IV may assist their buoyancy. Cholesterol, as a precursor for ecdysone, is essential for the normal growth and ecdysis of crustaceans, however, crustaceans cannot synthesis cholesterol *de novo* (Teshima and Kanazawa, 1971), and it has to be obtained from their diet (Holme et al., 2007c). Although the enriched *Artemia* meta-nauplii had the highest cholesterol content of the live foods used, cholesterol contents of *P. pelagicus* decreased significantly from zoea IV. This decrease may indicate megalopae and juvenile *P. pelagicus* had lower cholesterol requirements than the zoeal stage larvae. Moreover, during the moulting cycle, the early stage of crustacean larvae generally contains the lower total lipid levels than the middle and late moulting larvae (Limbourn and Nichols, 2009). Therefore, our sampled larvae had the low lipid levels in this study because those larvae were sampled from the early moulting stage.

While the fatty acid composition of newly hatched zoea I of *P. pelagicus* is similar to that of *P. trituberculatus* (Wu et al., 2010c) and *S. serrata* (Holme et al., 2009b), *P. pelagicus* zoea I contained substantially higher EPA level and had a lower DHA/EPA ratio. Our recent study has shown that although the mature ovary and newly spawned eggs contained higher DHA/EPA ratio, the significantly decreasing trend was found on the DHA/EPA ratio during the embryonic development of *P. pelagicus*, resulting in a lower DHA/EPA of newly hatched zoea I (See Chapter 2). This suggests EPA might be more important for *P. pelagicus* early larvae. A trend of increasing levels of 18:1n-9, DHA and ∑MUFA but decreasing 18:3n-3 and 18:4n-3 fatty acids were found from zoea IV onwards, which likely reflected fatty acid profile of their diet. As from zoea III onward, replacing un-enriched nauplii, enriched *Artemia* were fed to larvae,

and fatty acid analysis showed that enriched *Artemia* contained higher levels of 18:1n-9, DHA and ∑MUFA but lower levels of 18:3n-3 and 18:4n-3 fatty acids than newly hatched nauplii.

However, although the enrich *Artemia* and un-enriched nauplii contained similar levels of ARA (ca.1% total fatty acids), megalopae and first stage crabs contained significantly higher percentage of ARA than zoea IV (P <0.05). It indicates that megalopae and first stage crabs may have a higher ARA requirement than pelagic zoeal larvae. Sargent and Whittle (1981) had suggested that ARA, derived from the benthic algae and therefore more accessible to juvenile and adult crustaceans, is relatively low in planktonic crustacean larvae because their natural diets contain relatively low ARA (Sargent and Whittle, 1981). It has also been suggested that pelagic crustacean larvae may have a lower ARA requirements than that of juveniles (Ritar et al., 2003). The present study also showed that later larvae of *P. pelagicus* from zoea IV onward contained significantly higher levels of 18:2n-6 (6.74-7.26% total fatty acids) than newly hatched zoea I, which likely reflected the enriched *Artemia* containing higher 18:2n-6 than the rotifer and un-enriched nauplii. Although it is not clear whether 18:2n-6 is one of the essential fatty acids (EFA) for marine crustacean larvae (Sheen and Wu, 2002), more recently, Wu *et al.* (2010a) speculated that the accumulation of 18:2n-6 in *E. sinensis* tissues may serve as a mechanism to counter for negative effects caused by dietary HUFA deficiency (Wu et al., 2010a). Previous studies have also shown that an increase in ∑MUFA level is an indicator for essential fatty acids deficiency in crustaceans (Reigh and Stickney, 1989; Sheen and Wu, 2002; Suprayudi et al., 2004b). Therefore, the increasing ∑MUFA levels during larval ontogeny and lower ARA contents at later larval stages may suggest that later larvae of *P. pelagicus* in this study may not have an optimal supply of HUFA despite being fed enriched *Artemia*. Unfortunately, the HUFA requirements are completely unknown for blue swimmer crab larvae, which warrant further investigations.

4.5 Conclusion

In summary, our results provide a first description of the developmental patterns of larval growth for *P. pelagicus*. The highest increase in biomass and high SGR were recorded when zoea IV moulted to megalopa and when zoea I moulted to zoea II, respectively. Mass mortality occurred during the critical metamorphosis of zoea IV to megalopa and megalopa to the first crab stage, and this may be linked to insufficient energy and nutritional intakes during the previous stages (zoea IV and megalopa). Finally, the data on ontogenetic changes in lipid composition during larval development should provide important clues for understanding larval lipid nutrition and developing suitable dietary regimes for *P. pelagicus* larvae.

Chapter 5

Ontogenetic patterns of growth and lipid composition changes during early to mid-stages of development in *Panulirus ornatus* phyllosoma

5.1 Introduction

The rock spiny lobster, *Panulirus ornatus*, is widely distributed throughout the Indo-West Pacific region, particularly in the tropical and subtropical waters of the region, such as the Torres Straits and North Eastern Australia (Ye, 2008; Ye and Dennis, 2009). This lobster species is targeted in a sustainable fishery in this region with a managed annual harvest in Australia and Papua New Guinea of approximately 662 tonnes (Ye, 2008). *P. ornatus* has many favorable attributes for consumers, and hence an attractive aquaculture candidate, including non-aggressive communal behavior, high fecundity, rapid growth, and delicate flavor with global demand for the product increasing at 15% per annum (Murugan et al., 2005; Jones et al., 2007; Smith et al., 2009). However, it is evident that globally, the fishery for wild *P. ornatus* is fully exploited with landing yields plateauing during the past 10 years despite increased fishing effort (FAO, 2005; Hung and Tuan, 2009). It is likely that sustainable production of this species will only be possible through closed-life cycle aquaculture production through development of commercially robust hatchery technologies (Priyambodo and Sarifin, 2009; Smith et al., 2009).

Open-life cycle aquaculture of *P. ornatus* is established in Vietnam and Indonesia (Jones et al., 2007; Priyambodo and Sarifin, 2009). However, this form of aquaculture is reliant on the capture of wild planktonic puerulus and just settled benthic juveniles, which has the high likelihood that annual recruits from the wild will be overexploited resulting in detrimental impacts on the long term viability of natural populations (Jones et al., 2007). Over and above the potential impacts on local marine ecosystems, wild puerulus is known to vary in both quality and quantity, as well as being a vector of introducing pathogens into a grow-out system (Meng et al., 2009; O'Sullivan, 2010). The establishment of hatchery technologies based on closed larval cycle is the key to avoid overexploitation of wild stocks and the development of sustainable production for Palinurid lobsters as well as facilitating genetic selection for desirable traits, such as accelerated growth, colouration, flesh quality or pathogen resistance (Smith et al., 2009; O'Sullivan, 2010).

The larval cycle of *P. ornatus* is complex, consisting of eleven distinct morphological pelagic planktonic phyllosoma stages, a transitional planktonic post-larval puerulus stage and a

first instar benthic juvenile (also known as post-puerulus) (Smith et al., 2009b). The current bottleneck in the aquaculture production of all Palinurid marine lobster species is the lack of ability to rear large quantities larvae, from egg through the multiple planktonic phyllosoma stages to puerulus and the benthic juvenile stages. Successful larval production has been achieved for *P. ornatus* in Australia between 2006 and 2010, but only at small scales (Smith et al., 2009; O'Sullivan, 2010). Survival from egg to phyllosoma, puerulus and the first instar juvenile is currently less than 5% (Hall, pers comm), which in part is due to the limited information on larval feeding and nutritional requirements for this species (O'Sullivan, 2010). Larval nutrition, and in particular lipid nutrition, is deemed a critical area to the successful commercialization of this species (O'Sullivan, 2010).

Lipids, including phospholipids (PL), cholesterol (CHO), triacylglycerol (TG) and free fatty acids (FFA), play important roles in the survival and growth of crustacean larvae, by providing energy for metabolism, maintenance of structure and function of cellular biomembranes and precursors of eicosanoids (Anger, 1998; Ritar et al., 2003; Limbourn and Nichols, 2009). As critical nutritional components, the quality and quantity of dietary lipids have the potential to affect the development, survival and physiological status of crustacean larvae (Liddy et al., 2005; Holme et al., 2007a,b; Sui et al., 2007). However, the dietary requirement of phyllosomas is unknown. An examination of lipid composition of wild, naturally feeding crustacean larvae can provide valuable information of lipid requirements and an understanding of the relative importance of specific lipid classes and fatty acids during the different larval stages (Phleger et al., 2001; Phillips et al., 2006; Limbourn and Nichols, 2009). The range of natural food that larvae are exposed to in the wild is likely to provide a snapshot of the optimal nutrient composition, including the lipid fraction, required to provide a successful larval outcome (Danaher, 2003; Ritar et al., 2003; Jeffs et al., 2004; Jeffs, 2007).

Analysis of the ontogenetic changes in lipid composition during larval rearing has been used as a method for understanding larval nutrition requirements and for the development of suitable dietary regimes. This method has been used to understand lipid requirements for many crustacean species, including the marine shrimp, *Penaeus kerathurus* (Mourente et al., 1995), freshwater prawn, *Macrobrachium rosenbergii* (Roustaian et al., 1999), spider crab, *Maja brachydactyla* (Andrés et al., 2008), mud crab, *Scylla serrata* larvae (Holme et al., 2009b) and rock spiny lobster, *Jasus edwardsii* (Ritar et al., 2003; Smith et al., 2003). To date there is no published information on larval growth pattern or lipid composition of *P. ornatus* phyllosoma. Although collection of mid (Stages IV-VIII) to late stage (Stage VIII-XI) *P. ornatus* phyllosomas in the wild is possible, few early developmental stages are captured (Smith et al.,

2009a). For example, there were no Stage I or II and only one Stage III phyllosoma collected from two Australian Institute of Marine Science (AIMS) Coral sea collection cruises to Osprey Reef (13° 56' S to 14° 03' S and 144° 26' E to 146° 4' E) on 24 January - 09 February 2006 and 24 May - 9 June 2008, respectively (Smith et al., 2009a). Nevertheless, whole phyllosoma lipid analysis had been made on mid- to late Stage phyllosomas from the wild and provides insights into the natural diet of these larvae (Smith et al., pers comm).

The current study investigated the ontogenetic changes in the lipid composition (lipid classes and fatty acids) during the early larval development of *P. ornatus* as well as larval growth in terms of total body length (TBL), wet weight (WW) and dry weight (DW). The data gained in this study would provide baseline information on the basic lipid metabolism of early stage larvae and offer criteria for evaluating larval quality and rearing conditions as well as in the development of suitable dietary regimes for *P. ornatus*.

5.2 Materials and methods

5.2.1. Broodstock source and maintenance

Broodstock source and maintenance were described as Chapter 3 (Section 3.2.1). Females were examined weekly for the presence of eggs and the estimated date of hatching determined by inspection of embryo development. The broodstock were fed alternatively on a proprietary wet feed, blue mussel (*Mytilus galloprovincialis*) and squid (*Loligo opalescensat*) at a rate of 5-8% total body weight, adjusted based on daily observation of feed consumption. Berried females with eggs estimated to be within 1-3 days of hatching were removed from the holding tank and held in a 200 L tub for 4 h and treated with 25 mg L-1 formalin, 8 mg L-1 oxytetracycline, 9 mg L-1 erythromycin and 20 mg L-1 streptomycin for 4 h to partially surface sterilize the female and eggs (Bourne et al., 2004). After treatment, females were placed into a 1000 L hatching tank with the salinity of 33-36‰ and the temperature of 28±1 °C. Water in the hatching system was aerated and flow through with a turnover rate of once per hour. The berried females were not fed in the hatching tank with feces and discarded eggs siphoned daily till hatch.

5.2.2 Larvae culture and live food source

On the day of hatching, active and photopositive phyllosoma were collected from the surface of the broodstock spawning tank and transferred into a 20 L container of clean seawater for estimation of larval production, larvae density calculated and 10,000 newly hatched

phyllosoma volumetrically dispensed into 500 L mass culture vessel. Two to four replicate tanks were used for each hatching depending on the culture space and larval quality. Water temperature and salinity were same as for broodstock culture. Continuous aeration was provided using air-stones. Light was provided by overhead fluorescent ceiling lights set on a photoperiod of 14 h light: 10 h dark and light intensity of 5 ± 1 lx. Phyllosoma were fed twice daily at 9:00 and 16:00 h. The general rearing protocol was shown in Table 5.1. Each morning, *Artemia* remaining from the previous day were flushed from the culture tanks for 4 h prior to feeding. The larval culture tanks were siphoned each morning to remove dead larvae, faeces and other debris. Totally, three batches of larvae rearing were conducted during the experiment. Because the larvae quality varied from female to female, the data presented in the paper was from one female.

Table 5.1 General rearing protocol developed for ornate rock lobster *Panulirus ornatus* larvae at Australia Institute of Marine Science.

Larval stage	Live foods regime	Feed duration
Stage I-III (Instar 1- 5)	Newly hatched *Artemia* nauplii, 4 nauplli mL^{-1}	4- 5 weeks
Stage IV-VI (Instar 6-11)	Juvenile *Artemia* (body length 2.5-4.5 mm), 1 mL^{-1} Blue mussel (*M. galloprovincialis*) gonad, 20 mg L^{-1}	5- 8 weeks
Stage VII-XI (Instar 12- 24)	Juvenile/adult *Artemia* (body length 5.0-7.0 mm), 0.5- 1 mL^{-1} Blue mussel (*M. galloprovincialis*) gonad, 20 mg L^{-1}	9- 14 weeks

Artemia cysts (GSL strain, AAA+ grade, INVE Aquaculture Inc, Salt Lake City, UT, USA) were hatched daily in 50 L conical tanks at 28±1 ℃ in filtered seawater (33-36‰). After 20-24 h hatching, the newly hatched nauplii (size: 430-520 μm) was harvested by siphoning out free swimming photo positive nauplii, *Artemia* were treated with a 25 mg L^{-1} formalin in fresh algae (*Chaetoceros muelleri*, $2x10^6$ cells mL^{-1}) bath for 30 min before addition to larval-rearing tanks. For juvenile/adult *Artemia*, newly hatched nauplii were cultured for up to 10 days using mixed algal *spp*., i.e. *C. muelleri*, *Pavlova lutheri*, *Isochrysis aff. galbana* (Clone Tahiti Isochrysis) and *Tetraselmis chuii*, until they obtained the desired size required for larval feeding. The juvenile *Artemia* was 5-9 days old with body length of 2.5-4.5 mm while the adult *Artemia* was 10-11 days old with body length of 5-7 mm, but without berried eggs. Gonadal tissue from live blue mussel *M. galloprovincialis* was chopped into 0.2-0.3 mm pieces for subsequent feeding.

5.2.3 Sampling and data collection

During the experiment, 5-10 g wet weight samples of newly hatched *Artemia* nauplii, juvenile/adult *Artemia* and chopped blue mussel gonad were collected and stored separately at

-80 ℃ for later biochemical analysis. Phyllosoma stages and instars were identified using the morphological features (Smith et al., 2009a). When 70% of each tank population had attained the desired larval stage, samples of larvae were collected from the mass culture tanks and individuals staged under light microscopy rinsed three times with isotonic 0.5 M ammonium bicarbonate to remove traces of salts, then blotted on filter paper to remove excess moisture (Zhu and Lee, 1997) and then frozen at -80 ℃. For Stage I and II, there are only one instar within the stage, which were sampled for the late analysis. Although there are 2 - 3 instars within the stages for Stage III – VI phyllosoma, only the first instar of each Stage (i.e. instar 6, 8, 10) was sampled for the biomass measurement. Therefore, the majority instars were instar 1, 2, 3, 6, 8, 10, respectively to each Stage (I-V) for the experiments. Samples were collected until Stage VI, or day 65 after hatching.

Phyllosoma bulk samples were used to obtain mean individual phyllosoma body weight, due to their small initial weight immediately post hatch. A total number of between 20 - 200 phyllosoma, depending on the stage of development, were weighed and four replicates were used per sampling. Prior to weighing the phyllosoma were rinsed three times with 0.5 M ammonium bicarbonate. Phyllosoma WW were then determined using a Cahn C-33 micro-balance with a precision of 1μg (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). Phyllosoma DW were obtained after oven-drying for 24 h at 60 ℃. Individual mean WW and DW were calculated by the division of phyllosoma number for each larval Stage. Each replicate consisted of 50 Stage I phyllosoma, 25-30 Stage II and III phyllosoma, 10-15 Stage IV phyllosoma and 4-6 Stage V and VI phyllosoma, respectively. Total body length of the larvae was measured using light microscopy on 18-25 newly moulted Stage specific phyllosoma randomly selected from the mass culture tanks. Total body length was defined as the distance between from the anterior margin of the cephalic shield to the posterior edge of the pleon.

At the completion of the experiment, the percentage weight gain (%) and specific growth rate (SGR, % day^{-1}) was calculated according to the following formulae:

Percentage of weight gain (%) = 100 × (Wt-Wo)/Wo

Specific growth rate (SGR, % day^{-1}) = 100 × (lnWt- lnWo)/D.

Where Wt is the larval body weight after each moult, Wo is the initial body weight prior to a moult, D is the duration of the larval development in days.

5.2.4 Biochemical analysis

See section 4.2.4.

5.2.5 Statistical analysis

See section 4.2.5.

5.3 Results

5.3.1 Weight growth

The individual body WW and DW during the early larval development (Stage I-VI) increased exponentially with the number of Stages (Figure 5.1 and Table 5.2). There was a positive correlation between body WW and DW ($P < 0.001$, n = 24, see equation in Table 5.2). The greatest percentage increases in individual WW and DW were presented from Stage III - IV (WW:155.13%; DW: 166.64%) while the smallest percentage increase in DW and WW was present from Stage IV - V (63.34%) and Stage V - VI (72.67%), respectively (Fig. 5.2). There was a significant difference between the increase in individual wet weight (WW, 101.95%) and dry weight (DW, 63.34%) during the period between Stage IV and V ($P < 0.01$), where WW increase was significantly greater than DW increase.

Table 5.2 Equations of linear regressions and correlations between different biometric parameters.

	Equation	r	*P*	n
DW/WW	DW = 0.2241 × ww+9.9414	0.988	< 0.001	24
DW/X	ln(DW) = 0.7387 ×X + 3.1347	0.995	< 0.001	24
WW/X	ln(WW) = 0.7518 ×X + 4.5458	0.996	< 0.001	24
TBL/X	ln(TBL) = 0.3333 ×X + 0.0282	0.990	< 0.001	6
WW/TBL	ln(WW) = 2.2581 ×ln(TBL) + 4.4904	0.993	<0.001	6
DW/ TBL	ln(DW) = 2.2209 ×ln(TBL) + 3.0735	0.994	< 0.001	6

DW = Body dry weight; WW = Body wet weight; X= the number of stage; ln = natural logarithm; TBL = Total body length; r = correlation coefficient; *P* = level of significance; n = number of replicates.

The specific growth rate (SGR, % day^{-1}) of each Stage during early to mid-stage was reduced with subsequent development across both WW and DW (Figure 5.3). The exception to this was an increase in SGR of DW from Stage V - VI, and the significant differences noted between WW and DW for this period. Between the different stages the highest SGR could be found during the period from Stage I - II for both body WW and DW. The total SGR of phyllosoma from Stage I - VI during the study for WW and DW were 5.68 and 5.61, respectively.

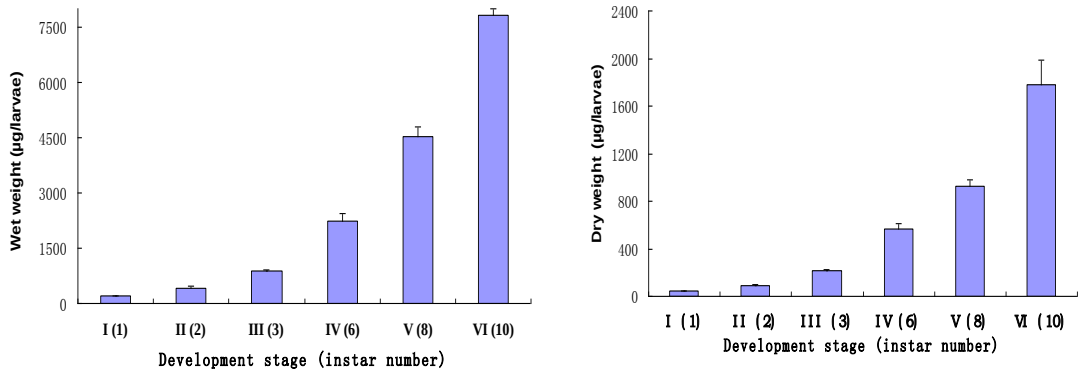

Figure 5.1 Developmental changes of larval wet weight (WW) and dry weight (DW) of *Panulirus ornatus* phyllosoma during the early to mid-larval development (Stage I - VI). Values are presented as means ±SD (n=4).

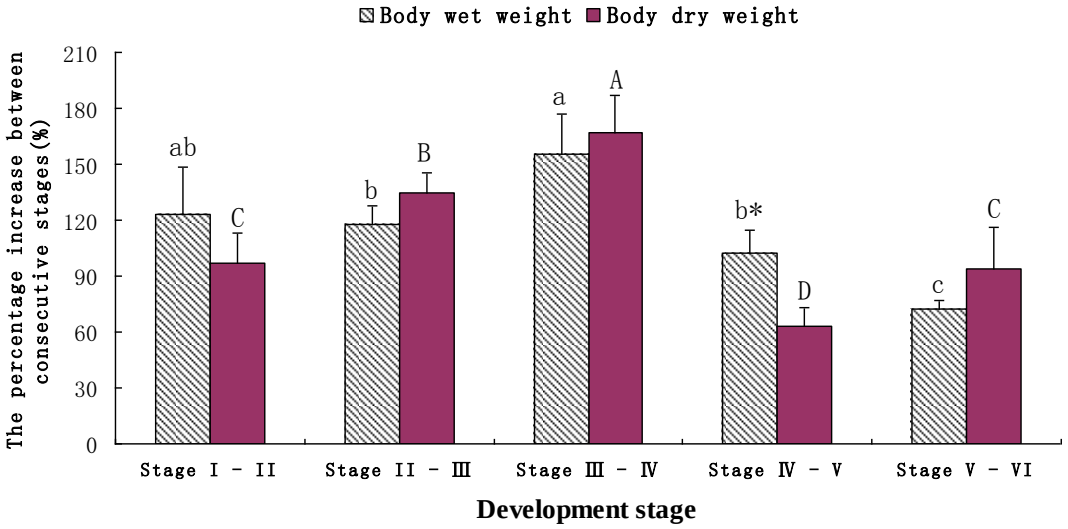

Figure 5.2 The percentage increase in body wet weight (WW) and dry weight (DW) between consecutive stages (% increase) of *Panulirus ornatus* phyllosoma during the early to mid-larval development (Stage I - VI). Values are presented as means ±SD (n=4). The bars that do not share a same letter are significantly different ($P<0.05$). "*" indicates significant differences between the growth ($P< 0.05$).

5.3.2 Total body length (TBL)

Newly hatched *P. ornatus* phyllosoma had a TBL of 1.50 ± 0.04 mm (Figure 5.4). After 65 days of rearing, the larvae had attained stage VI of development, and there had been an increase in the TBL to 7.56 ± 0.27 mm; a 504% increase from stage I. There was a significant increase in TBL with developmental Stage (Figure 5.4 and Table 5.2). Among the five transition periods during the early larval development, the greatest percentage increase in TBL was recorded for the development period between Stage III - IV (+82.04%), with the second greatest increase during the period from Stage V to VI (+42.11%) (Figure 5.4). A log transformation of body

weight (DW and WW) and TBL demonstrated significant non-linear relationships between TBL and WW as well as TBL and DW (Table 5.2).

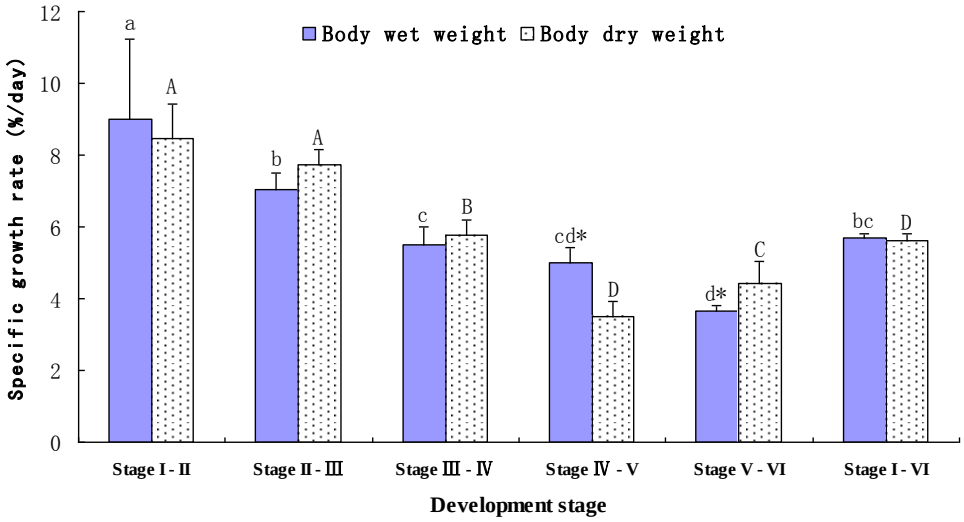

Figure 5.3 The specific growth rate (SGR, % day^{-1}) of both body wet weight (WW) and dry weight (DW) for each larval stage and total early to mid-larval development period (Stage I - VI) of *Panulirus ornatus* phyllosoma. Values are presented as means±SD (n=4). The bars that do not share a same letter are significantly different (P<0.05). "*" indicates significant differences between the SGR of WW and SGR of DW (P< 0.05).

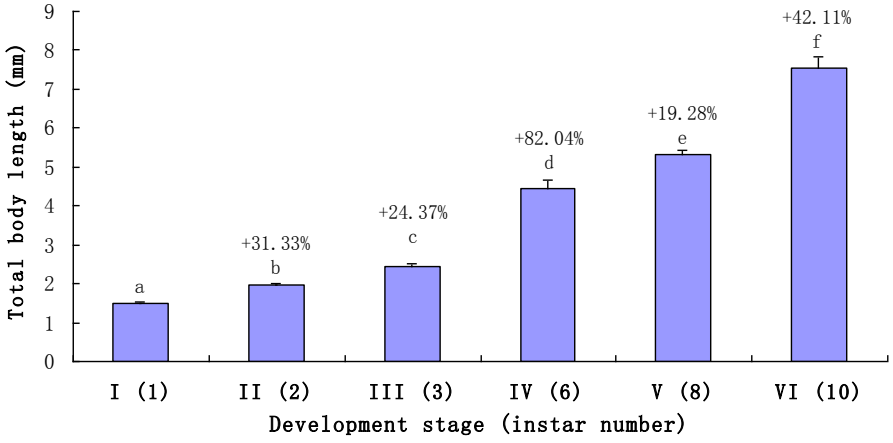

Figure 5.4 Changes of total body length (TBL) of *Panulirus ornatus* phyllosoma during the early to mid-larval development (Stage I - VI). Values are presented as means±SD (n=30).

5.3.3 Live food chemical composition

The proximate composition and lipid classes of the four different feed types demonstrated that juvenile *Artemia* contained the highest moisture content (89.64% WW, Table 3), and the lowest totals of highly unsaturated fatty acids (HUFA) (0.32% DW). Blue mussel gonad had the highest crude protein (69.38% DW) and HUFA (2.17% DW), and the lowest moisture (75.52%

WW) and total lipid contents (10.74% DW). As for the lipid classes of four live feeds, the newly hatched *Artemia* nauplii had the highest cholesterol (CHO) and free fatty acids (FFA) while the juvenile *Artemia* quantitatively contained the highest amount of phospholipids (PL) (Table 5.3). The adult *Artemia* contained the highest triacylglycerol (TG) content, but lowest PL (7.38%).

Table 5.3 Gross composition and lipid class composition (% DW) of newly hatched nauplii, enriched juvenile and adult *Artemia* and chopped blue mussel (*Mytilus galloprovincialis*) gonad used to feed early larval stages of *Panulirus ornatus*

	Newly hatched nauplii (n=2)	**Juvenile *Artemia* (n=2)**	**Adult *Artemia* (n=2)**	**Blue mussel gonad (n=2)**
Gross composition				
Moisture (% WW)	80.71 ± 0.42^{b}	89.64 ± 0.85^{d}	86.14 ± 0.85^{c}	75.52 ± 1.65^{a}
Crude protein (% DW)	54.63 ± 0.76^{b}	56.83 ± 0.39^{c}	50.11 ± 0.40^{a}	69.38 ± 0.46^{d}
Total lipid (% DW)	17.95 ± 0.53^{d}	12.55 ± 0.46^{b}	14.18 ± 0.53^{c}	10.74 ± 0.35^{a}
HUFA (% DW)	0.64 ± 0.09^{c}	0.32 ± 0.02^{a}	0.40 ± 0.03^{b}	2.17 ± 0.27^{d}
Lipid classes (% DW)				
Phospholipids	8.27 ± 0.38^{b}	9.02 ± 0.74^{c}	7.38 ± 0.12^{a}	8.34 ± 0.08^{b}
Monoacylglycerol	0.75 ± 0.21^{c}	0.34 ± 0.12^{b}	0.08 ± 0.00^{a}	0.28 ± 0.01^{b}
Cholesterol	0.81 ± 0.06^{c}	0.63 ± 0.07^{b}	0.58 ± 0.02^{a}	0.65 ± 0.07^{b}
Diacylglycerol	0.24 ± 0.03	--	--	--
Free fatty acids	2.77 ± 0.08^{c}	0.04 ± 0.01^{a}	0.25 ± 0.09^{b}	0.64 ± 0.31^{b}
Triacylglycerol	5.12 ± 0.01^{c}	2.42 ± 0.09^{b}	5.86 ± 0.55^{d}	0.83 ± 0.05^{a}

Values are presented as means ±SD. "n": the number of samples. Values in a same line that do not share the same superscripts are significantly different ($P<0.05$). -: values was not detectable or < 0.05%.

The fatty acid composition (% total fatty acids) of four feeds differed in the composition of saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA) (Table 5.4). Among SFA, 16:0 and 18:0 were the dominant across all feed groups. Among MUFA, 18:1n-9 and 18:1n-7, were the most dominant in all *Artemia* life stages while 18:1n-9 and 20:1n were dominated in blue mussel gonad. However, mussel gonad contained substantially lower MUFA than the other three live foods ($P<0.05$). The PUFA profile of juvenile *Artemia* was dominated by 18:2n-6 (24.23%) which was in significantly greater concentrations compared to found in the other foods, while newly hatched nauplii contained the highest 18:3n-3 (9.23%) among the four live foods. Blue mussel gonad was dominated by highly-unsaturated fatty acids (HUFA), with significantly higher concentrations of 20:5n-3 (EPA) and 22:6n-3 (DHA) and the only food source to have a DHA/EPA ratio greater than one ($P<0.05$, Table 5.4).

Table 5.4 Fatty acid compositions (% total fatty acids) of newly hatched nauplii, enriched juvenile and adult *Artemia* and blue mussel (*Mytilus galloprovincialis*) gonad used to feed early larval stages of *Panulirus ornatus*

Fatty acids	Newly hatched nauplii (n=2)	Juvenile *Artemia* (n=2)	Adult *Artemia* (n=2)	Blue mussel gonad (n=2)
14:0	1.27±0.18^{b}	0.87±0.06^{a}	1.82±0.21^{c}	1.70±0.37^{c}
15:0	0.92±0.05^{c}	0.23±0.00^{a}	0.24±0.06^{a}	0.73±0.13^{b}
16:0	15.42±0.47^{a}	16.06±0.30^{b}	14.23±1.08^{a}	16.31±2.00^{ab}
18:0	5.95±0.52^{b}	6.25±1.81^{ab}	5.77±0.02^{b}	4.48±0.68^{a}
∑SFA	23.56±0.17^{b}	23.41±1.46^{ab}	22.33±0.54^{a}	23.85±1.76^{ab}
14:1n-7	1.87±0.19^{c}	0.11±0.00^{a}	0.38±0.17^{b}	0.13±0.02^{a}
16:1n-7	3.97±0.26^{b}	3.30±0.10^{a}	7.03±0.84^{c}	3.11±0.59^{ab}
16:1n-5	0.96±0.02^{b}	0.34±0.01^{a}	1.94±0.54^{c}	2.94±1.35^{c}
18:1n-9	24.26±0.01^{c}	22.96±1.64^{c}	16.70±0.30^{b}	3.62±0.53^{a}
18:1n-7	9.32±0.87^{c}	4.74±0.15^{b}	9.01±0.38^{c}	1.67±0.26^{a}
20:1n	0.37±0.04^{ab}	0.55±0.14^{b}	0.28±0.05^{a}	5.34±0.33^{c}
22:1n	--	--	0.11±0.05^{a}	1.17±0.37^{b}
∑MUFA	40.75±0.43^{d}	31.99±1.63^{b}	35.42±0.97^{c}	17.98±1.54^{a}
16:2n-4	1.69±0.01^{b}	2.61±0.03^{c}	0.85±0.24^{a}	1.50±0.30^{b}
16:3n-4	1.65±0.05^{c}	0.79±0.00^{a}	1.71±0.17^{c}	1.21±0.07^{b}
18:2n-6	7.85±0.10^{b}	24.23±0.21^{d}	19.66±1.37^{c}	1.86±0.03^{a}
18:3n-6	0.69±0.05^{a}	9.35±0.01^{d}	7.93±0.31^{c}	1.63±0.13^{b}
18:3n-3	9.23±0.37^{d}	0.57±0.05^{a}	2.03±0.23^{b}	2.68±0.00^{c}
18:4n-3	0.70±0.05^{c}	0.24±0.05^{a}	0.27±0.09^{ab}	0.38±0.07^{b}
20:2n-6	--	0.61±0.01^{b}	0.35±0.02^{a}	0.72±0.10^{c}
20:3n-6	0.92±0.27^{c}	0.57±0.01^{b}	0.58±0.03^{b}	0.45±0.03^{a}
20:4n-6	1.18±0.10^{a}	1.14±0.00^{a}	1.24±0.13^{a}	1.95±0.28^{b}
20:3n-3	1.18±0.10	--	--	--
20:4n-3	1.37±0.19	--	--	--
20:5n-3	2.44±0.21^{a}	2.81±0.21^{ab}	3.12±0.23^{b}	15.64±1.43^{c}
22:2n-6	0.40±0.09	--	--	0.45±0.04
22:5n-3	--	--	--	1.04±0.26
22:6n-3	0.39±0.06^{b}	0.17±0.03^{a}	0.23±0.02^{a}	21.24±3.14^{c}
∑PUFA(≥ 18:2n)	25.39±1.30^{a}	39.73±0.38^{c}	35.85±1.43^{b}	48.28±4.65^{d}
n-3PUFA	15.31±0.67^{c}	3.83±0.19^{a}	5.65±0.08^{b}	40.98±4.76^{d}
n-6PUFA	10.08±0.61^{b}	35.90±0.19^{d}	29.76±1.18^{c}	7.07±0.04^{a}
n-3/n-6	1.52±0.03^{c}	0.11±0.00^{a}	0.19±0.02^{b}	5.80±0.64^{d}
∑HUFA(≥ 20:3n)	6.52±0.92^{b}	4.73±0.29^{a}	5.17±0.32^{ab}	40.32±5.08^{c}
DHA/EPA	0.16±0.01^{b}	0.06±0.01^{a}	0.08±0.01^{a}	1.35±0.08^{c}
Unkown	6.96±1.51^{c}	1.48±0.53^{a}	3.84±0.21^{b}	7.18±1.12^{d}

Values are presented as means±SD. "n": the number of samples. Values in a same line that do not share the same superscripts are significantly different ($P < 0.05$). -: values was not detectable or < 0.1%. SFA: saturated fatty acids. MUFA: mon-unsaturated fatty acids. PUFA: poly-unsaturated fatty acids. HUFA: highly unsaturated fatty acids.

The absolute contents of principal fatty acids of all diets are tabulated (Table 5.5). The quantitative/absolute contents of total fatty acids (TFA) increased from a low in blue mussel gonad (49.85 mg g^{-1} DW), followed by on-grown *Artemia* (65.53 mg g^{-1} DW), then adult *Artemia* (74.99 mg g^{-1} DW) and finally newly hatched nauplii (91.85 mg g^{-1}DW). A similar progressive increase in concentrations of total SFA and MUFA was also found in the respective diets. The concentrations of C-18 PUFA were greatest in juvenile and adult *Artemia*, particularly the 18:2n-6 and 18:3n-6 fatty acids, while 18:2n-6 and 18:3n-3 dominated in newly hatched nauplii (P<0.05, Table 5.5). The HUFA content of blue mussels were dominant among

the four feeds (21.65 mg/g DW), despite their low TFA content. Juvenile *Artemia* contained the lowest HUFA (3.15 mg/g DW).

Table 5.5 Principal fatty acid contents (mg g^{-1} DW) of newly hatched nauplii, enriched juvenile and adult *Artemia* and blue mussel (*Mytilus galloprovincialis*) gonad used to feed early larval stages of *Panulirus ornatus*

Fatty acids	Newly hatched nauplii (n=2)	Juvenile *Artemia* (n=2)	Adult *Artemia* (n=2)	Blue mussel gonad (n=2)
16:0	15.22±0.46^{d}	10.68±0.20^{b}	11.10±0.84^{c}	8.76±1.07^{a}
18:0	5.87±0.52^{c}	4.15±1.21^{bc}	4.50±0.01^{b}	2.41±0.36^{a}
∑SFA	23.26±0.17^{d}	15.57±0.97^{b}	17.42±0.42^{c}	12.81±0.95^{a}
16:1n-7	3.92±0.26^{c}	2.19±0.07^{b}	5.48±0.66^{d}	1.67±0.32^{a}
18:1n9	23.95±0.01^{d}	15.27±1.09^{c}	13.02±0.24^{b}	1.94±0.28^{a}
18:1n7	9.20±0.86^{d}	3.15±0.10^{b}	7.03±0.30^{c}	0.90±0.14^{a}
∑MUFA	40.23±0.42^{d}	21.28±1.09^{b}	27.62±0.75^{c}	9.66±0.83^{a}
18:2n-6	7.75±0.10^{b}	16.12±0.14^{c}	15.32±1.07^{c}	1.00±0.01^{a}
18:3n-6	0.70±0.05^{a}	6.22±0.01^{c}	6.19±0.24^{c}	0.87±0.07^{b}
18:3n-3	9.11±0.37^{c}	0.38±0.03^{a}	1.58±0.18^{b}	1.44±0.00^{b}
18:4n-3	0.68±0.05^{b}	0.16±0.03^{a}	0.21±0.07^{a}	0.20±0.04^{b}
20:4n-6	0.91±0.27^{ab}	0.76±0.00^{a}	0.97±0.10^{b}	1.05±0.15^{b}
20:5n-3	2.41±0.21^{b}	1.87±0.14^{a}	2.43±0.18^{b}	8.40±0.77^{c}
22:6n-3	0.38±0.06^{c}	0.11±0.02^{a}	0.18±0.03^{b}	11.40±1.69^{d}
∑PUFA(≥ 18:2n)	25.06±1.28	26.43±0.25	27.96±1.12	25.93±2.50
n-3PUFA	15.11±0.66^{c}	2.55±0.13^{a}	4.40±0.06^{b}	22.00±2.55^{d}
n-6PUFA	9.95±0.62^{b}	23.88±0.13^{c}	23.21±0.92^{c}	3.79±0.02^{a}
∑HUFA(≥ 20:3n)	6.43±0.90^{c}	3.15±0.19^{a}	4.03±0.25^{b}	21.65±2.73^{d}
Total fatty acid	91.85±1.50^{d}	65.53±0.35^{b}	74.99±1.80^{c}	49.85±0.60^{a}

Values are presented as means±SD. "n": the number of samples. Values in a same line that do not share the same superscripts are significantly different (P<0.05). SFA: saturated fatty acids. MUFA: mon-unsaturated fatty acids. PUFA: poly-unsaturated fatty acids. HUFA: highly unsaturated fatty acids.

5.3.4 Total lipid and lipid classes of larvae

Stage I had the lowest total lipids, PL and TG of all groups while Stage II phyllosomas contained higher total lipids, PL and FFA than the other stages (Table 5.6, % DW or mg g^{-1} DW). From stage II to stage V, there was a decreasing trend in the total lipid component based on DW (% DW, n=11, P<0.05). The qualitative lipid class composition was dominated by PL, with a range from 85.50% to 94.65% (Table 5.7). The second highest lipid class was cholesterol (2.15-10.49%total lipids), with Stage IV phyllosoma containing the highest percentage of cholesterol across all five phyllosoma stages examined (10.49%) (P < 0.05).

Table 5.6 Total lipids (% DW) and lipid class contents (mg g^{-1} DW) of early larval stages of *Panulirus ornatus*

	I (instar 1, n=4)	II (instar 2, n=3)	III (instar 3, n=3)	IV (instar 6, n=2)	V (instar 8, n=2)
Total lipids (% DW)	5.51±0.32^{a}	8.97±0.91^{c}	7.99±0.59^{bc}	7.36±0.61^{b}	7.13±0.06^{b}
Lipid classes (mg g^{-1} DW)					
Phospholipids	51.56±0.51^{a}	84.72±0.66^{d}	69.67±5.25^{c}	62.93±0.08^{b}	67.49±0.39^{c}
Monoacylglycerol	--	0.59±0.26^{a}	2.00±1.59^{a}	0.58±0.81^{a}	--
Cholesterol	2.56±0.32^{ab}	1.92±0.37^{a}	2.87±1.13^{ab}	7.72±0.65^{c}	2.85±0.44^{b}
Free fatty acids	0.44±0.22^{b}	1.46±0.10^{c}	1.19±0.48^{c}	0.07±0.05^{a}	0.32±0.00^{b}
Triacylglycerol	0.52±0.12^{a}	0.97±0.27^{b}	4.17±3.00^{bc}	2.31±0.15^{c}	0.65±0.06^{a}

Values are presented as means±SD. "n": the number of samples. Values in a same line that do not share the same superscripts are significantly different (P<0.05). -: values was not detectable or < 0.01%. Each samples were made of a certain number of larvae.

5.3.5 Fatty acids of larvae

The major constituents of the saturated fatty acids (SFA) for all stages were 16:0 and 18:0. Newly hatched phyllosoma larvae contained the highest percentage, 30.78% SFA, with 16:0 at 18.36% (P <0.05). Among MUFA, 18:1n-9 and 18:1n-7 were the two dominant fatty acids with Stage I phyllosoma having the lowest MUFA level (24.35%) (P <0.05, Table 5.8). From stage IV to VI, phyllosoma were fed on juvenile *Artemia* and blue mussel gonad and these larvae contained lower 18:1n-9, 18:1n-7 and total MUFA compared to Stage I - III which were fed on newly hatched *Artemia.* There were significant decreasing trends for 18:1n-9, 18:1n-7 and total MUFA from Stage III to V. Stage IV phyllosoma contained qualitatively the most 18:2n-6 which mirrored that of their juvenile/on-grown *Artemia* food, with a decline in prevalence in Stage V phyllosoma (4.96%). The lowest relative concentration of 18:2n-6 was in newly hatched phyllosomas (3.35%). Qualitatively 18:3n-3 was most prevalent in Stage II phyllosoma (13.39%) declining in stage III (9.18%) and IV (0.97%) phyllosoma, respectively. The HUFA's 20:4n-6 (ARA), EPA and DHA were present in significant concentrations in Stage I phyllosoma compared to their presence in the other stages (P<0.05). From Stage I – III, EPA, DHA and HUFA exhibited a decreasing trend while an increasing trend occurred in the DHA, HUFA and DHA/EPA ratios through Stage III to Stage V.

The principal quantitative fatty acid contents (mg/g dry weight) of early larval stage phyllosoma of *P. ornatus* are presented in Table 5.9. Stage I phyllosoma contained significantly lower concentrations of SFA, MUFA and PUFA and total fatty acids, but had the highest EPA and DHA content. From Stage II to Stage V, a decreasing trend could be found in MUFA while substantially lowest EPA, DHA and HUFA were found in the Stage III. From Stage I to IIII, both EPA and DHA decreased dramatically. Although the phyllosomas of Stage IV and V were

fed on the same diets (on-grown *Artemia* and blue mussel gonad), 16:0 and DHA increased significantly while 18:2n-6 and 20:4n-6 decreased (P<0.05).

Table 5.7 Lipid class composition (% total lipids) of early larval stages of *Panulirus ornatus*

Lipid classes	I (instar 1, n=4)	Ⅱ (instar 2, n=3)	Ⅲ(instar 3, n=3)	Ⅳ(instar 6, n=2)	Ⅴ (instar 8, n=2)
Phospholipids	93.57±0.93^{b}	94.49±0.74^{b}	87.19±6.58^{a}	85.50±0.10^{a}	94.65±0.54^{b}
Monoacylglycerol	--	0.65±0.29^{a}	2.50±1.99^{a}	1.09±0.79^{a}	--
Cholesterol	4.65±0.57^{b}	2.15±0.42^{a}	3.59±1.41^{ab}	10.49±0.88^{c}	4.00±0.62^{b}
Free fatty acids	0.80±0.44^{b}	1.63±0.11^{c}	1.50±0.60^{c}	0.07±0.10^{a}	0.44±0.01^{b}
Triacylglycerol	0.95±0.21^{a}	1.08±0.30^{a}	5.22±3.82^{b}	3.15±0.21^{b}	0.91±0.08^{a}

Values are presented as means±SD. "n": the number of samples. Values in a same column that do not share the same superscripts are significantly different (P<0.05). -: values was not detectable or < 0.01%. Each samples were made of a certain number of larvaes.

5.4 Discussion

This study is the first to describe the developmental patterns of larval growth, in terms of body weight, carapace length, specific growth rate (SGR) and the numbers of stages, for the laboratory-reared ornate rock spiny lobster *P. ornatus* during the early larval development (Stage I-VI). These developmental patterns allow the extrapolation of growth in size and biomass for successive developmental stages and can be applied in ecology and aquaculture to assess the quality of environmental conditions and food for larval growth (Anger et al., 2009).

The highest gain in body weight and carapace length was noted during the period from Stage III to Stage IV, but this included more instars, in which there is 2-3 instars, within stage III compared to only 1 or 2 instars in the other stages examined. This pattern of multiple instars during larval development has been already reported in a prior study on *P. ornatus* (Smith et al., 2009a) and in other spiny lobsters (Kittaka, 1997; Sekine et al., 2000). As in many other decapod crustacean species, the pattern of larval or early larval biomass growth could be described as an exponential relationship of the number of successive stages. After the natural logarithmic transformation, the slope parameters of the linear regression are 0.7387 for body dry weight and 0.7518 for body wet weight. These data are higher than many crustacean species, e.g. spider crab, *Maja brachydactyla* (slope of dry weight: 0.63) (Andrés et al., 2008), swimming crab, *Liocarcinus holsatus* (slope of dry weight: 0.62) (Harms, 1990), salt marsh crab, *Neohelice* (formerly *Chasmagnathus*) *granulata* (slope of dry weight: 0.69) (Anger and Ismael, 1997) and Amazon River prawn, *Macrobrachium amazonicum* (Anger et al., 2009), but lower than the swimming crab, *P. trituberculatus* larvae (slope of dry weight: 0.907) (Lim and Hirayama, 1991), sand crab, *P. pelagicus* (slope of dry weight: 0.8293) (Chapter 4).

Table 5.8 Fatty acid compositions (% total fatty acids) of early larval stages of *Panulirus ornatus*

Fatty acids	I (instar 1, n=4)	II (instar 2, n=3)	III (instar 3, n=3)	IV (instar 6, n=2)	V (instar 8, n=2)
14:0	0.99±0.32^{bc}	0.48±0.06^{a}	0.48±0.15^{a}	1.13±0.14^{c}	0.69±0.01^{b}
15:0	0.42±0.06^{a}	0.83±0.08^{c}	0.59±0.10^{b}	0.43±0.03^{a}	0.40±0.01^{a}
16:0	18.36±1.29^{c}	13.84±0.23^{a}	14.35±1.29^{ab}	13.63±1.06^{a}	15.14±0.28^{b}
18:0	11.01±0.41	11.09±0.98	11.39±0.49	9.99±0.78	10.93±0.20
∑SFA	30.78±1.24^{b}	26.24±0.61^{a}	26.81±1.31^{a}	25.19±2.01^{a}	27.51±0.50^{a}
14:1n-7	0.20±0.08^{a}	2.49±0.17^{d}	1.20±0.61^{c}	0.16±0.02^{a}	0.33±0.09^{b}
16:1n-7	2.94±0.42^{d}	3.95±0.46^{e}	1.29±0.13^{a}	1.60±0.14^{b}	2.08±0.04^{c}
16:1n-5	1.50±0.98^{ab}	0.84±0.04^{a}	1.12±0.16^{b}	0.62±0.09^{a}	0.90±0.02^{b}
18:1n-9	11.99±0.80^{a}	15.59±0.42^{b}	17.13±0.83^{c}	16.52±0.71^{bc}	15.65±0.52^{b}
18:1n-7	3.69±0.30^{a}	5.08±0.93^{b}	10.43±1.42^{d}	7.25±0.66^{c}	6.07±0.50^{b}
20:1n	4.03±0.23^{c}	1.78±0.05^{b}	1.87±0.53^{b}	1.85±0.36^{b}	1.19±0.01^{a}
∑MUFA	24.35±0.99^{a}	29.73±0.71^{d}	33.04±1.95^{e}	28.00±0.19^{c}	26.21±0.03^{b}
16:2n-4	0.65±0.05^{b}	1.14±0.00^{c}	1.33±0.13^{d}	0.30±0.00^{a}	0.62±0.01^{b}
16:3n-4	1.00±0.08^{a}	0.98±0.06^{a}	1.11±0.16^{a}	2.19±0.02^{b}	2.02±0.36^{b}
18:2n-6	3.35±1.22^{a}	6.01±0.22^{c}	7.70±1.32^{d}	12.17±0.64^{e}	4.96±0.09^{b}
18:3n-6	0.38±0.08^{bc}	0.26±0.07^{b}	0.55±0.18^{c}	1.80±0.12^{d}	0.18±0.00^{a}
18:3n-3	0.18±0.03^{a}	13.39±0.31^{d}	9.18±1.64^{c}	0.97±0.03^{b}	0.93±0.02^{b}
18:4n-3	0.91±0.11^{b}	0.14±0.02^{a}	1.21±0.50^{b}	0.99±0.67^{b}	1.44±0.29^{c}
20:2n-6	1.26±0.05^{b}	0.71±0.27^{a}	0.77±0.46^{a}	1.73±0.06^{c}	1.34±0.15^{b}
20:3n-6	--	0.41±0.17^{a}	0.32±0.13^{a}	0.64±0.02^{b}	0.68±0.01^{b}
20:4n-6	3.19±0.10^{b}	1.53±0.18^{a}	1.99±0.66^{a}	3.79±0.05^{c}	3.11±0.46^{b}
20:3n-3	0.71±0.15^{a}	2.44±0.28^{c}	3.57±0.50^{d}	1.08±0.02^{b}	1.10±0.02^{b}
20:4n-3	0.20±0.14^{a}	0.83±0.12^{d}	1.25±0.24^{e}	0.36±0.01^{b}	0.50±0.01^{c}
20:5n-3	14.33±0.41^{c}	7.11±1.16^{a}	5.18±1.21^{a}	9.10±0.18^{b}	9.05±0.55^{b}
22:2n-6	0.06±0.07^{a}	0.41±0.06^{c}	0.40±0.05^{c}	0.45±0.01^{c}	0.16±0.00^{b}
22:5n-3	0.34±0.07^{a}	0.37±0.03^{a}	--	0.31±0.04^{a}	0.63±0.01^{b}
22:6n-3	11.68±0.80^{e}	3.34±1.15^{b}	1.32±0.24^{a}	5.91±0.94^{c}	8.28±0.15^{d}
∑PUFA(≥ 18:2n)	36.54±0.69^{b}	36.94±1.02^{b}	33.44±0.94^{a}	39.29±0.59^{c}	32.37±0.26^{a}
n-3PUFA	28.31±1.17^{c}	27.61±1.55^{c}	21.71±1.03^{b}	18.71±1.46^{a}	21.93±0.05^{b}
n-6PUFA	8.23±1.05^{a}	9.33±0.53^{ab}	11.73±0.89^{c}	20.58±0.87^{d}	10.44±0.20^{b}
n-3/n-6	3.49±0.57^{d}	2.97±0.33^{d}	1.86±0.20^{b}	0.91±0.11^{a}	2.10±0.04^{c}
∑HUFA(≥ 20:3n)	30.41±1.21^{d}	16.03±1.53^{a}	13.63±2.82^{a}	21.18±0.79^{b}	23.35±0.81^{c}
DHA/EPA	0.81±0.03^{c}	0.46±0.09^{b}	0.26±0.03^{a}	0.65±0.12^{b}	0.92±0.07^{d}
Unkown	6.67±2.10^{a}	4.97±0.97^{a}	4.26±0.63^{a}	5.04±1.65^{a}	11.63±0.14^{b}

Values are presented as means±SD. "n": the number of samples. Values in a same column that do not share the same superscripts are significantly different (P<0.05). -: values was not detectable or < 0.1%. Each sample was made of a certain number of larvaes. SFA: saturated fatty acids. MUFA: mon-unsaturated fatty acids. PUFA: poly-unsaturated fatty acids. HUFA: highly unsaturated fatty acids.

The number of moults within a particular larval stage in crustaceans varies widely between species, as does the intermoult period of different larval stages of the same species (Sekine et al., 2000). Because of this it may be invalid to compare the growth rate based solely on the growth equations of biomass and the number of instars between the different species. Specific growth rate (SGR) is a commonly used criteria to evaluate the growth for the different diets and other rearing conditions of a same species (Guillaume, 2001; Romano and Zeng, 2006; Rotllant et al., 2010), compare the growth among the species (Mcandrew and Majumdar, 1989) and assess the growth rate of different life stages for a same species/inter-species (Folkvord, 2005; Rotllant et al., 2010) as both body weight increase and duration are taken into the calculation. We have used SGR, widely used in as a comparative measure of growth in aquaculture, to assess the larval growth of *P. ornatus* whilst acknowledging its limitations for organisms that grow in discrete periodic moults rather than continuously. Among phyllosomas SGR data for three Palinurid species, *Jasus edwardsii, Panulirus cygnus* and *P. ornatus*, *P. ornatus* has the highest SGR (Table 5.10). This difference may be due to *P. ornatus* originating in a tropical climate, with warmer water temperatures stimulating faster growth, compared to the temperate or sub-tropical Palinurid species, e.g. *Jasus edwardsii* and *Panulirus cygnus* (Ritar et al., 2003; Liddy et al., 2004a). More recently, Irvin et al. (2010) found the SGR of wet weight of juvenile *P. ornatus* (body weight 2.1-9.1g) was around 2.99 (calculated from the data provided in the reference), which is substantially lower than the SGR of early larvae of *P. ornatus* (5.68) (Irvin et al., 2010). This difference suggests the larval stages displays faster growth compared to that noted for juveniles of this species and is a general trend noted in many other marine species (Folkvord, 2005).

In the present study, the mean total lipid content for the newly hatched phyllosoma was 5.51% DW. Occasionally, the highest total lipid of newly hatched *P. ornatus* phyllosoma can reach 7.89% DW (Wu et al, unpublished data), and this may indicate the different hatching conditions, broodstock diets and physiological status between different larval batches (Phleger et al., 2001; Sachlikidis et al., 2010). The total lipid content of newly hatched *P. ornatus* phyllosoma is substantially lower than that of temperate species such as *J. edwardsii* (7-12% DW) (Ritar et al., 2003). Generally, crustaceans inhabiting tropical and subtropical regions contain lower body lipid contents compared to temperate and polar crustacean species (Wang et al., 1995a; Nates and McKenney, 2000; Kattner et al., 2003). In contrast, crustaceans from temperate and polar regions generally have higher body lipid reserve (Wang et al., 1995a; Wu et al., 2010b). This difference maybe explained that the high body lipids can improve the fluidity of cellular membranes, leading to enhance the cellular metabolic activity for temperate and polar crustacean species under low temperature (Kong et al., 2006).

Table 5.9 Principal fatty acid contents (mg g^{-1} DW larvae) of early larval stages of *Panulirus ornatus*

Fatty acids	I (instar 1, n=4)	Ⅱ (instar 2, n=3)	Ⅲ(instar 3, n=3)	Ⅳ(instar 6, n=2)	Ⅴ (instar 8, n=2)
16:0	4.86 ± 0.34^{a}	6.21 ± 0.10^{c}	5.90 ± 0.47^{bc}	4.95 ± 0.48^{a}	5.51 ± 0.25^{b}
18:0	2.91 ± 0.11^{a}	4.97 ± 0.44^{c}	4.69 ± 0.25^{c}	3.63 ± 0.35^{b}	3.98 ± 0.18^{b}
∑SFA	8.14 ± 0.33^{a}	11.77 ± 0.27^{d}	11.03 ± 0.48^{c}	9.15 ± 0.91^{b}	9.88 ± 0.45^{b}
16:1n-7	0.78 ± 0.11^{b}	1.77 ± 0.21^{c}	0.53 ± 0.05^{a}	0.58 ± 0.06^{a}	0.76 ± 0.03^{b}
18:1n9	3.17 ± 0.21^{a}	6.99 ± 0.19^{d}	7.05 ± 0.31^{d}	5.99 ± 0.14^{c}	5.69 ± 0.03^{b}
18:1n7	0.98 ± 0.08^{a}	2.28 ± 0.42^{b}	4.30 ± 0.65^{c}	2.63 ± 0.29^{b}	2.21 ± 0.24^{b}
∑MUFA	6.44 ± 0.26^{a}	13.33 ± 0.32^{d}	13.60 ± 0.98^{d}	10.16 ± 0.27^{c}	9.53 ± 0.25^{b}
18:2n-6	0.89 ± 0.32^{a}	2.70 ± 0.10^{c}	3.17 ± 0.56^{d}	4.42 ± 0.32^{e}	1.81 ± 0.08^{b}
18:3n-3	0.05 ± 0.01^{a}	6.00 ± 0.14^{d}	3.79 ± 0.76^{c}	0.35 ± 0.02^{b}	0.34 ± 0.02^{b}
18:4n-3	0.24 ± 0.03^{b}	0.06 ± 0.01^{a}	0.50 ± 0.21^{c}	0.36 ± 0.23^{bc}	0.53 ± 0.12^{c}
20:4n-6	0.84 ± 0.03^{b}	0.69 ± 0.08^{a}	0.81 ± 0.26^{bc}	1.38 ± 0.05^{d}	1.13 ± 0.14^{c}
20:5n-3	3.79 ± 0.11^{c}	3.19 ± 0.52^{bc}	2.12 ± 0.47^{a}	3.30 ± 0.13^{b}	3.29 ± 0.11^{b}
22:6n-3	3.09 ± 0.21^{d}	1.50 ± 0.51^{b}	0.54 ± 0.09^{a}	2.14 ± 0.30^{c}	3.01 ± 0.14^{d}
∑PUFA(≥ 18:2n)	9.67 ± 0.19^{a}	16.57 ± 0.46^{e}	13.76 ± 0.36^{c}	14.26 ± 0.06^{d}	11.77 ± 0.23^{b}
n-3PUFA	7.49 ± 0.31^{b}	12.39 ± 0.69^{d}	8.94 ± 0.50^{c}	6.79 ± 0.40^{a}	7.97 ± 0.20^{b}
n-6PUFA	2.18 ± 0.28^{a}	4.18 ± 0.24^{c}	4.82 ± 0.30^{d}	7.47 ± 0.46^{c}	3.79 ± 0.03^{b}
∑HUFA(≥ 20:3n)	8.04 ± 0.32^{c}	7.19 ± 0.69^{ab}	5.59 ± 1.09^{a}	7.69 ± 0.14^{b}	8.49 ± 0.06^{d}
Total fatty acid	24.68 ± 0.55^{a}	42.62 ± 0.44^{e}	39.39 ± 0.79^{d}	34.48 ± 1.27^{c}	32.13 ± 0.84^{b}

Values are presented as means ±SD. "n": the number of samples. Values in a same column that do not share the same superscripts are significantly different ($P<0.05$). Each sample was made of a certain number of larvaes. SFA: saturated fatty acids. MUFA: mon-unsaturated fatty acids. PUFA: poly-unsaturated fatty acids. HUFA: highly unsaturated fatty acids.

However, the total lipids of crustacean larvae is not only related to their inherited characters and environmental conditions (e.g. temperature and salinity), but their feeding foods (Sui et al., 2007; Wu et al., 2007c). In this study, the highest total lipid content was in Stage II and III phyllosoma, which reflect the feeding regime as these stages were fed solely on newly hatched *Artemia* nauplii, which contained a higher total lipids than the larval feeds available to more advanced phyllosoma stages. The gradual accumulation of total body lipids with development may be indicative that lipids are an important source of energy for larval development as well as precursors for metabolic processes (Ritar et al., 2003; Liddy et al., 2005).

Table 5.10 The comparison of body DW, growth (% increase) and SGR of different lobster species.

	Initial weight	Final weight	Growth (% increase)	Mean CDT (days)	SGR (% day^{-1})	Water temperature (℃)	Source
Panulirus ornatus Larvae	Stage I: 46.1μg	Stage VI: 1781μg	3763	65	5.62	28	This study
Jasus edwardsii Larvae	Stage I: 80.0μg	Stage VI: 1084.0μg	1255	63	4.14	18	Ritar et al (2003)
Panulirus cygnus Larvae	Stage I: 79.0μg	Stage VI: 222.0μg	181	26	3.97	23	Liddy et al (2004a,b)
Panulirus ornatus Juvenile	2.1 g	9.1g	333	49	2.99	28-30	Ivrin et al (2010)

Mean CDT: Mean cumulative development time; SGR: Specific growth rate.

Although newly hatched *Artemia*, juvenile and adult *Artemia* contained a higher content of TG (2.4-5.9% DW), PL was the dominant lipid class noted in early larval stage *P. ornatus*, accounting for more than 85% of total lipids, while TG was only a minor lipid fraction (0.9-5.2% total lipids). Similar findings have also been found in other Palinurid lobsters, including *J. edwardsii* (Phleger et al., 2001; Ritar et al., 2003) and *P. cygnus* (Liddy et al., 2004a; Liddy et al., 2005). Phleger et al. (2001) speculated that PL serves as the major lipid class for Palinurid phyllosomas because of its transparency character, in contrast to the more opaque neutral lipids such as TAG and FFA, as transparency in the planktonic phase may greatly reduce the risk of being detected by visually-hunting predators during the long larval development (Phleger et al., 2001; Limbourn and Nichols, 2009). Cholesterol is essential for the normal growth and ecdysis of crustacean species and as a precursor of ecdysone (Teshima and Kanazawa, 1971; Irvin et al., 2010) and is the second most abundant lipid class (up to 10.49% total lipid). However, it is unclear as to why Stage IV larvae contained the highest cholesterol among the five stages in a pattern similar to that present in wild *J. edwardsii* phyllosoma (Phleger et al., 2001). Although TG is a common short - term storage lipid for energy provision in many marine crustacean (Perez-Velazquez et al., 2003; Yao et al., 2008), it only represented a small proportions of the total lipids in phyllosoma in this study. This may indicated that TG is not a major energy source for early larval stages of *P. ornatus*. Previous studies have shown PL are the prominent energy source for late-stage *J. edwardsi*i (Ritar et al., 2003) and *P. cygnus* phyllosoma (Liddy et al., 2003; Liddy et al., 2004a; Liddy et al., 2004b; Liddy et al., 2005; Melville-Smith et al., 2007) and it is likely that a similar strategy is being utilized by *P. ornatus* phyllosoma. Interesting, despite ample free fatty acids (FFA) levels in their diet, early *P. ornatus* phyllosoma (Stage I to V) contained very low FFA levels (0.07-1.63% total lipid). A similar phenomenon was also found in *J. edwardsii* (Phleger et al., 2001) and *P. cygnus* phyllosoma (Liddy et al., 2003; Melville-Smith et al., 2007). Although the reason for this is

unclear, it may be explained by the conversion of FFA to PL or oxidation to be utilized as an energy source.

Overall, the fatty acid (FA) profile of newly hatched larvae of *P. ornatus* is similar to the phyllosomas of the southern spiny rock lobster, *J. edwardsii* (Phleger et al., 2001) and western spiny rock lobster, *P. cygnus* (Liddy et al., 2004a). However, the newly hatched larvae of *P. ornatus* contained higher 16:0 (18.36%) compared to *J. edwardsii* (13.9 -14.5% total fatty acids) (Phleger et al., 2001; Ritar et al., 2003) and *P. cygnus* larvae (14.69% total fatty acids) (Liddy et al., 2004a) and a very low percentage of ARA (3.19% total fatty acids) compared to *J. edwardsii* (7.0-11.6% total fatty acids) (Phleger et al., 2001; Ritar et al., 2003) and *P. cygnus* larvae (10.02% total fatty acids) (Liddy et al., 2004a). As a result of low ARA content in newly hatched *P. ornatus* larvae, their EPA/ARA ratio (4.49) was greater than that present in *J. edwardsii* (1.31-2.41) (Phleger et al., 2001; Ritar et al., 2003) and *P. cygnus* larvae (0.60-0.99) (Liddy et al., 2004a; Melville-Smith et al., 2007). To a certain extent, the FA profile of newly hatched larvae may reflect the broodstock diets, particularly the HUFAs (Wouters et al., 2001), although when an essential nutrient is required preferential sequestering may occur (Smith et al., 2004). For *P. cygnus* and *J. edwardsii*, previous studies have revealed that newly hatched larvae of wild broodstock contained higher percentage of ARA than the larvae from captive females fed a mixture diets, including mussel, squid, fish and artificial pellets (Phleger et al., 2001; Ritar et al., 2003; Smith et al., 2003a; Smith et al., 2004; Liddy et al., 2005; Melville-Smith et al., 2007b). This may indicate a higher presence of ARA in wild natural diets compared to a captive combination diet and/or low ability for the *de novo* synthesis of ARA (Kanazawa et al., 1979a; Wouters et al., 2001). In this study, the newly hatched larvae of *P. ornatus* had low percentage of ARA (3.19% total fatty acids), which may be explained by a small proportion of ARA in their broodstock diets, being primarily squid (2.17% total fatty acids) and mussel (1.98% total fatty acids) (Wu et al, unpublished data), but is also possible to reflect a fundamental differences in requirements for the tropical *P. ornatus* compared to temperate lobsters. There is little information on the effects of dietary ARA levels on ovarian development, reproductive performance and larval quality in Palinurid lobsters, and this is an area requiring further investigation.

Because larvae were only fed newly hatched *Artemia* nauplii from Stage I - III, it may be expected that their FA profile would reflect that of newly hatched *Artemia* (Ritar et al., 2003; Liddy et al., 2005). However, it was noted during this study that Stage II - III *P. ornatus* phyllosoma contained substantially higher percentages of 18:0, EPA, DHA, HUFA and a DHA/EPA ratio compared to newly hatched *Artemia* nauplii. This suggests preferential

accumulation and storage of essential fatty acids, particularly EPA and DHA, for the larval growth and development as noted in other spiny rock lobster phyllosoma (Smith et al., 2003b). As for the increasing percentage of 18:0, there are two possible explanations. Firstly, the early larvae (Stage I to Stage III) possess the capability of 18:0 synthesis as in some other crustacean (D'Abramo, 1997; Li, 2005); secondly, that 18:0 maybe not be used as the primary energy source during the early larvae development and moulting; therefore, its relative percentage increased compared to the newly hatched *Artemia* nauplii diet.

From Stage III to Stage V, a decreasing trend was detected for 18:1n-9, 18:1n-7 and 18:3n-3 while the percentage of ARA, DHA, EPA and DHA/EPA ratio exhibited an increased trend. This pattern may be indicative of the dietary shift from a diet of newly hatched *Artemia* nauplii to a diet of juvenile *Artemia* and blue mussel gonad. Surprisingly, Stage IV to Stage V phyllosoma contained a significantly higher percentage of ARA (3.11-3.79% total fatty acids) compared to their diets (1.14-1.95% total fatty acids) and suggests a higher ARA requirement and sequestering ability. Based on feeding structures, behavior and digestive capacity of *J. edwardsii* and *Sagmariasus verreauxi*, Jeff (2007) inferred wild phyllosoma probably undergo marked dietary shifts at around Stage III-IV and again at Stage VIII and IX. Furthermore, using lipid biomarkers and ecological association of zooplankton with the pelagic larvae, gelatinous zooplankton, such as jellyfish and small crustaceans, such as krill and mysids, were regarded as important prey items for the mid-Stages (V-VIII) of wild *J. edwardsii* phyllosoma (Jeffs et al., 2004; Jeffs, 2007). Similarly, gelatinous zooplankton, such as salps and chaetognaths, are considered to be putative food items for mid-Stage (IV-VIII) *P. ornatus* phyllosoma based on historic records, feeding structures, feeding behavior and field investigation (Smith et al., 2009a). As some gelatinous zooplankton contain a high ARA concentration (3.2-9.9% total fatty acids) (Danaher, 2003), it is possible that they would be a source for mid-Stage (IV-VIII) *P. ornatus* phyllosoma (Kittaka, 1997).

5.5 Conclusion

In summary, this study provides a first description of the developmental patterns of early larval growth for *P. ornatus* phyllosoma (Stage I-VI) in captivity. Overall, the body wet weight, dry weight and total body length increased exponentially with number of successive stages. The SGR of early phyllosoma of *P. ornatus* indicated this species grows faster than the temperate and subtropical Palinurid lobsters, *J. edwardsii* and *P. cygnus*. The lipid class profile of *P. ornatus* phyllosoma was dominated by PL for all early Stages (I-V) despite a high TG content of their primary diet of *Artemia*. Overall, total lipid, lipid class and fatty acid profile of phyllosoma reflects those of their diets. The elevated ARA content of Stage IV and V *P.*

ornatus phyllosoma suggested a higher ARA requirement compared to earlier stages. The phyllosomas can preferentially accumulate and store a range of essential fatty acids, including EPA and DHA. Finally, the ontogenetic changes of lipid composition during larval development may provide important cues for the understanding of larval lipid nutrition. Future research should be conducted to investigate the effects of varying dietary HUFA (ARA, EPA and DHA) levels on larval growth, survival and development of *P. ornatus* phyllosoma.

Chapter 6

Effects of starvation on survival, biomass and lipid composition of newly hatched larvae of the blue swimmer crab, *Portunus pelagicus*

6.1 Introduction

The blue swimmer crab, *Portunus pelagicus* (Linnaeus, 1758), is widely distributed in the coastal waters throughout the Indo-Pacific, particularly in the tropical and subtropical waters of the region (Dixon and Hooper, 2009). This crab supports important commercial and recreational fisheries in many countries of the region (Lestang et al., 2003; Dineshbabu et al., 2008; Romano and Zeng, 2008). Because of their large size, high meat yield and delicate flavor, there is an increasing market demand for *P. pelagicus* worldwide (Soundarapandian and Dominic Arul Raja, 2008; Wu et al., 2010d). However, wild *P. pelagicus* populations have already been fully exploited (Maheswarudu et al., 2008; Dixon and Hooper, 2009), hence, any further expansion on *P. pelagicus* output can only come from either aquaculture or stock enhancement efforts. During the past decade, many efforts have been spent on the development of hatchery techniques for *P. pelagicus* (Soundarapandian et al., 2007; Maheswarudu et al., 2008; Andrés et al., 2010). However, the survival of newly hatched larvae to the first crab stage is still variable (Soundarapandian et al., 2007). It is well known that larval survival and development of decapod crustaceans are affected by lipid nutrition (Takeuchi et al., 1999a; Liddy et al., 2005; Sui et al., 2007). Similarly, deficiencies in lipid nutrition are considered as one of major contributors to the inconsistency in larval survival of *P. pelagicus* (Maheswarudu et al., 2008; Romano and Zeng, 2008).

Previous studies have shown optimization of dietary lipid nutrition is highly important for achieving successful propagation for many decapod crustacean, including the Chinese mitten crab *Eriocheir sinensis* (Sui et al., 2007), the mud crab *Scylla paramamosain* (Nghia et al., 2007) and the swimming crab *Portunus trituberculatus* (Takeuchi et al., 1999a). Therefore, better understanding of lipid requirements is likely to facilitate the improvement in larval survival for hatchery culture of *P. pelagicus*. It proved very difficult to investigate lipid requirements by usual feeding experiments on newly hatched larvae as they are very small and fragile (Nates and McKenney, 2000; Ritar et al., 2003). Starvation experiments accompanied with lipid analysis on newly hatched larvae have thereby been considered as effective alternative for the elucidation of relative importance of various lipid classes and fatty acids in decapod crustacean larvae (Weng et al., 2002; Ritar et al., 2003).

In the wild, newly hatched *P. pelagicus* larvae with limited swimming ability are often carried by tides and currents (Weng et al., 2002; Bryars and Havenhand, 2004; Bryars and Havenhand, 2006). As the result, these pelagic larvae are likely to expose to highly variable conditions of food availability and could face prolonged starvation (Dawirs, 1984; Staton and Sulkin, 1991). Although starvation resistance capacity is species-specific and largely relates to nutrition reserves stored in newly hatched larvae, most of marine decapod larvae would eventually die without minimum food intakes (Dawirs, 1984; Staton and Sulkin, 1991). In aquaculture research, starvation has also been used to evaluate the quality of newly hatched larvae (Wu et al., 2007b). It is believed that those larvae could survive for longer period under starvation are of better quality (Djunaidah et al., 2003; Wu et al., 2010b). However, no information is so far available on the impacts of starvation on newly hatched *P. pelagicus* larvae. Therefore, the aims of current study were to investigate firstly, survival and body weight change of newly hatched larvae subjected to starvation; and subsequently by comparison of relative conservation and utilization of various lipid composition (lipid classes and fatty acids) during starvation to infer their comparative nutrition values. Such information may be useful for understanding starvation resistance and development of suitable dietary regime for the newly hatched *P. pelagicus* larvae.

6.2 Materials and methods

6.2.1 Broodstock source and maintenance

Broodstock source and husbandry were described in Chapter 4 (Section 4.2.1).

6.2.2 Larvae source and starvation resistance of newly hatched larvae

On the day of hatching, within 6 h of hatching, larvae were attracted to a light source and then collected. They were transferred to ten 300 L static rearing tanks and stocked at a density of ca. 150 larvae L^{-1}. Seawater used was filtered through three in-line cartridge filters (10, 5 and 1μm) and a UV sterilization unit. Water temperature and salinity were maintained at 28±1℃ and 22‰, respectively. Light was provided by overhead fluorescent ceiling lights (40 w/light) set on a photoperiod of 14 h light:10 h dark, light intensity reached larval tanks ranging from 350 to 450 lux. A daily 30-50% water exchange was performed for all larval tanks. The larval tanks were siphoned each morning to remove dead larvae and other debris. Aeration was provided to each tank using air-stones. The larvae were not fed during the experiment.

Additionally, 150 newly hatched zoea I larvae from one female crab were also randomly selected and transferred into five 500 mL glass beakers (30 larvae/beaker). The beaker was filled with 400 mL filtered seawater (filtered to 1μm). Water temperature and salinity were set the same as in the larval tanks. All beakers were gently aerated. Larval mortality was checked every 12 h with dead larvae removed during the checking until all larvae died. Daily 50% water exchange was performed for all beakers. At the end of experiment, larval mortality and average survival time under starvation for each replicate were calculated used the following formulae:

Mortality (%) = 100 × cumulative number of dead larvae at each checking time/30

Average duration of larval survival under starvation (day) = （ $0.5 \times N_{0.5} + 1 \times N_1 + 1.5 \times N_{1.5} + \cdots\cdots + T \times N_T$ ） / 30

Where $N_{0.5}$, N_1, $N_{1.5}$ … represent the number of dead larvae at each point of checking time at 12 h interval during starvation.

6.2.3 Sampling for lipid analysis and data collection

On day 0, 1, 2 and 3 post-hatching, all larvae from each of two tanks were taken using a net with a mesh size of ca.150μm for the following lipid analyis. The larval samples were then rinsed with fresh filtered seawater. After rinsing, each sample was blotted dry on an absorbent paper before beeing stored in a -70 ℃ freezer. At the same time, two small samples of approximately 50 larvae/sample were randomly taken from each of sampling tank for the measurement of body wet weight (WW) and dry weight (DW) of the larvae. These samples were briefly rinsed with 0.5 M ammonium bicarbonate to remove traces of salts and blotted dry on an absorbent paper before weight measurements. The use of 0.5 M ammonium bicarbonate for rinsing is based on the fact that it is an iso-osmotic solution to the seawater, hence can remove the trace salt from the larvae surface without leading to ionic loss (Zhu and Lee, 1997). Each sample was then weighed for total weight using a Cahn C-33 micro-balance (precision=1μg, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA) and total number of larvae in each sample counted under a microscope (Nikon SMZ645), which enabled the calculation of average weight per individual larva. The larval samples were then put into an over set at 60 ℃ for 24 h to dry before the dry weight (DW) of each sample was determined on the Cahn C-33 micro-balance. In this way, the mean individual wet weight (WW) and dry weight (DW) at each sampling point were calculated. After day 3, surviving larvae was not enough for taking further samples due to mass mortality between day 3 and day 4 post hatching. Then, four samplings were collected during the starvation of day 0, 1, 2 and 3.

6.2.4 Biochemical analysis

See section 4.2.4. For each starvation period, the samples were analyzed in duplicated.

6.2.5 Statistical analysis

See section 4.2.5.

6.3 Result

6.3.1 Starvation resistance and changes in larval body weight during starvation

Figure 6.1 shows cumulative mortality of newly hatched larvae of *P. pelagicus* with the progress of starvation duration. Clearly, the highest and the second highest mortality happened between day 3 to day 4 (61.9%) and day 4 to day 5 (24.2%), respectively. The average survival time of newly hatched larvae when subjected to starvation was 3.87 ± 0.13 days. No significant difference in individual larval wet weight (WW) was detected over the duration of starvation although a slight increasing in wet weight was found initially. In contrast, a significant decreasing trend in individual dry weight (DW) was evident (Figure 6.2) and regression showed that the relationship between individual larval DW and duration of starvation (days) can be described by a exponential curve (Figure 6.3). During starvation, the highest and the second highest DW loss occurred between day 0 to day 1 (8.84%) and day 1 to day 2 (5.05%), respectively. Overall, 2.62μg of larval dry weight was lost during 3-day starvation period, which accounted for 17.42% of initially larval DW.

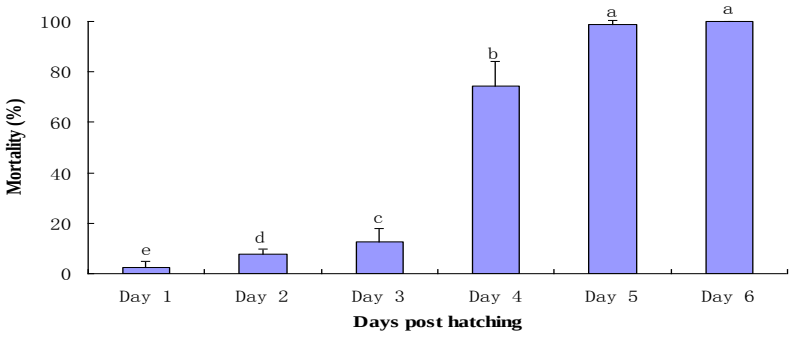

Figure 6.1 Cumulative mortality of newly hatched larvae of *Portunus pelagicus* subjected to starvation (n=5). The bars with different letters indicate significantly differences (P<0.05).

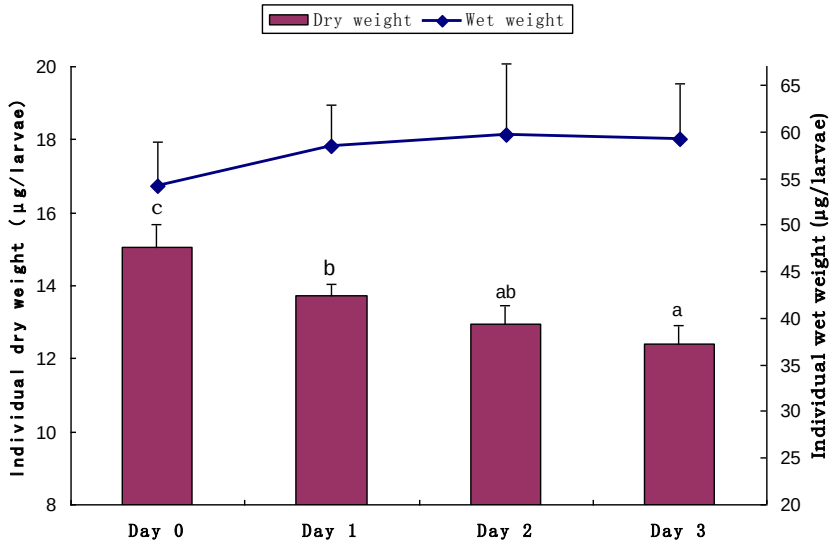

Figure 6.2 Changes in individual wet weight and dry weight of newly hatched larvae of *Portunus pelagicus* subjected to starvation. Data are presented as mean±SD (*n*=4). The bars with different letters indicate significantly differences (*P*<0.05).

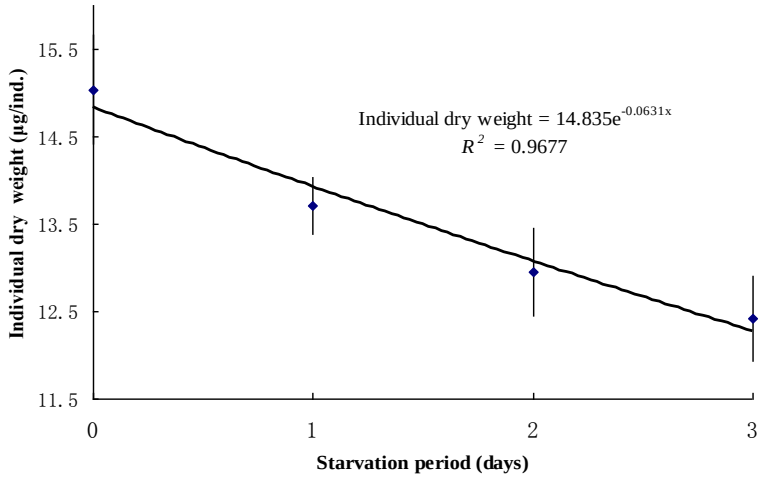

Figure 6.3 Regression analysis of individual dry weight of newly hatched *Portunus pelagicus* larvae in relation to days of starvation (n=5).

6.3.2 Larval total lipids and lipid classes

Both larval total lipid content (% larval dry weight) and individual total lipids (μg/larvae) decreased significantly over the duration of starvation and the highest loss of total lipid content (11.92%) and individual total lipids (19.16%) occurred during the first day starvation (i.e. from day 0 to day 1) (Figure 6.4). Over 3-day starvation, the total lipid content and individual total lipids decreased by 25.51% and 38.39%, respectively. Overall, 0.455μg of total lipids was lost per larvae over the 3-day period of starvation, which accounted for 17.36% of total body dry weight lose.

Table 6.1 Changes of lipid class contents (μg. larvae^{-1}) in newly hatched larvae of the blue swimmer crab, *Portunus pelagicus* subjected to starvation.

	Triacylglycerol	Free fatty acids	Cholesterol	Phospholipids	Total lipids
Day 0	0.020 ±0.003^{a}	0.017 ±0.001^{a}	0.039 ±0.002	1.109 ±0.064^{d}	1.185 ±0.060^{c}
Day 1	0.018 ±0.007^{a}	0.017 ±0.002^{a}	0.037 ±0.011	0.881 ±0.054^{c}	0.952 ±0.034^{b}
Day 2	0.045 ±0.011^{b}	0.039 ±0.012^{ab}	0.041 ±0.011	0.712 ±0.064^{b}	0.836 ±0.030^{ab}
Day 3	0.043 ±0.002^{b}	0.078 ±0.027^{b}	0.058 ±0.009	0.550 ±0.035^{a}	0.730 ±0.068^{a}
Change (%)	+116.54	+351.76	+49.66	- 50.39	- 38.46

Data are presented as mean ±SD (n=2). Values within a column with different superscript letters are significantly different (P<0.05).

Table 6.2 Changes of lipid class composition (% total lipids) of newly hatched larvae of the blue swimmer crab, *Portunus pelagicus* subjected to starvation.

	Triacylglycerol	Free fatty acids	Cholesterol	Phospholipids
Day 0	1.69 ±0.36^{a}	1.45 ±0.02^{a}	3.29 ±0.30^{a}	93.57 ±0.64^{c}
Day 1	1.88 ±0.77^{a}	1.76 ±0.31^{a}	3.87 ±1.25^{a}	92.49 ±2.33^{bc}
Day 2	5.39 ±1.56^{b}	4.64 ±1.60^{a}	4.87 ±1.49^{a}	85.08 ±4.65^{b}
Day 3	5.96 ±0.81^{b}	10.54 ±2.67^{b}	7.95 ±0.45^{b}	75.55 ±2.32^{a}

Data are presented as mean ±SD (n=2). Values within a column with different superscript letters are significantly different (P<0.05).

During starvation, among different lipid classes, phospholipids (PL) decreased significantly while triacylglycerol (TG) and free fatty acids (FFA) showed notable increases (P < 0.05, Table 6.1). Although there was an increasing trend for cholesterol content, the differences were not statistically significant (P >0.05). Over 3-day starvation, 50.39% of larval phospholipids were utilized while a 116.54%, 351.76% and 49.66% increase was detected on TG, FFA and cholesterol, respectively. In term of percentage composition, PL was the most dominant lipid fraction throughout despite a substantial decrease from 93.57% to 75.55% over the 3-day-starvation. In contrast to PL, the percentage of the other three lipid classes increased significantly over the duration of starvation (Table 6.2).

6.3.3 Larval fatty acids

A total of 27 fatty acids was identified for all larval samples. Of these 27 fatty acids, 13 had levels of more than 1% of total fatty acids throughout the starvation duration (Table 6.3). The five most abundant fatty acids (≥5%) in both newly hatched and starved larvae were 16:0 (13.02-15.58%), 18:0 (9.30-10.81%), 18:1n9 (7.17-8.24%), 20:5n3 (12.50-18.90%) and 22:6n3 (10.38-18.24%). These five fatty acids generally accounted for 55-66% of total fatty acids of the larvae. Among saturated fatty acids (SFA), 16:0 and 18:0 are the major constituents throughout the starvation duration and during starvation, the percentage of both 16:0 and ∑SFA increased

significantly (P<0.05). In contrast, the percentage of ∑PUFA and ∑HUFA in larvae decreased significantly during starvation. For mono-unsaturated fatty acids (MUFA), 14:1n, 18:1n9 and

Table 6.3 Fatty acid compositions (%total fatty acids) of newly hatched larvae of the blue swimmer crab, *Portunus pelagicus* subjected to starvation.

Fatty acid	Day 0	Day 1	Day 2	Day 3
14:0	1.02±0.13^{ab}	1.05±0.20^{ab}	0.90±0.09^{a}	1.10±0.09^{b}
15:0	0.32±0.07^{a}	0.34±0.09^{a}	0.42±0.04^{ab}	0.66±0.10^{b}
16:0	13.02±0.87^{a}	13.18±0.37^{a}	15.58±0.57^{b}	15.11±0.32^{b}
18:0	9.30±0.01^{a}	10.81±0.02^{b}	9.79±0.95^{a}	9.85±0.76^{a}
∑SFA	23.67±1.07^{a}	25.39±0.64^{b}	26.69±0.51^{c}	26.71±0.24^{c}
14:1n-7	3.03±0.42^{a}	5.77±1.41^{ab}	8.73±0.66^{bc}	11.17±1.61^{c}
16:1n-7	1.74±0.27^{ab}	1.53±0.41^{a}	2.12±0.21^{ab}	2.95±0.35^{c}
16:1n-5	0.55±0.12	0.71±0.18	0.65±0.06	0.77±0.36
18:1n-9	7.75±0.13	8.24±0.72	7.38±0.33	7.17±0.32
18:1n-7	4.62±0.04^{a}	4.83±0.17^{a}	5.52±0.16^{b}	5.46±0.19^{b}
20:1n-9	0.27±0.02^{a}	0.27±0.03^{a}	0.50±0.09^{b}	0.42±0.24^{b}
20:1n-7	0.63±0.04	0.58±0.35	0.36±0.06	0.49±0.14
∑MUFA	18.59±0.93^{a}	21.94±2.58^{ab}	25.25±0.64^{b}	28.43±2.50^{b}
16:2n-4	0.65±0.18^{a}	0.82±0.03^{b}	0.60±0.06^{a}	0.78±0.10^{b}
16:3n-4	1.17±0.41^{a}	1.37±0.47^{a}	2.57±0.10^{b}	1.23±0.50^{a}
18:2n-6	2.38±0.06^{a}	2.92±0.32^{b}	2.33±0.12^{a}	2.22±0.20^{a}
18:3n-6	0.16±0.05^{a}	0.41±0.01^{b}	0.40±0.04^{b}	0.34±0.12^{b}
18:3n-3	0.38±0.05^{a}	0.56±0.23^{b}	0.45±0.04^{ab}	0.51±0.00^{ab}
18:3n-4	0.24±0.07	--	--	--
18:4n-3	0.83±0.02^{a}	0.72±0.11^{a}	0.95±0.09^{b}	1.27±0.03^{c}
20:2n-6	0.62±0.10^{c}	0.45±0.14^{b}	0.22±0.02^{a}	0.50±0.05^{bc}
20:3n-6	1.46±0.15^{b}	1.10±0.43^{ab}	1.04±0.10^{a}	1.26±0.16^{ab}
20:4n-6	3.46±0.36^{c}	3.19±0.57^{bc}	2.53±0.25^{ab}	2.67±0.40^{a}
20:3n-3	0.26±0.00^{b}	0.19±0.01^{a}	0.32±0.03^{c}	0.51±0.02^{d}
20:4n-3	0.30±0.01^{a}	0.38±0.12^{ab}	0.36±0.04^{ab}	0.40±0.04^{b}
20:5n-3	18.90±1.03^{c}	17.83±1.00^{c}	15.40±0.11^{b}	12.50±1.50^{a}
22:2n-6	0.50±0.02^{a}	0.57±0.06^{a}	0.80±0.08^{b}	0.75±0.00^{b}
22:5n-3	1.73±0.24^{b}	1.06±0.28^{a}	0.98±0.09^{a}	1.19±0.27^{a}
22:6n-3	18.24±0.92^{d}	14.52±0.51^{c}	12.87±0.28^{b}	10.38±1.46^{a}
∑PUFA(≥ 18:2n)	49.53±2.71^{c}	43.90±3.04^{b}	38.64±1.04^{a}	34.50±3.53^{a}
∑n-3PUFA	40.64±2.21^{c}	35.25±2.29^{b}	31.32±0.68^{b}	26.75±3.24^{a}
∑n-6PUFA	8.65±0.57^{b}	8.65±0.75^{b}	7.32±0.36^{a}	7.75±0.29^{ab}
n-3/n-6	4.70±0.05^{c}	4.08±0.09^{b}	4.28±0.12^{b}	3.45±0.29^{a}
∑HUFA(≥ 20:3n)	44.34±2.68^{d}	38.25±2.94^{c}	33.50±0.89^{b}	28.91±3.78^{a}
DHA/EPA	0.97±0.00^{c}	0.81±0.02^{a}	0.84±0.01^{b}	0.83±0.02^{ab}
ARA/EPA	0.18±0.01^{b}	0.18±0.02^{ab}	0.16±0.01^{a}	0.21±0.01^{c}

Data are presented as mean±SD (n=2). Values within a row with different superscript letters are significantly different (P<0.05).

18:1n7 were the three dominant fatty acids. Interesting, of these three fatty acids, only 14:1n increased significantly by 268% during starvation. Among poly-unsaturated fatty acids (PUFA), starvation lead to higher levels of reduction in 20:4n6 (3.46% to 2.67%), 20:5n3 (18.90% to 12.50%) and 22:6n3 (18.24% to 10.38%). When changes in principal fatty acid contents during starvation were considered in term of absolute weight decreases per larvae (μg/larvae), except 14:1n, all principal fatty acids reduced substantially during starvation. The most significant reductions of between 57.69-68.88% were found for 20:4n6, 20:5n3 and 22:6n3, which were followed by 18:0, 18:1n9 and 18:2n6 with a reduction of between 40-50%. The rest of principal fatty acids decreased by a less extents of 7.23-36.56% (Table 6.4).

Table 6.4 Changes of principal fatty acid content (μg. Larvae^{-1}) in newly hatched larvae of the blue swimmer crab, *Portunus pelagicus* subjected to starvation.

Fatty acid	Day 0	Day 1	Day 2	Day 3	Change (%)
16:0	0.069 ±0.015^{d}	0.054 ±0.002^{c}	0.052 ±0.002^{b}	0.044 ±0.001^{a}	−36.56
18:0	0.050 ±0.000^{d}	0.044 ±0.000^{c}	0.033 ±0.003^{b}	0.029 ±0.002 ^{a}	−42.14
∑SFA	0.126 ±0.006^{d}	0.104 ±0.003^{c}	0.089 ±0.002^{b}	0.078 ±0.001^{a}	−38.28
14:1n	0.016 ±0.002^{a}	0.024 ±0.006^{b}	0.029 ±0.002^{c}	0.033 ±0.005^{c}	+101.59
16:1n-7	0.009 ±0.001^{b}	0.006 ±0.002^{a}	0.007 ±0.001^{a}	0.009 ±0.001^{b}	−7.23
18:1n-9	0.041 ±0.001^{d}	0.034 ±0.003^{c}	0.025 ±0.000^{b}	0.021 ±0.001^{a}	−49.41
18:1n-7	0.025 ±0.000^{c}	0.020 ±0.001^{b}	0.018 ±0.001^{b}	0.016 ±0.001^{a}	−35.38
∑MUFA	0.099 ±0.005^{b}	0.090 ±0.011^{b}	0.084 ±0.002^{a}	0.083 ±0.007^{a}	−16.36
18:2n-6	0.013 ±0.000^{c}	0.012 ±0.001^{c}	0.008 ±0.000^{b}	0.006 ±0.001^{a}	−48.86
20:4n-6	0.018 ±0.002^{c}	0.013 ±0.002^{b}	0.008 ±0.001^{a}	0.008 ±0.001^{a}	−57.69
20:5n-3	0.101 ±0.006^{d}	0.073 ±0.004^{c}	0.051 ±0.000^{b}	0.036 ±0.004^{a}	−63.82
22:6n-3	0.097 ±0.005^{d}	0.059 ±0.002^{c}	0.043 ±0.001^{b}	0.030 ±0.004^{a}	−68.88
∑PUFA	0.264 ±0.014^{d}	0.180 ±0.012^{c}	0.129 ±0.003^{b}	0.101 ±0.010^{a}	−61.91
∑HUFA	0.236 ±0.014^{d}	0.157 ±0.012^{c}	0.112 ±0.003^{b}	0.084 ±0.011^{a}	−64.35
Unknown	0.034 ±0.007^{a}	0.035 ±0.001^{a}	0.033 ±0.011^{ab}	0.045 ±0.004^{b}	+30.48
Total fatty acids	0.533 ±0.00^{d}	0.417 ±0.000^{c}	0.347 ±0.004^{b}	0.312 ±0.002^{a}	—41.53

Data are presented as mean ±SD (n=2). Values within a line with different superscript letters are significantly different (P<0.05).

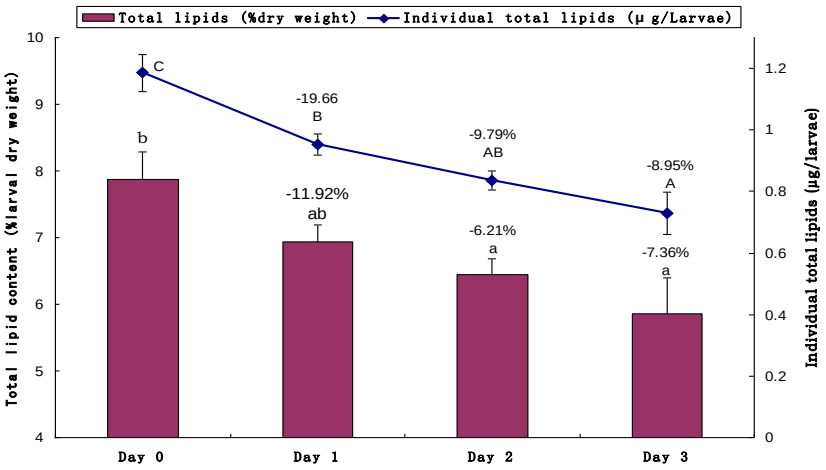

Figure 6.4 Changes of larval total lipids content (% larval dry weight) and individual total lipids (μg/larvae) of newly hatched larvae of *Portunus pelagicus* subjected to starvation. Data are presented as mean±SD (n=4). The bars with different letters indicate significantly differences (P<0.05). Values in the each column indicate percentage decreases in larval lipids as compared to the previous day.

6.4 Discussion

Under the similar water temperature, the average survival time of newly hatched *P. pelagicus* larvae subjected to starvation is generally close to the mud crabs, *Scylla serrata* and *S. paramamosain* (Li and Zeng, 2001; Djunaidah et al., 2003), but less than tropical lobster *Panulirus ornatus* (Smith et al., 2010). Average survival time under starvation has been used for evaluation starvation tolerances in crustacean larvae (Djunaidah et al., 2003; Wu et al., 2007b; Sui et al., 2009; Wu et al., 2010b,c). Generally, Point of no return (PNR) is also considered as a good measure for evaluation of starvation tolerance and nutritional condition of crustacean larvae for inter-species and intra-species (Anger and Dawirs, 1981; Calado et al., 2007). However, measurement of PNR is complicated and time-consuming (need several treatments for different starvation period and different feeding time) when there were several hatchings simultaneously (Li and Zeng, 2001; Djunaidah et al., 2003). It is worthy noting PNR is generally shorter than average survival time under starvation because mortality occurs after PNR has passed (Anger et al., 1981; Limbourn et al., 2008).

The present results show that the highest body dry weight and total lipid loss happened during the first day of starvation. This is similar to what has been reported for the swimming crab *P. trituberculatus* (Wu et al., 2006) and the mud crab *S. paramamosian* (Weng et al., 2002). One of likely reasons for this is that larvae are probably more active searching for food during their first day of starvation and their activity may tamper off as starvation continued and their energy

Table 6.5 Comparison of principal fatty acid profile (%total fatty acids) in the newly hatched larvae among various brachyuran crab species.

	16:0	18:0	SFA	14:1n	16:1n7	18:1n	MUFA	20:4n6	20:5n3	22:6n3	HUFA	Source
Portunus pelagicus	13.02	9.30	23.67	3.03	1.74	12.37	18.59	3.46	18.90	18.24	44.34	This study
Portunus trituberculatus	16.67	7.68	26.69	6.51	4.23	20.73	33.86	4.08	10.48	12.61	28.87	Wu et al. (2010b)
Scylla serrata	24.3	9.0	--	--	--	17.20	--	5.0	11.4	4.80	--	Weng et al. (2002)
Scylla serrata	20.86	6.9	30.90	0.15	4.91	22.43	31.05	5.09	11.74	10.25	28.68	Wu et al. unpublished data
Callinectes sapidus	22.36	8.29	35.31	1.0	9.38	13.24	33.17	3.04	9.52	9.24	23.51	Li et al. (2012)
Eriocheir sinensis	15.04	4.96	22.21	--	9.27	25.88	45.85	3.47	8.02	7.69	18.21	Sui et al (2007)

"—": undetectable or no data was provided in the cited literature.

reserve using up (Wu et al., 2006). During 3-day-starvation, larval body dry weight and total lipids decreased by 17.42% and 38.39%, respectively. However, the lipid loss only accounted for 15-20% of total body dry weight loss during starvation, which indicates that higher proportions of protein and/or carbohydrate were metabolised than lipids during the starvation. Newly hatched larvae of decapod crustaceans that are not lecithotrophy generally contain lower total lipids; therefore, they must start feeding immediately after hatching to avoid quick depletion of nutritional reserves (Staton and Sulkin, 1991; Calado et al., 2007). If such newly hatched larvae subjected to prolonged starvation, membrane structural proteins and lipids, and generally higher proportion of protein than lipids, will be oxidized for energy provision (Anger, 2001; Ritar et al., 2003; Wu et al., 2006). This prolonged starvation would eventually lead to point-of-no-return (Anger, 2001; Li and Zeng, 2001).

Phospholipids (PL) were the major lipid constitutes for newly hatched *P. pelagicus*, and it decreased by about 50% by the end of 3-day-starvation. It indicates that PL was the major lipid class utilized during the starvation by the newly hatched *P. pelagicus* larvae. PL is also known to serve as membrane structural lipids for brachyuran crabs (Anger, 2001). However, the oxidation of structural lipids suggests that prolonged starvation is highly detrimental to newly hatched larvae, which could lead to the point of no return (PNR) (Anger and Dawirs, 1981; Staton and Sulkin, 1991; Ritar et al., 2003). The increases in free fatty acids and cholesterol during starvation probably could be attributed to the decomposition of sterol-ester to free fatty acids and cholesterol while the increased level of triacyglycerol could be associated with the metabolic conversion of PL to TG (Liddy et al., 2004a).

Overall, the fatty acid profile of newly hatched larvae of *P. plegicus* is similar to other Portunidae crabs although they appeared to contain lower 16:0, 16:1n7 and 18:1n, and substantially higher percentage of 20:5n3 and 22:6n3 (Table 6.5). To certain extents, the fatty acid profile of newly hatched larvae reflects broodstock diets, particular for highly unsaturated fatty acids (HUFAs) (Wouters et al., 2001). In the present study, the broodstock *P. plegicus* were fed banana prawn (*Penaeus merguiensis*), green lipped mussel (*Perna canaliculus*) and California squid (*Loligo opalescens*), all of these diets contain high levels of EPA, DHA and HUFA. Additionally, the high HUFA percentage of newly hatched *P. plegicus* larvae may also been linked to the utilization of MUFA during the embryonic development (Rosa et al., 2003). The fatty acids, 18:3n3 and 18:2n6, have been identified as essential fatty acids for many crustaceans as they can not be synthesized *de novo* by decapod crustaceans but play important roles in larval survival, growth and development (Kanazawa et al., 1979a; Merican and Shim, 1996). However, relative low percentages of 18:3n3 (0.38-0.51% total fatty acids) and 18:2n6

(2.22-2.92%total fatty acids) were found in newly hatched larvae of *P. plegicus*, which might imply that they are less important to the newly hatched *P. plegicus* larvae.

During starvation, the increasing of 14:1n content (μg/larvae) may reflect the fact that this is an intermediate fatty acid of MUFA, such as 18:1n and 16:1n, catabolization/metabolization. It is a common strategy that SFA and MUFA are preferably metabolized during starvation while HUFA are conserved to maintain the structure of membranes and normal metabolic function during food deprivation in crustacean larvae (Ritar et al., 2003; Smith et al., 2003; Limbourn et al., 2008). However, interestingly it appeared that HUFA were preferentially utilized by newly hatched *P. plegicus* larvae when under starvation. This may relate to relative high HUFA content in the newly hatched *P. plegicus* larvae as compared to the other Portunidae crabs (Weng et al., 2002; Li et al., 2012). The higher levels of HUFA reduction under starvation could also indicate the oxidation of membrane structural PL since animal membrane PL generally contains high levels of HUFA (Liddy et al., 2004a; Holme et al., 2009b). Generally HUFA, in particular ARA, EPA and DHA, are considered to be more important for the functioning of the nervous system, membrane integrity, stress resistance, immunity to infections and parasitic disease than as a source of energy (Liddy et al., 2004a; Wu et al., 2007b). During food deprivation, preferentially depletion of particular fatty acids has been regarded as an indicator of limited nutritional value while retention of specific fatty acids is often interpreted as higher importance of those particular fatty acids (Weng et al., 2002; Smith et al., 2003). The high utilization of HUFA in newly hatched *P. plegicus* larvae might suggest those HUFA is less important in the larval tissue than previously suggested, or the requirement of HUFA for newly hatched *P. plegicus* larvae is lower than other Portunidae crabs.

6.5 Conclusion

In summary, when newly hatched *P. plegicus* larvae subjected to prolonged 3-day-starvation, their body dry weight and total lipids decreased by 17.42% and 38.39%, respectively. However, total lipids loss only accounted for 17.36% of total body dry weight reduction, which suggests protein and/or carbohydrate were the more important energy sources during the food deprivation. PL contributed to the majority of total lipids reduction during starvation. Meanwhile, the increases in free fatty acids and cholesterol during starvation maybe explained by the decomposition of sterol-ester to free fatty acids and cholesterol. The newly hatched larvae of *P. plegicus* contained substantially higher percentages of 20:5n3 and 22:6n3 than the other Portunidae crabs. The preferentially depletion of 20:5n3 and 22:6n3 suggest requirements of these HUFA by newly hatched *P. plegicus* larvae might be lower than other Portunidae crabs.

Chapter 7

Effects of starvation on survival, biomass (body weight) and lipid composition of newly hatched phyllosoma of the ornate rock lobster, *Panulirus ornatus*

7.1 Introduction

The spiny rock lobsters fishery is an important natural resource industry in Australia, with over AU$ 460 million of annual landing value (Mikami and Kuballa, 2004). Although there is an increasing market demand and high price (> $US 60/kg) around the world, most of commercially important spiny lobsters have been fully exploited (FAO, 2005). Therefore, aquaculture is one of the keys to expand production levels of them (Kittaka and Booth, 2000; Mikami and Kuballa, 2004; Smith et al., 2009a). Because of large size, fast growth, short larval period and delicate flavour, *Panulirus ornatus* become one prominent candidate for potential aquaculture (Irvin et al., 2010; O'Sullivan, 2010; Sachlikidis et al., 2010). One of the primary constraints for *P. ornatus* aquaculture is the lack of hatchery reared seed, particularly very low survival during the rearing period from phyllosoma to puerulus/juveniles. More recently, some breakthrough progress have been achieved in rearing *P. ornatus larvae* at the Australian Institute of Marine Science (AIMS), the M.G. Kallis Pty Ltd Exmouth hatchery facility and the Northern Fisheries Centre (NFC) of Queensland Primary Industries and Fisheries (QPI&F) from 2006 to 2010 (Smith et al., 2009a; O'Sullivan, 2010). However, the survival of newly hatched phyllosoma to puerulus/juveniles is very low (generally less than 1% at AIMS) and unstable during the larval rearing (Smith et al., 2009a; Wu et al., 2012). Larval nutrition, particularly lipid nutrition, is deemed as one of critical issues for the successful hatchery of this species (O'Sullivan, 2010).

Previous studies have shown optimization of dietary lipid nutrition is crucial to improve larval survival and shorten development period for western rock lobster, *Panulirus cygnus* (Liddy et al., 2004a; Liddy et al., 2005) and southern rock lobster, *Jasus edwardsii* (Nelson et al., 2003; 2004). Therefore, the better understanding of lipid requirements will facilitate the improvement of larval survival of *P. ornatus.* Starvation experiments and lipid analysis of newly hatched larvae have thereby been used as effective strategies to elucidate the relative importance of specific lipid compositions for crustacean larvae, with the consideration of the retention of specific lipid fractions and fatty acids (Weng et al., 2002; Ritar et al., 2003).

In field/open sea, newly hatched *P. ornatus* phyllosoma generally hatch in offshore water (depth 20 - 50m), and have limited swimming ability. Consequently, they have to rely on tides and currents for crossing continental shelf transportation (Jeffs, 2007). Therefore, similar to other marine crustacean, these pelagic larvae maybe exposed on the conditions of highly variable prey densities and face starvation (Dawirs, 1984; Staton and Sulkin, 1991). Although the ability of starvation resistance is species-specific depending on stored reserves of newly hatched larvae, most of marine decapod larvae are planktotrophic, and need immediate feeding after hatching (Dawirs, 1984; Staton and Sulkin, 1991; Calado et al., 2007). For a same species, starvation test was also used to evaluate the quality of newly hatched larvae. This is because the good quality larvae tend to have a longer survival period than the inferior quality larvae under the starvation (Djunaidah et al., 2003; Wu et al., 2010b). Moreover, the food deprivation of newly hatched decapod larvae will lead to a set of negative effects, including biomass loss, low survival, delayed development (Li and Zeng, 2001; Calado et al., 2007). Howver, no available information could be found on the impacts of starvation for the newly hatched *P. ornatus* phyllosoma.

Therefore, the aims of current study were to investigate (1) changes of survival and body weight of newly hatched larvae under the continued starvation; (2) which lipids (lipid classes and fatty acids) were of potentially important nutrition value by comparison of relative conservation or loss. Such information should be useful for the understanding of starvation resistance and development of suitable dietary regime for the newly hatched *P. ornatus* phyllosoma.

7.2 Materials and methods

7.2.1 Broodstock source and maintenance

Broodstock source and husbandry were described in Chapter 5 (Section3.2.1).

7.2.2 Larvae source and starvation resistance of newly hatched phyllosoma

On the day of hatching, active and photopositive phyllosomas from one berried female were collected from the surface of the broodstock spawning tank and transferred into a 20 L container of clean seawater for the estimation of larval production. Prior to stocking, the larvae were disinfected in 25 mg L^{-1} formalin solution for 4h. Then, larvae were placed into ten 64 L conical tubs at the density of 60 larvae L^{-1}. The tubs were water flow through system with rate of 1L/min. Water temperature and salinity were maintained at 29.9±1.1 ℃ and 34-36 ‰,

respectively. Prior to the use, seawater was settled, foam fractionated, ozonated (750Mv for 30 min), and carbon and cartridge filtered to 1μm, temperature adjusted and UV sterilized. Light was provided by overhead fluorescent ceiling lights set on a photoperiod of 14 h light: 10 h dark, and light intensity was around 180 lx. Continuous aeration was provided using air-stones. The larvae were not fed during the experiment. The larval culture tanks were siphoned each morning to remove dead larvae and other debris.

Additionally, one hundred and fifty newly hatched larvae were also randomly selected and transferred into five 500 mL glass beakers with twenty-five larvae per beaker. The beaker was filled with 400 mL filtered seawater. All beakers were water bath in the 64L conical tubs as described above. The mortality was checked and dead larvae was removed every 12 h until all larvae died. 100% water exchanges were performed for all beakers every morning. At the end of experiment, average survival time and mortality were calculated as the following formulae:

Average survival time (day) = $(1 \times N_1 + 1.5 \times N_{1.5} + 2 \times N_2 + \cdots\cdots + T \times N_T) / 30$, Where N_1, $N_{1.5}$, N_2 …represent the number of dead larvae during a 12 h period after 1, 1.5, 2… days of starvation.

Mortality (%) = 100 ×Number of cumulative dead larvae of each sampling period/30

7.2.3 Sampling for lipid analysis and data collection

During day 0, 1, 3 and 5 post hatching, all larvae sample (approximately 3-4 g wet weight) from 2 tanks were collected on nylon mesh (ca.200μm) and rinsed three times with 0.5 M Ammonium bicarbonate to remove traces of salts. 0.5 M Ammonium bicarbonate is an iso-osmotic solution to the seawater, which facilitates the removal of trace salt from the larvae surface but prevents the ionic loss of internal body fluids (Zhu and Lee, 1997). After rinsing, the samples were blotted on an absorbent paper and then frozen at -20 ℃ freezer. Prior to each sampling, two sub-samples (approximately 30 larvae/sample) were randomly selected from each tank for the measurement of body wet weight and dry weight. The samples were briefly rinsed with 0.5 M ammonium bicarbonate and blotted on an absorbent paper. Then, each sample was weighed by a Cahn C-33 micro-balance (precision=1μg, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA) and counted under a microscope (Nikon SMZ645). After 24 h oven-drying at 60 ℃, dry weight (DW) of each sample was determined. Therefore, the mean individual wet weight (WW) and dry weight (DW) of each sampling period were calculated by dividing the total sample wet and dry weight by the number of starved larvae. After day 5, most of phllosoma settle at the bottom and mix with dead larvae. Therefore, it is difficult to separate

and sample enough alive phyllosoma. Consequently, four samplings were collected during the starvation of day 0, 1, 3 and 5.

7.2.4 Biochemical analysis

See section 4.2.4. For each starvation period, the samples were analyzed in duplicated.

7.2.5 Statistical analysis

See section 4.2.5.

7.3 Results

7.3.1 Starvation resistance and changes of larval body weight

Figure 7.1 presented the cumulative mortalities of newly hatched larvae of *P. ornatus* during the starvation. Clearly, the highest mortality happened during day 8 to day 10 (from 38.54% to 85.34%) and only 20.22% of mortality was recorded from day 1 to day 7. Up to day12, 100% mortality happened. Under the starvation, average survival time of newly hatched larvae was 8.69 ± 0.92 days. During the starvation, the changes of individual wet weight and dry weight were shown in Figure 7.2. The significant decreasing trend could be detected on individual body wet weight and dry weight during the starvation period. As a consequence, a exponential curve could be found between individual body dry weight and starvation days (See Figure 7.3).

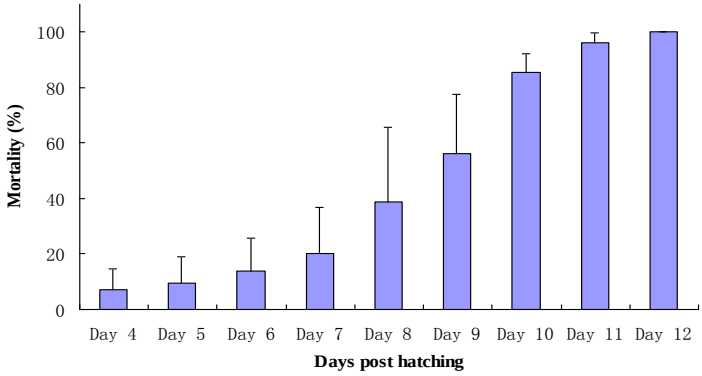

Figure 7.1. Time-course of cumulative mortalities of newly hatched larvae of the ornate rock lobster, *Panulirus ornatus* during the starvation period (n=5). The columns not having a same letter are significantly different (P <0.05).

During the starvation, the highest individual dry weight loss (12.38%) occurred during the day 0 to day 1, then the period of day 1 to day 3 accounted for the second highest individual dry weight loss (9.26%). Only 7.57% of individual dry weight loss existed during the period of day 3 to day 5. However, the highest individual wet weight loss (14.35%) occurred during the starvation of day 1 to day 3, then the period of day 0 to day 1 accounted for the second highest individual wet weight loss (9.26%). Only 2.05% of individual wet weight loss existed during the period of day 3 to day 5. Overall, 13.54μg of larval dry weight and 43.81μg of larval wet weight were lost during 5-days starvation, which accounted for 29. 21% and 22.03% of initially individual larvae dry weight and wet weight, respectively.

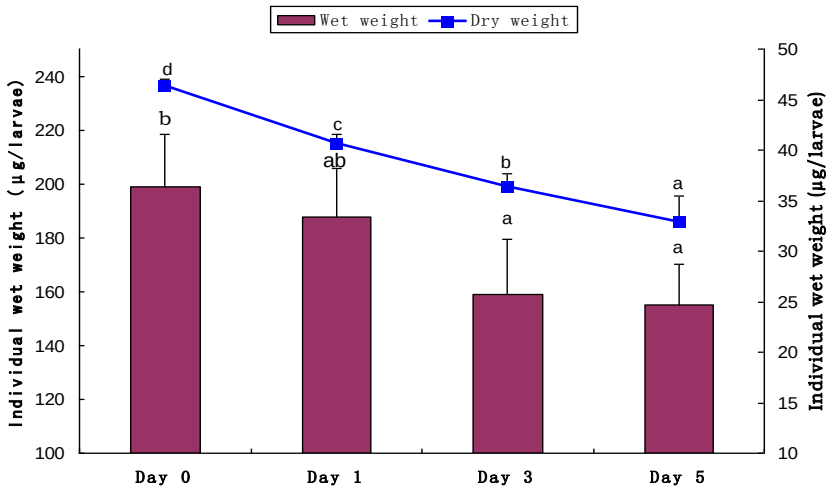

Figure 7.2 The changes of individual wet weight and dry weight of newly hatched larvae of *Panulirus ornatus* during the starvation period. Data are presented as mean ±SD (*n*=4). The columns that do not share a same letter are significantly different (*P*<0.05).

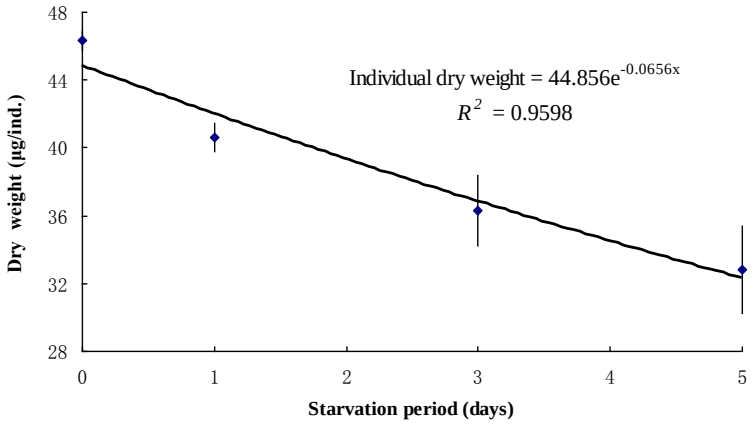

Figure 7.3 The regression of starvation period and individual dry weight of newly hatched larvae of the ornate rock lobster, *Panulirus ornatus* (n=4).

7.3.2 Changes of larval total lipids and lipid classes

Figure 7.4 presented the changes of larval total lipids and individual total lipids. The significant decrease could be detected on larval total lipids (% dry weight) and individual total lipids (μg/larvae) during the starvation. Although there was only one day starvation from day 0 to day 1, the highest individual total lipids loss (21.13%) was found on this period, then the period of day 1 to day 3 accounted for the second highest individual total lipids loss (19.05%). Only 13.84% of individual total lipids reduction existed during the period of day 3 to day 5. Overall, 1.97 μg total lipids per larvae was lost during 5-days starvation, which accounted for 53.97% of initially total lipids.

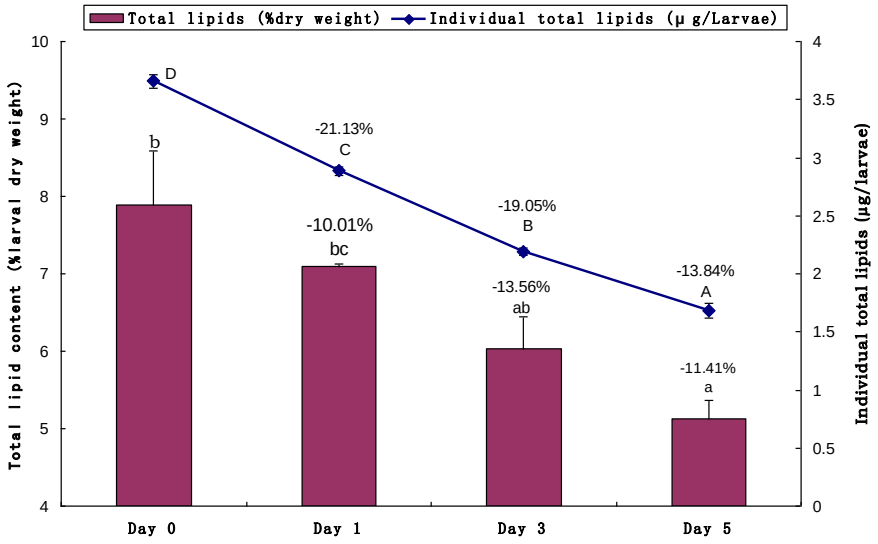

Figure 7.4 The changes of larval total lipids content (% larval dry weight) and individual total lipids (μg.Larvae^{-1}) of newly hatched larvae of *Panulirus ornatus* during the starvation period. Data are presented as mean ±SD (n=4). The columns that do not share a same letter are significantly different (P<0.05). Values in the each column indicate the percentage of decreasing lipids to that of newly hatched larvae during each starvation period.

During the starvation, phospholipids decreased significantly, and 55.47% of phospholipids per larvae was utilized (P<0.05, Table 7.1). Although there were 43.98% and 24.97% of decrease found on individual triacylglycerol and cholesterol contents, respectively, not statistic difference was found for them during 5-day starvation (P > 0.05). However, there was 22.77% of increase for individual free fatty acids. As for the percentage composition of lipid classes (Table 7.2), phospholipids was the dominant lipid fraction ranged from 92.70% to 95.90%. The dramatic decrease was only detected on the percentage of phospholipids, while the percentage of cholesterol increased significantly.

Table 7.1 Quantitative lipid class contents (μg. larvae^{-1}) of newly hatched larvae of the ornate rock lobster, *Panulirus ornatus* during the starvation period.

	Triacylglycerol	Free fatty acids	Cholesterol	Phospholipids	Total lipids
Day 0	0.031±0.014	0.021±0.015	0.105±0.045	3.506±0.270^{d}	3.657±0.322^{d}
Day 1	0.019±0.004	0.032±0.004	0.078±0.026	2.755±0.027^{c}	2.884±0.002^{c}
Day 3	0.026±0.016	0.019±0.007	0.082±0.013	2.062±0.113^{b}	2.189±0.149^{b}
Day 5	0.018±0.004	0.026±0.011	0.079±0.005	1.561±0.085^{a}	1.683±0.074^{a}
Change (%)	-43.98	+22.77	- 24.97	- 55.47	- 53.97

Data are presented as mean ±SD (n=2). Values within a column having different superscript letter are significantly different (P<0.05).

Table 7.2 Qualitative lipid class composition (% total lipids) of newly hatched larvae of the orante rock lobster, *Panulirus ornatus* during the starvation period.

	Triacylglycerol	Free fatty acids	Cholesterol	Phospholipids
Day 0	0.88±0.45	0.54±0.38	2.84±0.97^{ab}	95.90±1.06^{b}
Day 1	0.65±0.14	1.10±0.13	2.72±0.90^{b}	95.54±0.89^{ab}
Day 3	1.18±0.63	0.85±0.28	3.73±0.33^{ab}	94.23±1.24^{ab}
Day 5	1.05±0.31	1.55±0.71	4.70±0.08^{a}	92.70±0.93^{a}

Data are presented as mean ±SD (n=2). Values within a column having different superscript letter are significantly different (P<0.05).

7.3.3 Changes of larval fatty acids

The fatty acid compositions (% total fatty acids) of newly hatched and starved *P. ornatus* phyllosomas were presented in Table 7.3. A total of 28 individual fatty acids were identified in all samples. Of the 28 fatty acids identified, 13 fatty acids had relative levels of more than 1% of total fatty acids at all starvation points. There were five most abundant fatty acids (≥5%) in newly hatched larvae and starved larvae, i.e. 16:0 (12.06-18.33%), 18:0 (9.29-14.24%), 18:1n-9 (6.51-10.04%), 20:5n-3 (14.36-16.31%) and 22:6n-3 (10.58-15.51%). Those five fatty acids generally accounted for 58-68% of total fatty acids in larvae. Among saturated fatty acids (SFA), 16:0 and 18:0 are the major constituents of SFA for all stages, and their percentages decreased significantly during the starvation. For mono-unsaturated fatty acids (MUFA), 14:1n, 16:1n-7, 18:1n-9 and 18:1n-7 were four dominant fatty acids. Interesting, only dramatic increase was found on 14:1n, which increase by 12.2 times during the starvation. Of poly-unsaturated fatty acids (PUFA), the percentages of 18:2n-6 and 18:3n-3 decreased significantly while 20:4n-6 and 22:6n-3 increased significantly, particularly during the day 3 to day 5. Consequently, the percentage of ∑PUFA, ∑HUFA and DHA/EPA ratio increased significantly during the starvation of day 3 to day 5. Table 7.4 presented the changes of principal fatty acid contents (μg/larvae) during the starvation. Except for 14:1n, principal fatty acid contents reduced dramatically during the starvation. There are six fatty acids with reduction of more than 70%,

including 16:0, 18:0, 16:1n-7, 18:1n-9, 18:1n-7 and 18:2n-6. In contrast, the lowest loss was detected on 20:4n-6, and 22:6n3 while 20:5n-3 decreased by 63.59% during 5-days starvation.

Table 7.3 Qualitative fatty acid composition (%total fatty acids) of newly hatched larvae of the ornate rock lobster, *Panulirus ornatus* during the starvation period.

Fatty acid	Day 0	Day 1	Day 3	Day 5
14:0	0.68 ±0.03^{a}	0.48 ±0.03^{a}	0.99 ±0.05^{b}	1.13 ±0.18b
15:0	0.48 ±0.02	0.39 ±0.01	0.60 ±0.28	0.50 ±0.02
16:0	18.33 ±0.75^{c}	16.42 ±0.00^{b}	15.04 ±1.24^{b}	12.06 ±0.79^{a}
18:0	13.58 ±2.04^{b}	14.24 ±0.84^{b}	13.25 ±0.88^{b}	9.29 ±0.59^{a}
23:0	0.38 ±0.05	0.24 ±0.07	0.37 ±0.03	0.34 ±0.05
∑SFA	33.46 ±2.80^{b}	31.76 ±0.81^{b}	30.24 ±0.10^{b}	23.32 ±1.63^{a}
14:1n	1.45 ±0.42^{a}	3.22 ±1.06^{a}	11.69 ±0.27^{b}	13.20 ±0.70^{b}
16:1n-7	4.25 ±0.13^{b}	3.69 ±0.02^{b}	2.64 ±0.55^{a}	2.62 ±0.18^{a}
16:1n-5	0.58 ±0.10^{ab}	0.45 ±0.03^{a}	0.81 ±0.16^{b}	0.74 ±0.14^{ab}
18:1n-9	9.76 ±0.65^{b}	10.04 ±0.32^{b}	7.78 ±0.79^{a}	6.51 ±0.47^{a}
18:1n-7	2.97 ±0.45^{b}	2.73 ±0.19^{ab}	2.46 ±0.07^{ab}	2.21 ±0.14^{a}
20:1n-9	0.28 ±0.04	0.22 ±0.04	0.52 ±0.04	0.31 ±0.022
20:1n-7	1.40 ±0.22	1.36 ±0.18	1.28 ±0.09	1.20 ±0.02
∑MUFA	20.69 ±1.77^{a}	21.70 ±0.47^{a}	27.18 ±1.26^{b}	26.80 ±0.25^{b}
16:2n-4	0.52 ±0.10^{a}	0.25 ±0.01^{a}	1.16 ±0.13^{c}	0.83 ±0.13^{b}
16:3n-4	1.35 ±0.06	1.02 ±0.11	1.93 ±1.02	2.07 ±0.10
18:2n-6	1.94 ±0.01^{b}	1.63 ±0.10^{ab}	1.53 ±0.14^{a}	1.48 ±0.18^{a}
18:3n-6	0.21 ±0.13	0.28 ±0.06	0.43 ±0.01	0.27 ±0.11
18:3n-3	1.00 ±0.05^{b}	0.80 ±0.02^{ab}	0.73 ±0.14^{a}	0.59 ±0.10^{a}
18:3n-4	0.31 ±0.07	0.25 ±0.02	0.43 ±0.24	0.16 ±0.08
18:4n-3	0.64 ±0.07^{a}	0.68 ±0.05^{a}	0.96 ±0.14^{b}	0.94 ±0.01^{b}
20:2n-6	0.77 ±0.07^{b}	0.49 ±0.03^{a}	0.51 ±0.03^{a}	0.44 ±0.15^{a}
20:3n-6	1.11 ±0.11	1.20 ±0.08	1.08 ±0.15	1.14 ±0.12
20:4n-6	2.96 ±0.37^{a}	3.51 ±0.20^{ab}	3.75 ±0.13^{a}	3.98 ±0.17^{ab}
20:3n-3	0.35 ±0.06	0.36 ±0.06	0.49 ±0.08	0.28 ±0.09
20:4n-3	0.53 ±0.02^{b}	0.37 ±0.14^{ab}	0.25 ±0.02^{a}	0.40 ±0.01^{ab}
20:5n-3	14.73 ±0.35	16.31 ±0.95	14.67 ±0.07	14.36 ±1.61
22:2n-6	0.27 ±0.10^{ab}	0.21 ±0.05^{a}	0.27 ±0.04^{ab}	0.42 ±0.07^{b}
22:5n-3	0.38 ±0.08^{a}	0.67 ±0.14^{a}	1.46 ±0.85^{ab}	2.78 ±0.65^{b}
22:6n-3	11.48 ±0.44^{a}	11.32 ±0.95^{a}	10.58 ±1.00^{a}	15.51 ±0.67^{b}
∑PUFA(≥ 18:2n)	36.67 ±1.84^{a}	38.08 ±0.68^{ab}	37.13 ±0.03^{ab}	42.75 ±3.48^{b}
∑n-3PUFA	29.11 ±0.97^{a}	30.50 ±0.13^{a}	29.13 ±0.16^{a}	34.88 ±3.12^{b}
∑n-6PUFA	7.26 ±0.79	7.33 ±0.53	7.56 ±0.11	7.72 ±0.44
n-3/n-6	4.03 ±0.30	4.17 ±0.28	3.84 ±0.04	4.52 ±0.14
∑HUFA(≥ 20:3n)	31.53 ±1.44^{a}	33.73 ±0.35^{a}	32.27 ±0.16^{a}	38.46 ±2.30^{b}
DHA/EPA	0.78 ±0.01^{a}	0.70 ±0.10^{a}	0.72 ±0.06^{a}	1.08 ±0.08^{b}
ARA/EPA	0.20 ±0.02^{a}	0.22 ±0.02^{a}	0.26 ±0.01^{b}	0.28 ±0.02^{b}

Data are presented as mean ±SD (n=2). Values within a line having different superscript letter are significantly different (P<0.05).

7.4 Discussion

Under the starvation, the average survival time of newly hatched *P. ornatus* phyllosoma is 8.69 ± 0.92 days. This is dramatically longer than the other tropical crustacean, including mud crabs, *Scylla serrata* (3.5days) and *Scylla paramamosain* (ca.3 days) (Li and Zeng, 2001; Djunaidah et al., 2003) and blue swimmer crab, *P. pelagicus* (3.87days, see Chapter 6). This

difference is likely to be due to their specie-specific abilities of starvation resistance and their different nutrient reserves in the newly hatched larvae (Abrunhosa and Kittaka, 1997; Liddy et al., 2003). However, for a same species, the newly hatched larvae also displayed a significant variability of starvation resistance (Anger, 1995; Wu et al., 2006). This could be explained by a number of factors, including hatching conditions, broodstock diets and its physiological status (Garcia-Guerrero et al., 2003; Garcia-Guerrero, 2010; Wu et al., 2010b). Previous studies have shown those factors may play an important role in the quantity and quality of reserves of newly hatched larvae for crustacean, consequently, resulting in the different starvation resistance of newly hatched larvae (Garcia-Guerrero et al., 2003; Wu et al., 2010b).

Table 7.4 Principal fatty acid content (µg. Larvae^{-1}) of newly hatched larvae of the ornate rock lobster, *Panulirus ornatus* during the starvation period.

Fatty acid	Day 0	Day 1	Day 3	Day 5	Change (%)
16:0	0.355 ±0.015^{d}	0.232 ±0.000^{c}	0.146 ±0.012^{b}	0.087 ±0.006^{a}	-75.44
18:0	0.263 ±0.040^{c}	0.201 ±0.012^{c}	0.129 ±0.009^{b}	0.067 ±0.004^{a}	-74.46
∑SFA	0.648 ±0.054^{d}	0.449 ±0.011^{c}	0.293 ±0.001^{b}	0.169 ±0.012^{a}	-73.98
14:1n	0.028 ±0.008^{a}	0.045 ±0.015^{a}	0.113 ±0.003^{b}	0.096 ±0.005^{b}	+240.76
16:1n-7	0.082 ±0.002^{c}	0.052 ±0.000^{b}	0.026 ±0.005^{a}	0.019 ±0.001^{a}	-77.02
18:1n-9	0.189 ±0.013^{d}	0.142 ±0.005^{c}	0.075 ±0.008^{b}	0.047 ±0.003^{a}	-75.09
18:1n-7	0.058 ±0.009^{d}	0.039 ±0.003^{c}	0.024 ±0.001^{b}	0.016 ±0.001^{a}	-72.23
∑MUFA	0.401 ±0.034^{d}	0.307 ±0.007^{c}	0.264 ±0.012^{b}	0.194 ±0.002^{a}	-51.63
18:2n-6	0.038 ±0.000^{d}	0.023 ±0.001^{c}	0.015 ±0.001^{b}	0.011 ±0.001^{a}	-71.68
20:4n-6	0.057 ±0.007^{c}	0.050 ±0.003^{c}	0.036 ±0.001^{b}	0.029 ±0.001^{a}	-49.77
20:5n-3	0.286 ±0.007^{d}	0.230 ±0.013^{c}	0.142 ±0.001^{b}	0.104 ±0.012^{a}	-63.59
22:6n-3	0.223 ±0.009^{c}	0.160 ±0.013^{b}	0.103 ±0.010^{a}	0.112 ±0.005^{a}	-49.53
∑PUFA	0.711 ±0.036^{d}	0.538 ±0.017^{c}	0.360 ±0.002^{b}	0.309 ±0.025^{a}	-56.47
∑HUFA	0.611 ±0.028^{d}	0.477 ±0.022^{c}	0.313 ±0.002^{b}	0.278 ±0.024^{a}	-54.45
Unknown	0.142 ±0.015^{b}	0.102 ±0.034^{b}	0.023 ±0.002^{a}	0.031 ±0.014^{a}	-78.36
Total fatty acids	1.797 ±0.015^{d}	1.311 ±0.034^{c}	0.947 ±0.002^{b}	0.693 ±0.014^{a}	-61.42

Data are presented as mean ±SD (n=2). Values within a line having different superscript letter are significantly different (P<0.05).

Table 7.5 Qualitative comparison of principal fatty acid profile (%total fatty acids) of newly hatched phyllosoma among the different lobster species.

	16:0	**18:0**	**SFA**	**16:1n7**	**18:1n**	**MUFA**	**20:4n6**	**20:5n3**	**22:6n3**	**HUFA**	**Source**
Nephrops norvegicus	21.43	5.28	30.18	7.59	28.29	40.09	2.88	11.26	9.88	25.44	Rosa et al (2003)
Panulirus hormarus	12.22	7.35	23.86	4.36	28.91	37.14	4.96	5.34	1.07	12.3	Chakraborty et al (2010)
Panulirus cygnus	13.7	13.6	28.2	2.7	16.9	24.7	9.9	13.7	10.5	35.6	Liddy et al (2004a)
Jasus edwardsii	14.5	6.9	27.6	3.7	17.7	27.6	11.6	15.1	8.1	38.3	Phleger et al (2003)
Panulirus ornatus	18.33	13.58	33.46	4.25	12.73	20.69	2.96	14.73	11.48	31.53	This study

Fifty percentage of point of no return (PNR_{50}) was considered as a valuable measure for evaluation of starvation tolerances and nutritional condition of crustacean larvae for inter-species and intra-species (Anger and Dawirs, 1981; Calado et al., 2007). However, measurement of PNR is more complicated and time-consuming (need several treatments for different starvation period and different feeding time) when there were several hatchings simultaneously (Li and Zeng, 2001; Djunaidah et al., 2003; Liddy et al., 2003). Therefore, average survival time under starvation was recommended and used to evaluate starvation tolerances for crustacean larvae (Djunaidah et al., 2003; Wu et al., 2007b; Sui et al., 2009; Wu et al., 2010b). It is worth noting PNR_{50} is generally shorter than average survival time under starvation because the death will normally occur after they have passed the PNR (Anger et al., 1981; Limbourn et al., 2008). For instance, the current study showed average survival time under starvation was 8.69 ± 0.92 days while PNR_{50} is 3.8 days for the newly hatched *P. ornatus* phyllosoma (Smith et al., 2010).

Although there was only one day of starvation during the first day starvation, the highest body dry weight and total lipids loss happened. Although a clear decreasing trend for the individual dry weight and total lipids reduction during the starvation of day 1 – day 3 to day 3 – day 5, the individual body dry weight loss of later period is less than that of the first day starvation. This is similar to swimming crab *P. trituberculatus* (Wu et al., 2006) and mud crab *Scylla serrata* (Weng et al., 2002). This is probably because the larvae were more active and restless in the first day starvation than the later. The later inactive/tranquil seclusion of larvae is effective strategy to reduce energy consumption during the starvation (Wu et al., 2006). During the starvation, reduction of the dry weight and total lipids per larvae were 29.21% and 53.97% to the initial values, respectively. However, the lipid loss only accounted for 13.47-16.20% of total body dry weight reduction during the starvation, and mean percentage was 17.36%. This is similar to southern rock lobster, *Jasus edwardsii* (17.8%) (Smith et al., 2003a), but higher than the western rock lobster, *P. cygnus* (6.5%) (Liddy et al., 2005). Those authors further suggested more protein, and to a lesser extent carbohydrate and lipids, was preferentially catabolized during the starvation of newly hatched spiny rock lobster phyllosoma (Ritar et al., 2003; Smith et al., 2003a). For those newly hatched larvae absent of lecithotrophy, they generally contained the lower body reserves, particularly for total lipid levels; therefore, they must start feeding immediately after hatching and avoid the deleterious effects induced by starvation (Staton and Sulkin, 1991; Anger, 1995; Calado et al., 2007). If those newly hatched larvae are subjected to starvation, they will be oxidized membrane structural proteins and lipids for the energy provision, leading to beyond the point of no return (Anger, 2001; Ritar et al., 2003; Wu et al., 2006).

Phospholipid (PL) is the major lipid constitutes (92-95%) for newly hatched *P. ornatus*, and PL was the major lipid class utilized during the starvation. Although TAG is a common short-term storage lipid for energy provision in many marine crustacean (Perez-Velazquez et al., 2003; Yao et al., 2008), it only accounted for 0.88-1.18% of total lipids in the newly hatched *P. ornatus* phyllosoma. Therefore, TAG could not be as one of major energy source during the starvation experiment on newly hatched *P. ornatus* phyllosoma. This is similar to the other spiny rock lobsters, including western rock lobster, *P. cygnus* (Liddy et al., 2005; Limbourn and Nichols, 2009) and southern rock lobster, *Jasus edwardsii* (Smith et al., 2003). PL commonly serves as membrane structural lipids for crustacean (Anger, 2001). Therefore, oxidation of structural lipids may suggest the continuous starvation of newly hatched *P. ornatus* larvae leads to a detrimental damage to successful development, resulting in a point of no return (PNR) (Anger and Dawirs, 1981; Staton and Sulkin, 1991; Ritar et al., 2003). During the starvation experiment, the increasing of free fatty acids may be due to the hydrolysis of sterol-ester and PL to free fatty acids, cholesterol and glycerol (Liddy et al., 2004a).

Overall, the fatty acid profile of newly hatched phyllosoma of *P. ornatus* is similar to the other lobsters (Table 7.5). However, the newly hatched phyllosoma of *P. ornatus* and *Nephrops norvegicus* had higher SFA, but lower 20:4n-6 than other lobsters. To some extent, the fatty acid profile of newly hatched phyllosoma can reflects that of their broodstock diets, particular for highly unsaturated fatty acids (HUFAs) (Wouters et al., 2001). Therefore, this difference of fatty acid profile may indicate their different broodstock diets as well as fatty acid requirements (Ritar et al., 2003; Smith et al., 2003a; Melville-Smith et al., 2007). 18:3n-3 and 18:2n-6 have also been identified as essential fatty acids for many crustaceans as they cannot be synthesized *de novo* and play important roles for survival, growth and development (Kanazawa et al., 1979a; Merican and Shim, 1996b). However, the relative low percentages of 18:3n-3 (1.00% total fatty acids) and 18:2n-6 (1.94%total fatty acids) may suggest they have inferior effect to newly hatched *P. ornatus* phyllosoma.

During the starvation experiment, the percentages of four major fatty acids, 16:0, 18:0, 18:1n and 16:1n, decreased dramatically. This indicated those fatty acids were preferentially utilized during the starvation of newly hatched *P. ornatus*. It is common phenomenon that SFA and MUFA are initially catabolized during the starvation and HUFA are conserved to maintain the structure of membranes and normal metabolic function during the food deprivation (Ritar et al., 2003; Smith et al., 2003; Limbourn et al., 2008). However, 20:5n-3, as an important HUFA, was not relatively conserved during the starvation. Therefore, its percentage remained around

14% of total fatty acids. The high utilization of 20:5n-3 might suggest this HUFA is less important for newly hatched *P. ornatus* than previously suggested, or the requirement of this fatty acid for newly hatched *P. ornatus* is lower than previously expected. In addition, the increasing 14:1n content (μg/larvae) maybe reflect this is an intermediate fatty acid during the catabolization of MUFA, such as 18:1n and 16:1n.

7.5 Conclusion

In summary, during the starvation of newly hatched *P. ornatus*, the individual body dry weight and total lipids decreased by 29.21% and 34.89% to the initial, respectively. However, total lipids loss only accounted for 14.58% of total body weight reductions. This suggests protein and/or carbohydrate are also important energy source during the food deprivation. The lipid class profile was dominated by PL, and PL contributed the major total lipids reduction during the starvation. The newly hatched phyllosoma of *P. ornatus* contained high percentages of 20:5n-3 and 22:6n-3, but low 20:4n-6. During the starvation experiment, the preferentially conservation of DHA and ARA might suggest those HUFAs are very important for newly hatched *P. ornatus*. Therefore, more attention should be focused on their HUFA requirements, storage and utilization for *P. ornatus* phyllosoma.

Chapter 8

Dietary arachidonic acid level affects larval performance and the occurrence of ing death syndrome in the blue swimmer crab, *Portunus pelagicus*

8.1 Introduction

The blue swimmer crab, *Portunus pelagicus* (Linnaeus, 1758), is a commercially important species that is widely distributed in the Indo-Pacific, particularly in tropical and subtropical waters (Stephenson and Campbell, 1959). This species has been sold worldwide in various product forms, including traditional hard-shell crab, and more recently as soft-shell crab and various pasteurized crabmeat products (Romano and Zeng, 2008; O'Sullivan and Savage, 2008). Because of their high nutritional value, large size and delicate flavor, there is an increasing market demand for this species (Wu et al., 2010d). It is believed that wild *P. pelagicus* stocks have been fully exploited, therefore, any further increase in *P. pelagicus* production is likely to have to come from either aquaculture or stock enhancement programs (Maheswarudu et al., 2008; Dixon and Hooper, 2009), both of which rely on successful hatchery seed production. Larval development of *P. pelagicus* includes four zoeal instars and one megalopal stage before settling as the first stage crab (Josileen and Menon, 2004; Andres et al., 2010). Generally, larval culture of *P. pelagicus* has the better survival than that of other commercially important portunid crabs, such as the mud crab *Scylla* spp. and the blue crab, *Callinectes sapidus* (Zmora et al., 2005; Nghia et al., 2007). However, high incidences of moulting death syndrome (MDS) during the period when zoea IV metamorphoses to megalopa is still commonly observed (Soundarapandian et al., 2007; Castine et al., 2008), which signficantly impacts the final yield of seed production.

Previous research has shown that unbalanced dietary highly unsaturated fatty acids (HUFA) could lead to low larval survival as well as a high incidence of ing death syndrome (MDS) in larval culture of the mud crab, *S. serrata* (Suprayudi et al., 2004c; Dan and Hamasaki, 2011) and the swimming crab, *Portunus trituberculatus* (Takeuchi et al., 1999a,b; Arai et al., 2004). For *P. pelagicus*, larval nutrition, particular fatty acid nutrition, has also been suggested as a major contributor to incidents of unsuccessful larval rearing (Maheswarudu et al., 2008). However, there is no published information on the HUFA requirements of *P. pelagicus* larvae. This undoubtedly will hinder the development of hatchery culture protocols as well as potential formulation of cost-effective larval feeds for *P. pelagicus*.

More importantly, despite extensive research on n-3 HUFA (e.g. EPA and DHA) requirement for larval crustaceans, very limited research efforts have focused on n-6 HUFA requirements, particularly those for arachidonic acid (ARA) (Bell and Sargent, 2003; Nghia et al., 2007). Interestingly, our recent study revealed that there were positive correlations between larval ARA level and higher survival and shorter intermoult period in the newly hatched larvae of the swimming crab *P. trituberculatus* (Wu et al., 2010c). It is known that ARA generally serves as the preferred precursor for biosynthesis of eicosanoids, such as prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which play important roles in the modulation of growth, metamorphosis and stress resistance in crustacean and fish larvae (Koskela et al., 1992; Bell and Sargent, 2003; Van Anholt et al., 2004a,b). This current study was designed and carried out to assess the effects of dietary ARA on survival, development, growth and occurrence of MDS, and to identify the optimal dietary ARA level and ARA/EPA ratio for *P. pelagicus* larvae.

8.2 Materials and methods

8.2.1 Broodstock source and maintenance

Broodstock source and husbandry were described in Chapter 4 (Section 4.2.1).

8.2.2 Artemia enrichment

Five *Artemia* enrichment emulsions containing different ARA levels were formulated by incorporating 0% (ARA1), 8% (ARA 2), 16% (ARA 3), 24% (ARA 4) and 32% (ARA 5) of concentrated ARA oil (containing 41.19% ARA) (Table 8.1). Because the concentrated ARA oil used for formulation also contained 6.79% of 18:2n-6, the addition of different quantities of the ARA oil could lead to differences in 18:2n-6 contents of these emulsions. To resolve this problem, various amounts of corn oil (rich in 18:2n-6) and macadamia oil (rich in 18:1n-9) were added to maintain similar levels of 18:2n-6 in all enrichment emulsions (Bransden et al., 2005b). Concentrated n-3 HUFA oil (DHA/EPA=1.51, n-3 HUFA=67.53% total fatty acids) was also added to the emulsions to provide DHA (22:6n-3) and EPA (20:5n-3). Finally, 10% of soy lecithin was added as an emulsifier while 2% of α-tocopherol acetate was added as an antioxidant (Estévez et al., 2001; Villalta et al., 2005). Each emulsion was then emulsified with equal amounts of distilled water using an Ultra-turrax T25 homogenizer (IKA Works Inc, Wilmington, North Carolina, USA) operating at 10, 000 rpm for 60 seconds. They were then stored in bottles and kept in a refrigerator at 4^{o}C.

To obtain newly hatched *Artemia* nauplii for the experiment, *Artemia* cysts (EG grade, INVE (Thailand) Ltd, Thailand) were hatched daily in 1-μm filtered seawater with a salinity of

25‰ at 28°C. After 24 h, nauplii (size between 430-520μm) were harvested for subsequent enrichment. Six-hour-old *Artemia* nauplii were enriched in 300 mL containers at a density of 200-250 *Artemia*/mL for 24 h with 0.6 g/L of each emulsion oil (Sui et al., 2007). During enrichment, temperature and salinity were maintained at 28 °C and 25‰, respectively. Enriched *Artemia* were then harvested and rinsed with freshwater to remove traces of the emulsion before being fed to *P. pelagicus* larvae. Any enriched *Artemia* that were not fed to larvae were immediately placed at 4℃ in a refrigerator to minimize fatty acid catabolism (Merchie, 1996), they were to be used within 24 h and any leftovers discarded after this period. Enriched *Artemia* (ca.1-2 g/sample) in each treatment were sampled three times during the larval culture for lipid and fatty acid analysis. All samples were stored at -70 ℃ before analysis took place.

Table 8.1 The formulation (mg/g) of five experimental emulsion oils for *Artemia* enrichment

	Source	ARA1	ARA 2	ARA 3	ARA 4	ARA 5
ARA oil	A	0	80	160	240	320
Macadamia oil	B	440	370	300	230	160
Corn oil	C	40	30	20	10	0
n-3 HUFA oil	D	400	400	400	400	400
Soy Lecithin	C	100	100	100	100	100
α-tocopherol acetate	E	20	20	20	20	20

Source of the ingredients:
A. Cargill health and food Technologies, MA, USA, contained 41.19% arachidonic acid (20:4n-6);
B. Macadamias (Aust) Ltd, Gympie, Queensland, Australia;
C. Sigma-Aldrich Pty Ltd, Castle hill, NSW, Australia;
D. Obsidian Research Ltd, U.K., contained 67.53% of n-3 HUFA; DHA/EPA=1.51;
E. Blackmores Ltd, Warriewood, New South Wales, Australia.

8.2.3 Experimental design and setup

On the day of larval hatching, within 8 h of hatching, healthy and actively swimming zoea I larvae origated from one female were collected and randomly transferred into 600 mL glass beakers filled with 500 mL UV- treated, 1 μm filtered seawater at a salinity of 28‰. Twenty-five larvae were stocked into each beaker as a replicate. There were five treatments (Treatment 1 to 5, defined as T1-T5) with larvae fed *Artemia* enriched with self-prepared ARA1- ARA5 emulsions, respectively. Every treatment had 6 replicates and among them, one replicate was used exclusively for sampling of zoea IV for measurement of chela length (CHL) and carapace length (CL) to calculate the ratio of chela length (CHL) to carapace length (CL) (CHL/CL), which has been reported as a good index of morphogenesis from final zoea to megalopae in portunid crabs (Hamasaki et al., 2002a; Arai et al., 2004). Throughout the experiment, water temperature was maintained at 28±1 ℃ using a water bath. Photoperiod was set at 14 h light :10 h dark, and light was provided by fluorescent lights with light intensity ranging between 500 - 600 lux. At the beginning of the experiment, three samples of newly

hatched zoea I (50 larvae/sample) were collected and briefly rinsed with 0.5 M ammonium bicarbonate to remove trace of salt before being blotted dry on a filter paper. They were then oven-dried at 60℃ for 24 h before being weighed to determine the average dry weight of individual newly hatched zoea I larvae.

Previous research in this laboratory has shown that when cultured in beakers with daily 100% water exchange, newly hatched larvae of *P. pelagicus* fed *Artemia* alone, could show comparable survival to those fed on rotifers (Josileen and Zeng, unpublished data). As a result, throughout this experiment, the larvae were fed enriched *Artemia* containing five levels of ARA (Table 8.2). *Artemia* were held at a density of 4 ind. mL^{-1} for all treatments. Each morning, total water exchange was carried out when surviving larvae in each beaker were counted and staged before being transferred to a new beaker with clean seawater and fresh enriched *Artemia*. Mortality and moulting of larvae in each beaker were checked again 12 h later (i.e. every 12 h). By doing this, the average intermoult period of larval instars could be estimated more accurately. Cannibalism typically occurs after larvae metamorphose to megalopae when they develop two chelipeds (Castine et al., 2008). To avoid cannibalism, any newly developed megalopae were removed from beakers as soon as they were located. The experiment terminated when all larvae had either moulted to megalopae or died.

8.2.4 Data collection

Larval survival, development and specific growth rate (SGR)

Larval survival was expressed as cumulative survival to a particular larval stage, which was calculated by dividing the total number of larvae that moulted successfully to a particular larval stage with the initial number of larvae stocked into each replicate (i.e. 25). Similarly, larval development was expressed as the mean cumulative development time required reaching a particular larval stage from the newly hatched zoea I. The specific growth rate (SGR, % day^{-1}) was calculated for each replicate using the following formulae:

$SGR = 100 \times (\ln Wt - \ln Wo)/D$

where Wo is the mean dry weight of newly hatched zoea I larvae while Wt is the mean dry weight of newly moulted megalopae; D is the cumulative development time (in days) from newly hatched zoea I to megalopae.

Carapace length and dry weight of newly settled megalopae

The carapace length (CL) and dry weight of each newly moulted megalopa were measured. Firstly the carapace length of a newly developed megalopa was measured using a microscope (precision=0.01mm). The megalopa was then briefly rinsed with 0.5 M ammonium

bicarbonate to remove traces of salt and blotted dry on a filter paper before oven-drying at 60 ℃ for 24 h. The dry weight of individual megalopa was subsequently determined using a Cahn C-33 micro-balance (precision=1μg, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA).

The ratio of chela length to carapace length (CHL/CL) of Zoea IV

All zoea IV larvae (i.e. the final zoeal instar) in one replicate beaker of a treatment designed for measuring chela length (CHL) and carapace length (CL) were sampled for such measurements. These zoea IV larvae were fixed with 10% formalin for 24 h and then preserved in 70% ethanol solution. Their CHL and CL were then measured under a microscope and CHL/CL was calculated as an index of morphogenesis from zoea IV to megalopae (Hamasaki et al., 2002a; Arai et al., 2004).

Occurrence of moult**ing death syndrome (MDS)**

Toward the end of the experiment, it was obvious that some of the mortalities occurring during the zoea IV metamorphosis moulted to megalopae were linked to moulting death syndrome (MDS), i.e. mortality due to larvae being incapable of completely shedding their old exoskeleton (Arai et al., 2004). In order to quantity the incident of MDS, all mortality of the larvae was examined using a microscope to determine if MDS was involved and the percentage of MDS was calculated using the following formula based on Hamsasaki et al. (2002a), Arai et al. (2004) and Dan et al. (2011):

MDS (%) =100%×Total incidents of MDS during zoea IV metamorphosis moulting/(Total number of zoea IV mortalities during metamorphosis + total number of survival megalopae).

8.2.5 Total lipid and fatty acid analysis

See section 4.2.4. For enriched *Artemia,* three sub-samples of each treatment were pooled for freeze-drying. Then, enriched *Artemia* of each treatment was analyzed in duplicate for total lipid and fatty acid composition. The average of the two values was used as the final value. For each megalopal samples, because they were very limited for each treatment, only one sample was analysed for each treatment.

8.2.6 Data analysis and statistics

Homogeneity of variance of all data was first tested with Levene's test. Where necessary, arcsine-square root or logarithmic transformation was performed prior to analysis. One-way ANOVA was used to determine significant differences for various parameters among treatments. If any significant difference was detected, Tukey's multiple range test was used as the means

separation procedure. Pearson-test was used to determine statistical relationship between dietary ARA level and different biological parameters measured. The relationships between dietary ARA level and different biological parameters were further evaluated with regression analysis. When a normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Kruskal-Wallis H non-parametric test followed by the Games-Howell non-parametric multiple comparison test. $P < 0.05$ was regarded as the statistically significant level. The coefficient of variation was calculated as the percentage of standard deviation divided by the mean. For any treatment, all CHL/CL ratio data were measured from one beaker, the CHL/CL ratio from one beaker was considered as the unreplicated data. Therefore, only Pearson Chi-square was performed to test the correlations between zoea IV CHL/CL ratio and dietary ARA levels as well as the zoea IV CHL/CL ratio and the percentage of MDS during the metamorphosis moult to megalopa (Hurlbert, 1984). All statistics were performed using SPSS package (version 12.0).

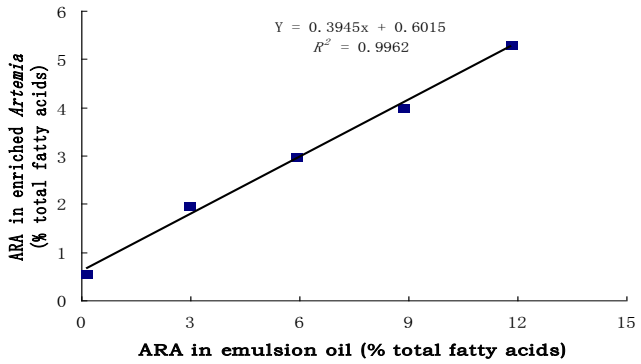

Figure 8.1 The relationship between ARA contents of enriched *Artemia* and the emulsion oils

8.3 Results

8.3.1 Total lipid and fatty acid profile of enriched Artemia

The total lipid and fatty acid compositions of *Artemia* enriched with five emulsion oils containing different levels of ARA are presented in Table 8.2. Total lipid contents were similar for all five treatments. While all *Artemia* contained similar levels of saturated fatty acid (SFA), with the increasing ARA level of the emulsion oils from ARA1 to ARA5 (Table 8.1), a decreasing trend of 16:1n-7, 18:1n-9 and total monounsaturated fatty acid (MUFA) was evident in the *Artemia* enriched with these emulsion oils, respectively (Table 8.2). For the polyunsaturated fatty acids (PUFA), all enriched *Artemia* generally had similar contents of 18:2n-6, 18:3n-3, 20:5n-3 (EPA) and 22:6n-3 (DHA). As expected, the five experimental emulsions oils with graduated 20:4n-6 (ARA) contents resulted in increasing ARA level and ARA/EPA ratio of enriched *Artemia* and a significant positive correlation was detected between

ARA contents of enriched *Artemia* and ARA levels of the emulsion oils (Figure 8.1, n=5, P <0.01). Consequently, the ratio of n-3PUFA/n-6PUFA decreased from 2.61 for *Artemia*

Table 8.2 Total lipid contents (% dry weight) and fatty acid composition (% total fatty acids) of *Artemia* enriched with five emulsions with graduated ARA concentrations

	Artemia enriched with ARA1	*Artemia* enriched with ARA2	*Artemia* enriched with ARA3	*Artemia* enriched with ARA4	*Artemia* enriched with ARA5
Total lipid	28.19	27.18	27.79	27.19	27.23
Fatty acids					
C14:0	0.62	0.55	0.60	0.58	0.62
C15:0	0.78	0.72	0.76	0.86	0.93
C16:0	8.75	8.47	9.13	9.09	9.31
C18:0	5.37	3.95	4.27	4.21	4.20
SFA	15.52	13.70	14.76	14.74	15.06
C14:1n7	1.75	1.70	1.74	1.72	2.08
C16:1n7	5.16	5.03	4.53	3.94	3.58
C16:1n5	0.62	0.59	0.58	0.62	0.69
C18:1n9	30.30	29.52	28.53	25.65	23.95
C18:1n7	4.29	4.14	4.49	4.31	4.31
C20:1n9	0.30	0.28	0.31	0.23	0.38
C20:1n7	0.89	0.94	0.94	0.82	0.76
MUFA	43.31	42.19	41.14	37.28	35.76
C16:2n4	0.57	0.54	0.59	0.60	0.61
C16:3n4	0.60	0.56	0.58	0.60	0.62
C18:2n6	8.78	9.20	8.42	9.43	9.40
C18:3n6	0.16	0.14	0.24	0.28	0.36
C18:3n3	14.84	14.40	14.71	16.47	16.16
C18:3n4	2.29	2.15	2.20	2.33	2.37
C20:2n6	0.18	0.22	0.16	0.16	0.46
C20:3n6	0.20	0.20	0.33	0.30	0.39
C20:4n6	**0.53**	**1.95**	**2.97**	**3.99**	**5.30**
C20:3n3	0.41	0.35	0.44	0.41	0.46
C20:4n3	0.411	0.43	0.38	0.43	0.45
C20:5n3	5.20	6.10	5.64	5.46	5.28
C22:2n6	0.20	0.17	0.25	0.23	0.25
C22:5n3	0.40	0.54	0.49	0.50	0.39
C22:6n3	3.73	4.46	4.11	4.06	3.63
∑PUFA (≥18:2n)	36.83	40.30	40.32	44.06	44.87
∑n-3PUFA	24.98	26.28	25.77	27.33	26.34
∑n-6PUFA	9.56	11.88	12.36	14.39	16.16
n-3/n-6	2.61	2.21	2.08	1.90	1.63
∑HUFA (≥20:3n)	10.87	14.03	14.36	15.15	15.87
DHA/EPA	0.72	0.73	0.73	0.74	0.68
ARA/EPA	0.10	0.32	0.53	0.73	1.00

enriched with ARA1 to 1.63 for those enriched with ARA5 while the ratio of ARA/EPA increased from 0.10 to 1.00, respectively. Table 3 shows the absolute contents (mg/g DW) of

principal fatty acids of *Artemia* enriched with the five emulsion oils and similar trends were found when fatty acid compositions were expressed as % of total fatty acids (Table 8.2). The ARA contents ranged from 1.10 mg/g DW in the *Artemia* enriched with ARA1 to 11.12 mg/g DW of the *Artemia* enriched with ARA5, while the total fatty acid contents remained very similar among all treatments (205.79 – 211.20 mg/g DW) (Table 8.3).

Table 8.3 Principal fatty acid contents (mg/g DW) of *Artemia* enriched with five emulsions with graduated ARA concentrations

	Artemia enriched with ARA1	*Artemia* enriched with ARA2	*Artemia* enriched with ARA3	*Artemia* enriched with ARA4	*Artemia* enriched with ARA5
C16:0	18.01	17.50	19.28	19.03	19.53
C18:0	11.06	8.17	9.03	8.82	8.80
SFA	31.93	28.30	31.18	30.86	31.58
C16:1n7	10.61	10.41	9.56	8.25	7.51
C18:1n9	62.36	61.97	60.27	53.69	50.22
C18:1n7	8.83	8.54	9.49	9.03	9.03
MUFA	89.12	87.15	84.88	78.06	74.97
C18:2n6	17.05	19.00	17.78	19.75	19.71
C18:3n3	30.53	29.74	31.06	34.49	33.89
C18:3n4	4.72	4.40	4.64	4.88	4.96
C20:4n6	**1.10**	**4.03**	**6.27**	**8.36**	**11.12**
C20:5n3	10.70	12.61	11.91	11.44	11.06
C22:6n3	7.67	9.21	8.68	8.51	7.55
∑PUFA (≥18:2n)	75.79	83.25	85.17	92.24	94.07
∑n-3PUFA	51.40	54.28	54.42	57.23	55.23
∑n-6PUFA	19.67	24.53	26.11	30.13	33.88
∑HUFA (≥20:3n)	22.37	28.98	30.33	31.72	33.28
Total fatty acids	205.79	206.57	211.20	209.36	208.31

Table 8.4 Cumulative survival (%) to subsequent larval stages of newly hatched *Portunus pelagicus* zoea I larvae fed enriched *Artemia* containing different levels of ARA

Treatments	**Cumulative survival (%)**			
	Zoea II	Zoea III	Zoea IV	Megalopa (CV)
T1	90.2 ± 3.5^{a}	85.3 ± 4.4^{ab}	76.3 ± 4.0^{ab}	36.3 ± 7.4^{bc} (20.4)
T2	95.2 ± 6.6^{ab}	90.9 ± 6.2^{abc}	83.4 ± 4.2^{b}	15.8 ± 6.5^{a} (41.1)
T3	96.8 ± 3.3^{b}	92.7 ± 3.3^{bc}	87.1 ± 5.9^{b}	45.6 ± 3.8^{c} (8.3)
T4	99.0 ± 2.0^{b}	95.9 ± 3.4^{c}	87.1 ± 9.1^{b}	33.5 ± 14.6^{bc} (43.6)
T5	97.3 ± 4.5^{b}	82.8 ± 11.3^{a}	69.3 ± 17.4^{a}	31.7 ± 11.6^{b} (36.6)

Data are presented as mean ±SD (n=5). Values within a same column with different superscript letters are significantly different ($P < 0.05$). CV: coefficient of variation (%) = 100 ×standard deviation/mean.

8.3.2 Larval survival, development and growth

For all treatments, larval survival to the megalopa stage was successfully achieved. However, larval survival showed significant differences between treatments as early as the zoea

II Stage with T1 showing significantly lower survival than T3 - T5 (Table 8.4, $P < 0.05$). The cumulative survival was similar and no significant difference was found among T2, T3 and T4 until zoea IV Stage. By the end of the experiment, larvae in the T3 treatment has the highest overall zoeal survival (i.e. cumulative survival to megalopa = 45.6%), followed by T1, T4, T5 and T2, while larvae in the T2 treatment had significant lower survival than those in other treatments. The lowest coefficient of variation (CV) of zoeal survival was also found in T3 (8.33%), indicating that survival was most consistent among replicates for the treatment.

Table 8.5 Cumulative development time (days) to subsequent larval stages of newly hatched *Portunus pelagicus* zoea I larvae fed enriched *Artemia* containing different ARA levels

Treatments	Cumulative development period (days)			
	Zoea II	Zoea III	Zoea IV	Megalopa (CV)
T1	2.2 ± 0.1^{a}	4.5 ± 0.1^{bc}	7.6 ± 0.2^{d}	11.4 ± 0.2^{d} (1.7)
T2	2.2 ± 0.2^{a}	4.4 ± 0.1^{ab}	6.5 ± 0.1^{b}	9.8 ± 0.2^{b} (2.2)
T3	2.2 ± 0.1^{a}	4.2 ± 0.1^{a}	6.2 ± 0.1^{a}	9.3 ± 0.1^{a} (1.0)
T4	2.1 ± 0.0^{a}	4.3 ± 0.1^{ab}	7.2 ± 0.2^{c}	10.9 ± 0.2^{c} (1.8)
T5	2.1 ± 0.1^{a}	4.6 ± 0.3^{c}	7.9 ± 0.3^{e}	11.5 ± 0.6^{d} (5.2)

Data are presented as mean ±SD (n=5). Values within a same column with different superscript letters are significantly different ($P < 0.05$). CV: co-efficient of variation (%) = 100 ×standard deviation/mean.

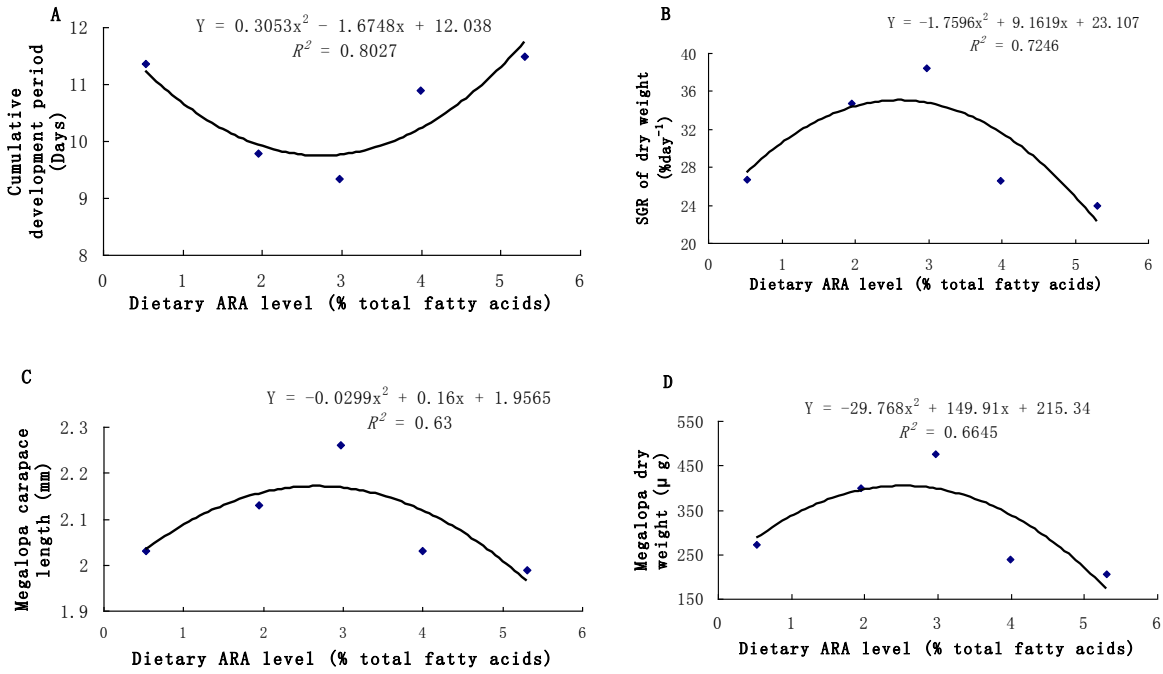

Figure 8.2 Regression between dietary ARA level and zoeal development time to megalopa (A); specific growth rates (SGR) on larval dry weight (B); mean carapace length (C) and dry weight of newly metamorphosed megalopae (D) of *Portunus pelagicus* larvae fed enriched *Artemia* containing different ARA levels

Table 8.5 shows mean development time from newly hatched zoea I to each subsequent zoeal stage and finally to the megalopal stage for each treatment. Significant differences in larval cumulative development time were firstly detected at the zoea III stage and the differences appeared to increase with progressive larval development. Overall, from zoea III onward, larvae of T3 consistently had the shortest development time ($P < 0.05$) and showed the most synchronized metamorphosis moulting to megalopae among all treatments. Among other treatments, larvae in T2 had the second shortest overall zoeal development time (9.8 days), which was followed by larvae in T4 (10.9 days), and both development times were significantly shorter than that of larvae in T1 (11.4 days) and T5 (11.5 days) treatments ($P <0.05$, Table 8.5). When larval dietary ARA level was plotted against overall zoeal development time, it fitted into an upper parabolic curve (Figure 8.2A, n=5, $P <0.01$). Among treatments, T3 achieved the highest specific growth rate (SGR, % day^{-1}) of dry weight (DW) over zoeal development while T5 showed the lowest SGR (Figure 8.3). Regression showed that the correlation between dietary ARA level and SGR could be described as downer parabolic (Figure 8.2B, n=5, $P <0.05$).

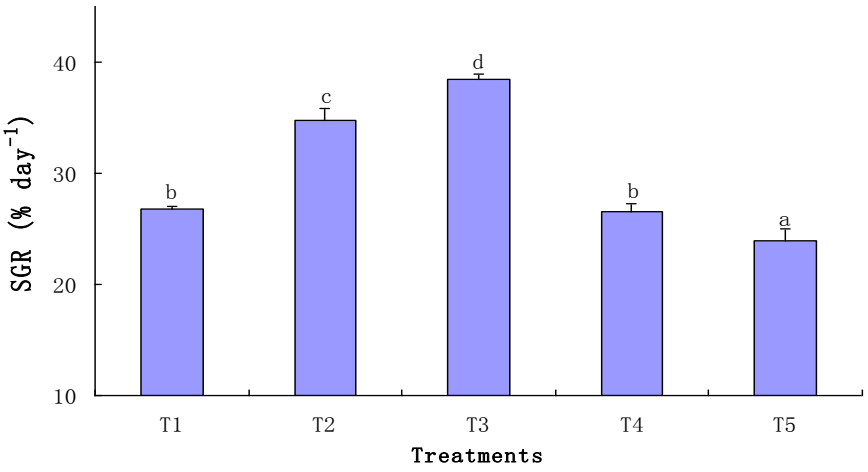

Figure 8.3 The specific growth rates (SGR, % day^{-1}) of *Portunus pelagicus* larvae fed enriched *Artemia* containing different ARA levels. SGRs are calculated based on dry weight of newly settled megalopae. Data are presented as mean ±SD (n=5). Bars with different letters on the top are significantly different ($P < 0.05$)

Table 8.6 Carapace length and dry weight of newly metamorphosed megalopae of *Portunus pelagicus* larvae fed enriched *Artemia* containing different ARA levels

Treatments	Carapace length (mm)	Dry weight (μg.ind.$^{-1}$)
T1	2.03 ± 0.05^{a}	273.54 ± 14.35^{b}
T2	2.13 ± 0.02^{b}	394.80 ± 39.16^{c}
T3	2.26 ± 0.06^{c}	475.07 ± 3.58^{d}
T4	2.03 ± 0.05^{a}	238.38 ± 18.22^{ab}
T5	1.99 ± 0.05^{a}	206.40 ± 25.80^{a}

Data are presented as mean ±SD (n=5). Values within a same column having different superscript letters are significantly different ($P < 0.05$).

8.3.3 Carapace length and dry weight of newly settled megalopae

The carapace length (CL) and dry weight (DW) of newly metamorphosed megalopae are shown in Table 8.6. The larvae from T3 had the highest CL and DW among all treatments and these were significantly higher than those in all other treatments (P <0.05). The second highest CL and DW was found for larvae in the T2 treatment (P <0.05). Interestingly, the lowest CL and DW were recorded for larvae in the T5 treatment in which larvae were fed *Artemia* containing the highest level of ARA at 5.30% total fatty acids. Regression showed that the relationship between dietary ARA level and both CL and DW of newly settled megalopa could be described as downer parabolic (Figure 8.2C and 8.2D, n=5, P<0.05).

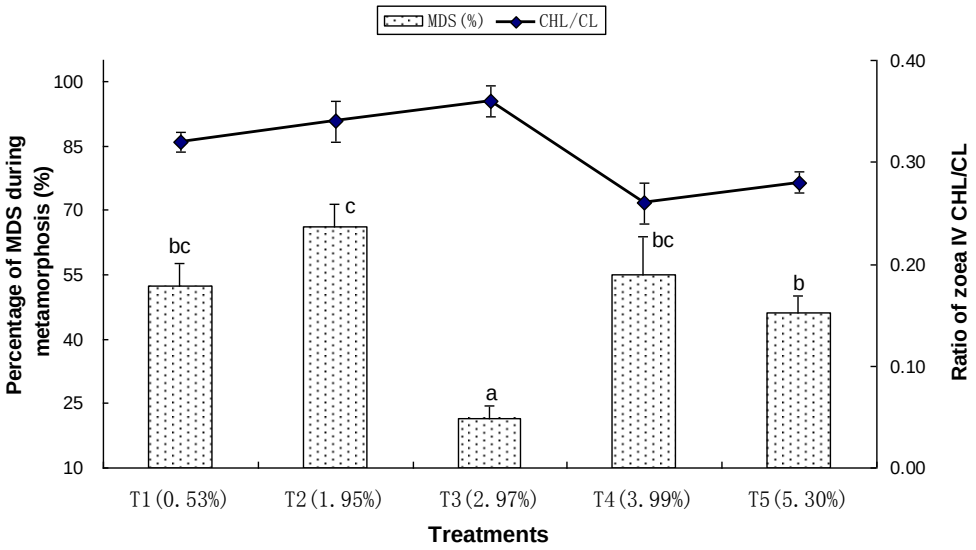

Figure 8.4 The zoea IV chela length to carapace length (CHL/CL) ratio (diamonds) and percentage of moulting death syndrome (MDS) (bars) during metamorphosis of *Portunus pelagicus* larvae fed enriched *Artemia* containing different levels of ARA. Data are presented as mean ±SD. Bars with different letters on the top are significantly different (P <0.05).

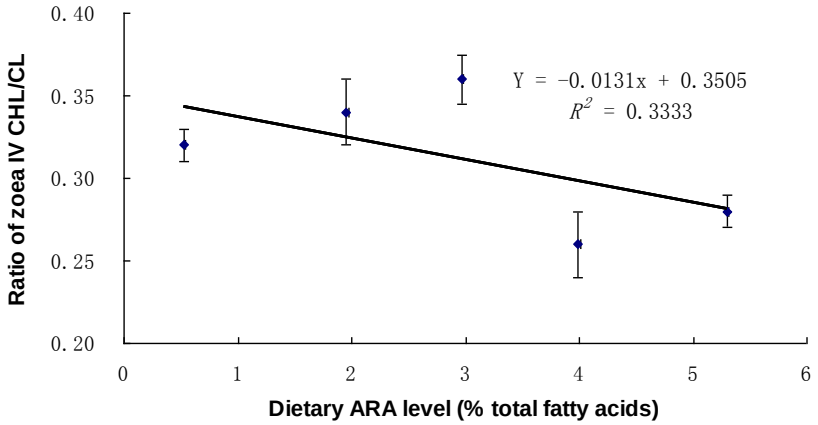

Figure 8.5 Regression between dietary ARA level and the ratio of zoea IV CHL/CL of *Portunus pelagicus* larvae fed enriched *Artemia* containing different ARA levels. Data are presented as mean ±SD (n=5).

8.3.4 Zoea IV CHL/CL ratio and its relationship to MDS

Figure 8.4 shows the ratio of chela length to carapace length (CHL/CL) of zoea IV larvae and the percentage of MDS during the metamorphosis moult to megalopa in different treatments. Of all treatments, larvae from T3 had the highest CHL/CL ratio but the lowest percentage of MDS during the metamorphosis moult to megalopa. In contrast, the lowest CHL/CL ratio and the highest percentage of MDS were found for larvae in T4 and T2, respectively. Pearson Chi-square test suggested a negative linear correlation was found between zoea IV CHL/CL ratio and dietary ARA levels (Figure 8.5, r = -0.577, n=5, P <0.01). Furthermore, no significant correlation was identified between zoea IV CHL/CL ratio and the percentage occurrence of MDS (n=5, P >0.05).

Table 8.7 Fatty acid composition (% total fatty acids) of newly moulted *Portunus pelagicus* megalopae fed by enriched *Artemia* containing different ARA levels

	T1	T2	T3	T4	T5
Fatty acid					
C14:0	1.90	1.07	1.22	0.79	0.44
C15:0	0.75	0.62	0.45	0.52	0.59
C16:0	15.68	16.99	15.40	15.03	15.61
C18:0	8.11	9.01	8.01	9.06	8.42
C20:0	0.96	0.85	0.89	1.18	1.02
SFA	27.40	28.54	25.97	26.58	26.08
C14:1n7	0.37	0.31	0.42	0.45	0.39
C16:1n7	5.90	3.50	3.01	1.86	1.72
C16:1n5	0.38	0.56	0.51	0.50	0.65
C18:1n9	18.52	19.32	15.89	13.21	13.76
C18:1n7	3.91	4.65	4.38	3.90	3.46
C20:1n9	0.64	0.39	0.49	0.39	0.64
C20:1n7	1.26	0.91	1.04	0.89	1.99
MUFA	30.98	29.64	25.74	21.20	22.61
C18:2n6	9.67	7.15	6.30	4.87	5.91
C18:3n3	1.70	3.63	3.60	3.90	2.25
C18:4n3	0.37	0.81	0.50	0.77	0.76
C20:4n6	0.53	2.28	3.26	3.90	4.58
C20:3n3	0.39	0.48	0.72	0.78	0.66
C20:5n3	5.79	7.91	7.39	6.54	4.09
C22:6n3	4.86	4.45	5.45	4.20	4.81
∑PUFA (≥18:2n)	23.31	27.01	27.58	24.96	23.54
∑HUFA (≥20:3n)	11.57	15.12	16.82	15.42	14.62
∑n-3PUFA	13.11	17.28	17.66	16.19	13.05
∑n-6PUFA	10.20	9.43	9.56	8.77	10.49
n-3/n-6	1.29	1.83	1.85	1.85	1.24
ARA/EPA	0.09	0.29	0.44	0.60	1.12

8.3.5 Fatty acid profile of newly moulted megalopae

The fatty acid profile of newly moulted megalopae showed that there were six dominant fatty acids that contributed to more than 4% of the total fatty acids, which were 16:0, 18:0, 18:1n9, 18:2n6, 20:5n3 (EPA) and 22:6n3 (DHA) (Table 8.7). The percentage of these dominant fatty acids was generally reflective of larval dietary fatty acid compositions (Table 8.2), particularly for the 18:2n6, ARA, EPA and DHA. For example, the ARA percentage of newly moulted megalopae increased significantly from T1 (0.53%) to T5 (4.58%).

8.4 Discussion

A search of the literature suggests that this study is the first to use a dose-response design to examine the effect of dietary ARA on larval survival, development, growth and occurrence of MDS for a crustacean. For zoeal larvae of portunid crabs, microbound or microencapsulated diets are generally insufficient to support good survival and normal development through larval ontogeny, particularly for the early zoeal stages (Jones, 1998; Holme et al., 2006). Use of formulated diets is therefore not a feasible option for lipid nutrition studies for larvae of portunid crabs (Holme et al., 2006). This study utilized enriched *Artemia* for this purpose and the results showed that ARA in self-prepared lipid enrichment emulsions was readily absorbed/assimilated by *Artemia*. Therefore, by careful manipulation of ARA levels in lipid emulsions, graded ARA levels could be achieved in enriched *Artemia*, which can then be used to feed the crab larvae to access their dietary ARA requirements. Although similar dose-response experiments using enriched *Artemia* have been used to examine EPA and DHA requirement of crustacean larvae (Suprayudi et al., 2004b; Nghia et al., 2007; Sui et al., 2007), no published literature appears to exist for such study on ARA requirements. The outcomes of this study suggested that *Artemia* enrichment based, dose-response design, is a suitable approach for investigating the ARA requirements of crustacean larvae.

Survival data showed that for all treatments, mass mortality occurred during metamorphosis of zoea IV to megalopae (mortality rates between 40-70%). A high percentage of such mortalities could be attributed to ing death syndrome (MDS), i.e., larval mortality due to their inability to completely shed the old exoskeleton (Hamasaki et al., 2002a; Wu et al., 2007c). MDS is a common phenomenon found in brachyuran crab larvae during metamorphosis moulting, and mass mortalities due to MDS have been reported for various portunid crabs targeted for aquaculture, including the mud crabs, *Scylla serrata* (Hamasaki et al., 2002a), *S. paramamosain* (Takano et al., 2004), *S. tranquebarica* (Baylon, 2009) and the swimming crab, *P. trituberculatus* (Arai et al., 2004; Dan et al., 2013). More recent studies have revealed that an imbalance of dietary fatty acids is one of the major contributors to MDS in larval mud crabs

(Hamasaki et al., 2002b; Suprayudi et al., 2004a). The present results demonstrated, for the first time, that dietary ARA level also had a significant effect on the occurrence of MDS for *P. pelagicus* larvae. *P. pelagicus* larvae fed enriched *Artemia* containing 2.97% (of total fatty acids) of ARA (or 6.27 mg/g DW) had the highest survival with the lowest percentage of MDS. This treatment also recorded the shortest zoeal development time and most synchronized metamorphosis moult to megalopae.

In the mud crab *S. serrata*, excessive dietary n-3HUFA (DHA and EPA) has been reported to result in larvae with more advanced morphological features, i.e. showing morphological features of megalopae, such as large rudimentary chelipeds and plumose setae on pleopods, in the last zoeal stage larvae (zoea V) (Hamasaki et al., 2002b; Suprayudi et al., 2004a; Dan et al., 2013). Hamasaki et al. (2002a) defined such phenomenon as 'hyper-morphogenesis' and reported that *S. serrata* zoea V larvae with hyper-morphogenesis often led to MDS. These authors used the ratio of chela length to carapace length (CHL/CL) to quantify the degree of hyper-morphogenesis in *S. serrata* zoea V larvae. Since a positive correlation between dietary n-3 HUFA (particularly DHA) level and the occurrence of MDS was identified, both dietary n-3 HUFA level and the occurrence of MDS were positively correlated to the ratio of CHL/CL of zoea V, confirming that CHL/CL ratio is a good predictor of MDS (Hamasaki et al., 2002b; Hamasaki et al., 2002a; Suprayudi et al., 2002; Suprayudi et al., 2004a; Dan and Hamasaki, 2011). It was subsequently reported that for *S. serrata* and *P. trituberculatus*, if the CHL/CL ratios of the final zoeal stage larvae are higher than 0.45 or 0.37, respectively, MDS is likely to occur during metamorphosis (Arai et al., 2004; Dan and Hamasaki, 2011; Dan et al., 2013). Interestingly, in the current study, different results were shown because, in general, both dietary ARA levels higher or lower than 2.97% of total fatty acids, resulted in decreasing CHL/CL ratios of *P. pelagicus* zoea IV larvae. Moreover, in contrast to what was reported for *S. serrata* and *P. trituberculatus* (Hamasaki et al., 2002b; Hamasaki et al., 2002a; Arai et al., 2004; Dan and Hamasaki, 2011), lower CHL/CL ratios did not result in signficantly reduced MDS, and no significant correlation was identified between zoea IV CHL/CL ratio and the percentage occurrence of MDS. Understandably, dietary ARA deficiency is likely to affect larval growth and hence lead to lower CHL/CL ratio in zoea IV larvae. The present results suggested that excessive dietary ARA could also depress larval growth and lead to a reduced ratio of CHL/CL; and in both cases, increased incidences of MDS as the result. The present results were different to the previous reports for *S. serrata* and *P. trituberculatus* (Hamasaki et al., 2002a,b; Arai et al., 2004; Dan and Hamasaki, 2011; Dan et al., 2013). The possible reasons might include different roles of ARA and n-3 HUFA during crab larval development and growth, as well as species-specific differences (Dan and Hamasaki, 2011; Dan et al., 2013).

P. pelagicus larvae fed enriched *Artemia* containing ARA at a level of 2.97% of total fatty acids not only had the higest survival and shortest larval development time, but resulting newly settled megalopae also had the longest carapace length and the heaviest dry weight. All these point to the importance of supplying appropriate dietary ARA during larval culture of *P. pelagicus*. The nutritional significant of ARA might firstly be explained by the fact that the moulting process in crustaceans is regulated by prostaglandin production and their ratios (Koskela et al., 1992; Brante et al., 2003). It is well known ARA and EPA are the precursors of series-2 (e.g. prostaglandin E_2, PGE_2) and series-3 prostaglandin (e.g. prostaglandin E_3, PGE_3), respectively (Yang et al., 2002). Optimal dietary ARA levels are therefore likely to result in increased PGE_2 synthesis and concentration in crustaceans (Reddy et al., 2004; Meunpol et al., 2010). Indeed, previous research has shown that brown tiger prawn *Penaeus esculentus* injected with PGE_2, a series-2 prostaglandin derived from ARA, displayed shorter moult cycles compared to the controls (Koskela et al., 1992). It is therefore possible that when dietary ARA is below an optimal level, increased dietary ARA would lead to increased PGE_2 synthesis and concentration, hence enhancing larval development. However, since there exists a competitive inhibition between conversion of ARA to PGE_2 and EPA to PGE_3 (Bell and Sargent, 2003), further increase of dietary ARA beyond an optimal level may lead to excessive PGE_2 being produced with decreased PGE_3 production, resulting in an imbalanced PGE_2/PGE_3 ratio. The imbalanced PGE_2/PGE_3 ratio could lead to extended development time and high occurrence of MDS in *P. pelagicus* larvae. If this is true, then it suggested that like DHA/EPA ratio, ARA/EPA ratio is another important ratio that needs to be considered in crustacean larval nutrition and this aspect warrants further research.

In black tiger prawn *Penaeus monodon* juveniles, excessive dietary ARA has also been reported to reduce hepatopancreas palmitic acid (16:0) and total saturated fatty acid (SFA) contents (Glencross et al., 2001). Since 16:0 is one of the most important fatty acids for energy storage in crustaceans, low 16:0 levels have been suggested to have negative effects on moulting and growth of this species (Teshima et al., 1977; Teshima, 1998). Excessive levels of ARA in larval diets for *P. pelagicus* may have similar effects, resulting in reduced carapace length and dry weight of newly settled megalopae, high incidence of MDS and extended larval development.

Furthermore, experiments on mammals have shown that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) have positive effects on protein synthesis while in contrast, PGE_2 inhibits muscle fiber formation and stimulates protein degradation. As a result, muscle growth could be modulated by altered ratios

of these two prostaglandins (Palmer, 1990; Velica and Bunce, 2008). Although both $PGF_{2\alpha}$ and PGE_2 are derived from ARA and have been detected in crustaceans (Hampson et al., 1992; Spaziani et al., 1993; Reddy et al., 2004), the ratio of $PGF_{2\alpha}$ to PGE_2 might be regulated by exogenous ARA (dietary or injected ARA) because of preferred bioconversion or competitive inhibition of the same enzyme system for their synthesis. Prostaglandin H_2 (PGH_2) is an intermediate product for $PGF_{2\alpha}$ and PGE_2, that converts ARA to either $PGF_{2\alpha}$ or PGE_2 (Tocher, 2003; Reddy et al., 2004). Therefore, it is possible that the effects of dietary ARA on the larval growth of *P. pelagicus* observed in the present study are regulated by the ratio of $PGF_{2\alpha}$ to PGE_2. The optimal dietary ARA level might represent the level that produced most appropriate ratio of $PGF_{2\alpha}$ to PGE_2, resulting in the greatest carapace length and dry weight of newly metamorphosed megalopae as well as the highest SGR.

8.5 Conclusion

This study is the first report on quantitative larval ARA requirement of a crustacean. The present results suggested that dietary ARA level has significant effects on survival, development, growth as well as occurrence of MDS in *P. pelagicus* larvae. The results indicate that both deficient and excessive levels of ARA in larval diets could negatively affect larval performance. The optimal dietary ARA level for *P. pelagicus* zoeal larvae was identified to be around 2.97% of total fatty acids (or 6.27 mg/g DW) and the ARA/EPA ratio around 0.53. The results highlight ARA as an importance fatty acid for *P. pelagicus* larval survival and development and provide knowledge basis for the development of formulated diets for seed production of *P. pelagicus* in the future. The mechanisms underlying these observed effects of dietary ARA on *P. pelagicus* larvae warrant further research.

Chapter 9

The effects of dietary arachidonic acid levels on survival, growth and fatty acid composition of early stage phyllosoma of ornate rock lobster, *Panulirus ornatus*

9.1 Introduction

The spiny rock lobster, *Panulirus ornatus*, is widely distributed throughout the Indo-West Pacific region, particularly in the tropical and subtropical waters of the region, such as the Torres Straits and Northeastern Australia (Ye, 2008; Ye and Dennis, 2009). This lobster species is targeted in a sustainable fishery in this region with a managed annual harvest in Australia and Papua New Guinea of approximately 662 tonnes (Ye, 2008). Although global demand for *P. ornatus* product is increasing at 15% per annum (Murugan et al., 2005; Jones et al., 2007; Smith et al., 2009a), the fishery for wild *P. ornatus* is fully exploited with landing yields plateauing during the past 10 years despite increased fishing effort (FAO, 2005; Hung and Tuan, 2009). Among the rock lobsters, *P. ornatus* is recognized as a prime candidate for aquaculture due to its short planktonic phyllosoma phase (<6 months), rapid growth (attaining 1 kg after 18 months post-settlement) and a delicate flavor (Johnston et al., 2008; Smith et al., 2009a). It is likely that sustainable production of this species will only be possible through closed-life cycle aquaculture production through development of commercially robust hatchery technologies (Priyambodo and Sarifin, 2009; Smith et al., 2009a).

The larval cycle of *P. ornatus*, is complex, consisting of eleven distinct morphological pelagic planktonic phyllosoma stages, a transitional planktonic post-larval puerulus stage and the first instar benthic juvenile (also known as post-puerulus) (Smith et al., 2009a). The current bottleneck in the aquaculture production of all Palinurid marine lobster species is the lack of ability to rear large quantities larvae, from egg through the multiple planktonic phyllosoma stages to puerulus and the benthic juveniles. Successful larval production has been achieved for *P. ornatus* in Australia, but only at small scales (Smith et al., 2009a; O'Sullivan, 2010). High mortalities during culture of the phyllosoma stages to puerulus, and the first instar juvenile, linked to a number of aspects, including poor nutrition (Johnston et al., 2008; O'Sullivan, 2010). Larval nutrition, particular fatty acid nutrition, has also been suggested as one of major contributor to incidents of unsuccessful larval rearing of this species (Wu et al., 2012).

Highly unsaturated fatty acids (HUFA), having carbon chain lengths of ≥ 20 and with ≥ 3 double bonds (Tocher, 2003), has been identified as essential fatty acids (EFA) for many

crustacean species (Xu et al., 1994a; Merican and Shim, 1996b; Suprayudi et al., 2004b). Because many crustaceans have limited capacity to synthesize HUFAs and therefore require certain amount of HUFAs in their diet (Kanazawa et al., 1979a,b). Previous research has shown that unbalanced dietary HUFA composition often leads to low larval survival and growth during larval culture of the rock lobsters, such as *Panulirus cygnus* (Liddy et al., 2005), and *Panulirus japonicus* (Kanazawa and Koshio, 1994). Our recent examination of the fatty acid composition, for early-mid Stages *P. ornatus* phyllosoma, has provided valuable insights into lipid nutrition for *P. ornatus* phyllosoma, and the results showed the Stage II-III phyllosoma accumulated relatively higher 20:4n6 (ARA) and 20:5n3 (EPA) than their live prey: *Artemia* (Wu et al., 2012). However, there is no published information on the HUFA requirements of *P. ornatus* phyllosoma. This undoubtedly will hinder the development of hatchery protocols as well as potential formulation of cost-effective larval feeds for *P. ornatus*.

More importantly, despite extensive research on n-3 HUFA (e.g. 20:5n3 and 22:6n3) requirement for larval crustaceans, very limited research efforts have focused on n-6 HUFA requirements, particularly those for ARA (Bell and Sargent, 2003; Liddy et al., 2005). It is well known that ARA generally serves as the preferred precursor for biosynthesis of eicosanoids, such as prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which play important roles in the modulation of growth, metamorphosis and stress resistance in crustacean and fish larvae (Koskela et al., 1992; Bell and Sargent, 2003; Van Anholt et al., 2004a). Currently, *Artemia* have been used as major food source for early-Stage phyllosoma rearing as formulated diets could not support a good survival (Johnston et al., 2008; Wu et al., 2012). Although *Artemia* nauplii or meta-nauplii generally lacks HUFAs, enrichment can be used to improve the HUFA contents of the *Artemia*, which in turn affects the lipid composition of early stage phyllosoma for other rock lobster species (Ritar et al., 2004; Liddy et al., 2005). This study was designed and carried out to assess the effects of dietary ARA on survival, development, growth and fatty acid composition, and to identify the optimal dietary ARA levels for early-Stage *P. ornatus* phyllosoma.

9.2 Materials and methods

9.2.1 Broodstock source and maintenance

Broodstock source and husbandry were described in Chapter 5 (Section 5.2.1).

9.2.2 Artemia enrichment

Five *Artemia* enrichment emulsions containing different ARA levels were formulated by incorporating 0% (ARA1), 8% (ARA 2), 16% (ARA 3), 24% (ARA 4) and 32% (ARA 5) of concentrated ARA oil (containing 41.19% ARA) (see Table 8.1). Because the concentrated ARA oil used for formulation also contained 6.79% of 18:2n-6, the addition of different quantities of the ARA oil could lead to differences in 18:2n-6 contents of these emulsions. To resolve this problem, various amounts of corn oil (rich in 18:2n-6) and macadamia oil (rich in 18:1n-9) were added to maintain similar levels of 18:2n-6 in all enrichment emulsions (Bransden et al., 2005b). Concentrated n-3 HUFA oil (DHA/EPA=1.51, n-3 HUFA=67.53% total fatty acids) was also added to the emulsions to provide DHA (22:6n-3) and EPA (20:5n-3). Finally, 10% of soy lecithin was added as an emulsifier while 2% of α-tocopherol acetate was added as an antioxidant (Estévez et al., 2001; Villalta et al., 2005). Each emulsion was then emulsified with equal amounts of distilled water using an Ultra-turrax T25 homogenizer (IKA Works Inc, Wilmington, North Carolina, USA) operating at 10, 000 rpm for 60 seconds. They were then stored in bottles and kept in a refrigerator at 4^{o}C.

To obtain newly hatched *Artemia* nauplii for the experiment, *Artemia* cysts (GSL strain, AAA+ grade, INVE Aquaculture Inc, Salt Lake City, UT, USA) were hatched daily in 50 L conical tanks at 28±1 ℃ in filtered seawater (33-36‰). After 20-24 h hatching, the newly hatched nauplii (size: 430-520 μm) was harvested for subsequent enrichment. The newly hatched *Artemia* were disinfected in 400 μl L^{-1} formalin bath for 1 hour before enrichment, and then they were enriched in 400 mL containers at a density of 200-250 *Artemia*/mL for 24 h with 0.6 g/L of each emulsion oil (Sui et al., 2007). During enrichment, temperature and salinity were maintained at 28 ^{o}C and 33‰, respectively. Enriched *Artemia* were then harvested and rinsed with UV treated freshwater to remove traces of the emulsion before being fed to *P. ornatus* larvae. Any enriched *Artemia* that were not fed to larvae were immediately placed at 4℃ in a refrigerator to minimize fatty acid catabolism (Merchie, 1996), they were to be used within 24 h and any leftovers discarded after this period. Enriched *Artemia* (ca.1-2 g/sample) in each treatment were sampled three times during the larval culture for lipid and fatty acid analysis. All samples were stored at -70℃ before analysis took place.

9.2.3 Experimental design and setup

On the day of hatching, within 8 h of hatching, active and photopositive phyllosoma originated from one female were collected from the surface of the broodstock spawning tank and transferred into a 20 L contain clean seawater for estimation of larval production. Experimental culture system was comprised of 20 x 250 ml mini round vessels (kriesels)

stocked at 25 phyllosoma per vessel. All experimental vessels were supplied with flow-through water equivalent to 300% water exchanges per hour. Water was introduced at the vessel bottom, providing minor levels of turbulence, but sufficient to maintain phyllosoma and feed in suspension. Seawater was supplied from a shoreline pump house with water being settled for 1-4 days followed by sequential passage through bag filters (100, 50 and 10 μm), foam fractionation, ozonation (700-750 mV), UV (254 nm) and particulate/carbon filtration to 1μm. Seawater within this system was delivered to the point of usage within 20 minutes of processing. Salinity and water temperature were maintained at 32‰ and 28ºC, respectively, during the period of the experiment. Light was provided by fluorescent light set on a photoperiod of 14 h light: 10 h dark and light intensity of 5 lx.

The culture experiment was conducted using four replicates, with 25 phyllosoma per replicate. There were five treatments (Treatment 1 to 5, defined as T1-T5) with phyllosoma fed *Artemia* enriched with self-prepared ARA1- ARA5 emulsions, respectively. At the beginning of the experiment, three samples of newly hatched Stage I phyllosoma (50 phyllosoma/sample) were collected and briefly rinsed with 0.5 M ammonium bicarbonate to remove trace of salt before being blotted dry on a filter paper. They were then oven-dried at 60 ℃ for 24 h before being weighed to determine the average dry weight of individual newly hatched Stage I phyllosoma. Throughout the experiment, the phyllosoma were fed enriched *Artemia* containing five levels of ARA (Table 9.2). *Artemia* were held at a density of 4 ind. mL^{-1} for all treatments. Each morning, surviving phyllosoma were counted and staged before being transferred to a new culture vessel, while old *Artemia* were removed. Fresh enriched *Artemia*, were then placed into each vessel at the density of 4 ind. mL^{-1}. Mortality and moulting of larvae in each vessel were checked again 12 h later (i.e. every 12 h). By doing this, the average development time (intermoult period) of larval instars could be estimated more accurately. When any newly moulted stage III phyllosoma was found, it was taken out for the later measurement and lipid analysis. The experiment terminated when all larvae either moulted to Stage III phyllosoma or died.

9.2.4 Data collection

Phyllosoma survival, development and specific growth rate (SGR)

Phyllosoma survival was expressed as cumulative survival to a particular larval Stage, which was calculated by dividing the total number of phyllosoma that moulted successfully to a particular stage with the initial number of phyllosoma stocked into each culture vessel (i.e. 25). Similarly, phyllosoma development was expressed as the mean cumulative development time

required reaching a particular stage from the newly hatched S tage I. The specific growth rate (SGR, % day^{-1}) was calculated for each replicate using the following formulae:

$SGR = 100 \times (\ln Wt - \ln Wo)/D$

where Wo is the mean dry weight of newly hatched Stage I phyllosoma while Wt is the mean dry weight of newly moulted stage III phyllosoma; D is the cumulative development time (in days) from Stage I to Stage III phyllosoma.

Total body length and dry weight of newly moulted stage III phyllosoma

The body length (BL) and dry weight of each newly moulted stage III phyllosoma were measured. Firstly the BL of a newly developed Stage III phyllosoma was measured using a microscope (precision=0.01mm). BL was defined as the distance between from the anterior margin of the cephalic shield to the posterior edge of the pleon. The phyllosoma was then briefly rinsed with 0.5 M ammonium bicarbonate to remove traces of salts, and blotted dry on a filter paper. A total number of 3-5 individual Stage III phyllosoma were randomly sampled and then dried in an oven at 60℃ for 24 h. The phyllosoma dry weight (DW) was subsequently weighed using a Cahn C-33 micro-balance (precision=1μg, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). Individual mean DW of Stage III phyllosoma was calculated by the division of phyllosoma number. The remaining samples were kept in a -70℃ freezer for the later fatty acid analysis.

9.2.5 Total lipid and fatty acid analysis

See section 4.2.4. For enriched *Artemia*, three sub-samples of each treatment were pooled for freeze-drying and homogenizing. Then, enriched *Artemia* of each treatment was analyzed in duplicate for total lipid and fatty acid composition. The average of the two values was used as the final value. For Stage III phyllosoma samples, because the survival numbers in each culture vessel were very limited, the survival phyllosoma from two vessels of each treatment were pooled for lipid analysis. Therefore, there were two replicates of each treatment for lipid analysis.

9.2.6 Statistical analysis

Homogeneity of variance of all data was first tested with Levene's test. Where necessary, arcsine-square root or logarithmic transformation was performed prior to analysis. One-way ANOVA was used to determine significant differences for various parameters among treatments. If any significant difference was detected, Tukey's multiple range test was used as the means

separation procedure. Pearson-test was used to determine statistical relationship between dietary 20:4n-6 level and different biological parameters measured. The relationships between dietary

Table 9.1 Total lipid contents (% dry weight) and fatty acid composition (%total fatty acids) of *Artemia* meta-nauplii enriched with five emulsions with elevated ARA concentrations

	Artemia enriched with ARA1	*Artemia* enriched with ARA2	*Artemia* enriched with ARA3	*Artemia* enriched with ARA4	*Artemia* enriched with ARA5
Total lipids	26.28	25.86	25.97	25.62	26.01
Fatty acids					
C14:0	0.90	0.82	0.97	0.95	0.82
C15:0	0.70	0.71	0.80	0.78	0.73
C16:0	9.21	8.86	9.43	10.00	8.66
C18:0	4.16	4.38	3.91	4.47	3.77
SFA	14.97	14.76	15.11	16.20	13.97
C14:1n7	1.34	1.47	1.71	1.78	1.43
C16:1n7	4.65	4.22	3.73	3.67	3.33
C16:1n5	0.35	0.44	0.41	0.45	0.50
C18:1n9	30.41	27.41	25.46	23.00	22.83
C18:1n7	4.23	4.28	4.16	4.07	4.15
C20:1n9	0.28	0.43	0.39	0.37	0.23
C20:1n7	0.98	0.93	0.90	0.78	0.76
MUFA	42.23	39.18	36.76	34.12	33.23
C16:2n4	0.42	0.38	0.67	0.49	0.67
C16:3n4	0.48	0.49	0.47	0.52	0.46
C18:2n6	7.79	7.94	7.16	7.44	7.80
C18:3n6	0.20	0.28	0.28	0.35	0.44
C18:3n3	16.63	15.96	16.53	17.81	16.78
C18:3n4	2.53	2.43	2.43	2.60	2.43
C18:4n3	0.33	0.13	0.12	0.08	0.10
C20:2n6	0.10	0.23	0.13	0.26	0.14
C20:3n6	0.29	0.24	0.27	0.33	0.30
C20:4n6	0.66	2.58	4.43	5.45	8.36
C20:3n3	0.51	0.52	0.51	0.53	0.47
C20:4n3	0.55	0.60	0.54	0.56	0.56
C20:5n3	4.19	5.26	4.70	4.48	4.99
C22:2n6	0.86	1.31	1.31	1.07	1.25
C22:5n3	0.27	0.50	0.35	0.40	0.42
C22:6n3	4.01	4.78	4.57	3.99	4.40
∑PUFA (≥18:2n)	38.92	42.79	43.32	45.34	48.43
∑n-3PUFA	26.49	27.76	27.31	27.84	27.71
∑n-6PUFA	9.90	12.59	13.58	14.90	18.29
n-3/n-6	2.68	2.20	2.01	1.87	1.52
∑HUFA (≥20:3n)	10.48	14.49	15.36	15.73	19.50
DHA/EPA	0.96	0.91	0.97	0.89	0.88
ARA/EPA	0.16	0.49	0.94	1.22	1.68

20:4n-6 level and different biological parameters were further evaluated with regression analysis. When a normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Kruskal-Wallis H non-parametric test followed by the Games-Howell non-parametric multiple comparison test. $P < 0.05$ was regarded as the statistically significant level. The coefficient of variation was calculated as the percentage of standard deviation divided by the mean. All statistics were performed using SPSS package (version 12.0).

9.3 Results

9.3.1 Total lipid and fatty acid profile of enriched Artemia

The total lipid and fatty acid compositions of *Artemia* enriched with five emulsion oils containing different levels of ARA are presented in Table 9.1. Total lipid contents were similar for all five treatments. While all *Artemia* contained similar levels of saturated fatty acid (SFA), with the increasing ARA level of the emulsion oils from ARA1 to ARA5 (see Table 8.1), a

Table 9.2 Principal fatty acid contents (mg/g DW) of *Artemia* meta-nauplii enriched with five emulsions with graduated ARA concentrations

	Artemia enriched with ARA1	*Artemia* enriched with ARA2	*Artemia* enriched with ARA3	*Artemia* enriched with ARA4	*Artemia* enriched with ARA5
C16:0	18.40	17.64	18.86	19.98	17.57
C18:0	8.32	8.71	7.82	8.94	7.64
SFA	29.90	29.40	30.21	32.37	28.35
C16:1n7	9.29	8.40	7.46	7.34	6.76
C18:1n9	60.73	54.57	50.90	45.97	46.32
C18:1n7	8.44	8.52	8.32	8.13	8.42
MUFA	84.34	78.01	73.50	68.18	67.42
C18:2n6	15.56	15.81	14.32	14.86	15.83
C18:3n3	33.21	31.77	33.05	35.60	34.04
C20:4n6	1.31	5.13	8.85	10.90	16.97
C20:5n3	8.37	10.48	9.40	8.96	10.12
C22:6n3	8.01	9.52	9.13	7.98	8.92
∑PUFA (≥18:2n)	77.73	85.20	86.63	90.61	98.26
∑n-3PUFA	52.91	55.28	54.61	55.64	56.22
∑n-6PUFA	19.77	25.08	27.66	29.78	37.11
∑HUFA (≥20:3n)	20.93	28.86	30.72	31.44	39.56
Total fatty acids	193.79	194.36	192.63	193.18	196.33

decreasing trend of 16:1n-7, 18:1n-9 and total monounsaturated fatty acid (MUFA) was evident in the *Artemia* enriched with these emulsion oils, respectively (Table 9.1). For the polyunsaturated fatty acids (PUFA), all enriched *Artemia* generally had similar contents of 18:2n-6, 18:3n-3, 20:5n-3 (EPA) and 22:6n-3 (DHA). As expected, the five experimental

emulsions oils with graduated 20:4n-6 (ARA) contents resulted in increasing ARA level and ARA/EPA ratio of enriched *Artemia* and a significant positive correlation was detected between ARA contents of enriched *Artemia* and ARA levels of the emulsion oils (r=0.998, P <0.01). Consequently, the ratio of n-3PUFA/n-6PUFA decreased from 2.68 for *Artemia* enriched with ARA1 to 1.52 for those enriched with ARA5 while the ratio of ARA/EPA increased from 0.16 to 1.68, respectively. Table 9.2 shows the absolute contents (mg/g DW) of principal fatty acids in enriched *Artemia*, and similar trends were found in absolute contents of fatty acids as fatty acid percentages (% of total fatty acids) (Table 9.1). The ARA contents ranged from 1.31 mg/g DW in the *Artemia* enriched with ARA1 to 16.97 mg/g DW of the *Artemia* enriched with ARA5, while the total fatty acid contents remained very similar among all treatments (192.63 – 196.33 mg/g DW) (Table 9.2).

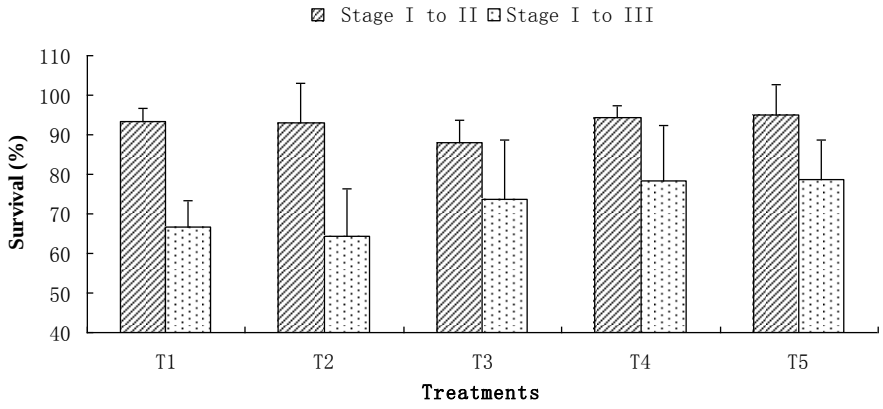

Figure 9.1 Cumulative survivals (%) of ornate rock lobster, *Panulirus ornatus* phyllosoma from Stage I to II and Stage I to III fed enriched *Artemia* containing different ARA levels. Data are presented as mean ±SD (n=4).

Table 9.3 Development time (days) from hatching to Stage II and Stage III of ornate rock lobster, *Panulirus ornatus* phyllosoma fed enriched *Artemia* containing different ARA levels

Treatments	Stage I to II (CV)	Stage I to III (CV)
T1	8.15 ± 0.08^{c} (0.98)	14.65 ± 0.22^{ab} (1.50)
T2	7.58 ± 0.12^{a} (1.58)	14.39 ± 0.12^{a} (0.83)
T3	8.22 ± 0.20^{bc} (2.43)	14.88 ± 0.14^{bc} (0.94)
T4	8.23 ± 0.09^{c} (1.09)	15.10 ± 0.23^{c} (1.52)
T5	7.92 ± 0.17^{b} (2.15)	14.95 ± 0.35^{bc} (2.34)

Data are presented as mean ±SD (n=4). Values within a column having different superscript letter are significantly different (P <0.05). CV: co-efficient of variation (%) = 100×standard deviation/mean.

9.3.2 Survival, development and growth

For all treatments, the similar survivals were found from Stage I to Stage II and Stage I to Stage III (Figure 9.1). The survival of Stage I to Stage II were approximately 90% for all treatment while cumulative survival from Stage I to Stage III ranged from 64.5% (T2) to 78.8% (T5). Although a slight higher survival was found in T3, T4 and T5 than that of T1 and T2, no

significant differences were detected among all treatments (Figure 9.1, $P > 0.05$). Table 9.3 showed mean development time from newly hatched phyllosoma to Stage II and III for each treatment. Significant differences in larval development time were detected for both stage I to II and stage I to III. Overall, phyllosoma of T2 consistently had the shortest development time ($P < 0.05$) and showed the most synchronized metamorphosis moult among all treatments. Among treatments, T2 achieved the highest specific growth rate (SGR, % day^{-1}) of dry weight (DW) over the period of Stage I to Stage III while T5 showed the lowest SGR (Figure 9.2). Further broken line regression analysis suggested that the optimal ARA content was estimated at 3.69 mg/g DW based on SGR of early Stage *P. ornatus* phyllosoma (Figure 9.3).

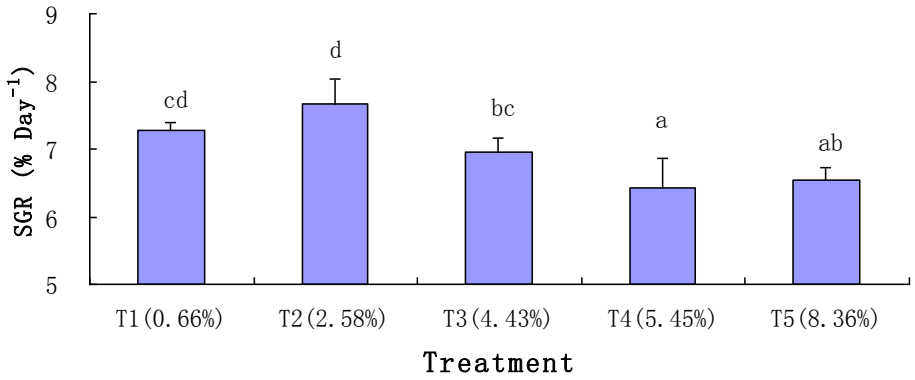

Figure 9.2 The specific growth rates (SGR, % day^{-1}) of ornate rock lobster, *Panulirus ornatus* phyllosoma from Stage I to Stage III fed enriched *Artemia* containing different ARA levels. SGRs are calculated based on dry weight of newly moulted Stage III phyllosoma. Data are presented as mean ±SD (n=4). Bars with different letters on the top are significantly different ($P < 0.05$).

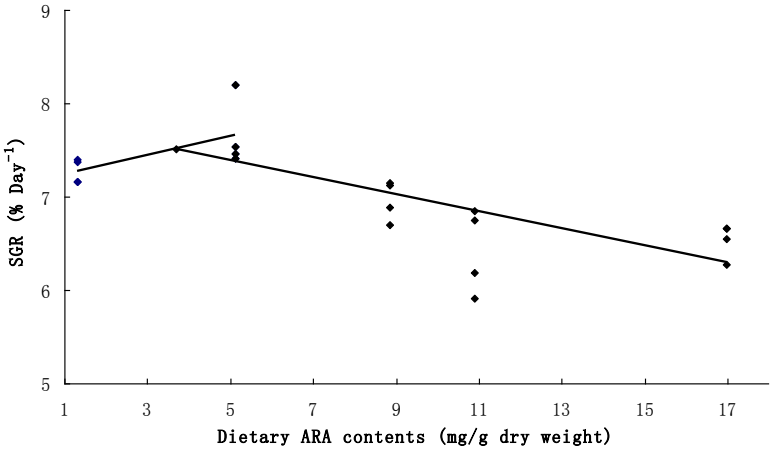

Figure 9.3 Broken line regression analysis of specific growth rates (SGR, % day^{-1}) and dietary ARA contents (mg/g DW) for ornate rock lobster, *Panulirus ornatus* phyllosoma from Stage I to Stage III fed enriched *Artemia* containing different ARA levels.

9.3.3 Body length and dry weight of newly moulted Stage III phyllosoma

The body length (BL) and dry weight (DW) of newly moulted phyllosoma are shown in Table 9.4. The phyllosoma from T5 had the lowest BL among all treatments and these were significantly lower than that in all other treatments (P <0.05). The second lowest BL was found in phyllosoma from T4 (P <0.05) while T1, T2 and T3 had the similar BL. Interestingly, although the highest body DW were recorded for phyllosoma in T2, no significant difference was detected on the body DW among T1, T2 and T3 treatments.

Table 9.4 Mean body length and dry weight of newly moulted Stage III *Panulirus ornatus* phyllosoma fed enriched *Artemia* containing different ARA levels.

Treatments	Body length (mm)	Dry weight (μg.ind.$^{-1}$)
T1	2.43 ± 0.02^{c}	130.97 ± 4.00^{b}
T2	2.41 ± 0.03^{bc}	135.81 ± 6.73^{b}
T3	2.41 ± 0.02^{bc}	127.10 ± 3.70^{ab}
T4	2.37 ± 0.03^{b}	119.35 ± 9.79^{a}
T5	2.31 ± 0.02^{a}	119.92 ± 4.45^{a}

Data are presented as mean ±SD (n=4). Values within a column having different superscript letter are significantly different (P<0.05). CV: co-efficient of variation (%) = 100×standard deviation/mean.

9.3.4 Fatty acid profile of newly moulted Stage III phyllosoma

Twenty-seven fatty acids were identified in Stage III phyllosoma larvae with levels ≥ 0.30% of total fatty acids while only 8 fatty acids were more than 5% of total fatty acids, including 16:0, 18:0, 16:1n-7, 18:1n-9, 18:2n-6, 18:3n-3, 20:5n3(EPA) and 22:6n3(DHA) (Table 9.5). The percentage of dominant fatty acids was generally reflecting the dietary fatty acid composition, particularly for 18:1n-9 and 20:4n-6. As expected, the ARA percentage in the phyllosoma larvae increased significantly from T1 (2.29%) to T5 (5.89%). Although the 18:2n-6 percentages were in a close range for five enriched *Artemia* treatments, significant lower percentages of 18:2n-6 was found in the Stage III phyllosoma from T3, T4 and T5 treatments. Table 9.6 showed the relative retention ratios (phyllosoma body PUFA percentage/dietary PUFA percentage) of PUFA for the newly metamorphosed Stage III *P. ornatus* phyllosoma fed enriched *Artemia*. The decreasing trends were found in 18:2n-6 and ARA. Among the five PUFAs, 18:3n-3 had the lowest relative retention ratio while relative retention ratios of EPA and DHA were close to 1 for all treatments.

9.4 Discussion

To the best of my knowledge, this study is the first to use a dose-response design to examine the effect of dietary ARA on larval survival, development, growth and fatty acid composition of early stage larvae for a crustacean species. For early stage phyllosoma of ornate

rock lobsters, formulated diets as replacement for live or fresh feeds are generally insufficient to support good survival and normal development through larval ontogeny (Johnston et al., 2008).

Table 9.5 Fatty acid composition (%total fatty acids) of newly moulted Stage III *Panulirus ornatus* phyllosoma fed enriched *Artemia* containing different ARA levels.

	T1	T2	T3	T4	T5
C14:0	1.52 ±0.06^{b}	1.61 ±0.16^{b}	1.65 ±0.14^{b}	1.56 ±0.49^{ab}	1.20 ±0.17^{a}
C15:0	0.72 ±0.13^{b}	0.55 ±0.06^{a}	0.71 ±0.28^{ab}	0.70 ±0.21^{ab}	0.68 ±0.20^{ab}
C16:0	14.67 ±0.28^{c}	13.50 ±0.76^{ab}	13.65 ±0.73^{ab}	13.54 ±0.04^{b}	13.13 ±0.32^{a}
C18:0	7.98 ±0.00^{b}	7.75 ±0.11^{a}	8.00 ±0.68^{abc}	7.69 ±0.44^{ab}	8.47 ±0.07^{c}
SFA	24.89 ±0.47^{b}	23.41 ±0.66^{a}	24.01 ±0.47^{ab}	23.49 ±0.19^{a}	23.48 ±0.22^{a}
C14:1n7	0.95 ±0.08^{a}	1.01 ±0.23^{ab}	1.45 ±0.08^{c}	1.05 ±0.14^{a}	1.25 ±0.11^{b}
C16:1n7	4.67 ±0.66	5.47 ±0.95	5.15 ±0.72	5.20 ±1.51	3.99 ±1.27
C16:1n5	1.77 ±0.18^{d}	1.15 ±0.11^{ab}	1.26 ±0.05^{b}	1.39 ±0.06^{c}	0.98 ±0.08^{a}
C18:1n9	17.87 ±0.76	15.50 ±0.17	14.67 ±0.13	14.53 ±1.36	13.75 ±0.74
C18:1n7	4.19 ±0.05^{b}	3.86 ±0.12^{a}	4.18 ±0.06^{b}	4.23 ±0.31^{ab}	4.35 ±0.17^{b}
C20:1n9	0.47 ±0.01^{b}	0.33 ±0.12^{a}	0.46 ±0.18^{b}	0.33 ±0.12^{a}	0.42 ±0.13^{b}
C20:1n7	1.12 ±0.05^{b}	1.02 ±0.05^{a}	1.18 ±0.04^{b}	0.81 ±0.26^{ab}	1.12 ±0.04^{b}
MUFA	31.02 ±0.19^{d}	28.32 ±0.71^{bc}	28.33 ±0.47^{c}	27.53 ±0.06^{b}	25.86 ±0.16^{a}
C16:2n4	0.78 ±0.08^{b}	0.68 ±0.05^{ab}	0.64 ±0.04^{a}	0.65 ±0.03^{a}	0.71 ±0.01^{b}
C16:3n4	0.76 ±0.05^{d}	0.36 ±0.01^{a}	0.57 ±0.02^{b}	0.60 ±0.01^{b}	0.69 ±0.01^{c}
C18:2n6	8.44 ±0.02^{b}	8.90 ±0.04^{c}	6.56 ±0.63^{a}	6.04 ±0.52^{a}	6.17 ±0.72^{a}
C18:3n6	0.77 ±0.03^{b}	0.73 ±0.08^{ab}	0.63 ±0.09^{a}	0.62 ±0.23^{ab}	0.60 ±0.07^{a}
C18:3n3	5.30 ±0.11^{b}	5.51 ±0.06^{c}	5.12 ±0.04^{a}	5.34 ±0.42^{ab}	5.67 ±0.16^{c}
C18:3n4	0.89 ±0.08^{a}	1.10 ±0.04^{b}	0.85 ±0.15^{a}	0.90 ±0.09^{a}	1.17 ±0.03^{c}
C18:4n3	0.48 ±0.01^{c}	0.45 ±0.01^{b}	0.42 ±0.02^{a}	0.46 ±0.00^{b}	0.62 ±0.04^{d}
C20:2n6	0.78 ±0.06^{b}	0.76 ±0.18^{ab}	0.59 ±0.08^{a}	0.97 ±0.43^{ab}	0.69 ±0.07^{a}
C20:3n6	0.98 ±0.30	0.66 ±0.07^{ab}	0.74 ±0.04^{b}	0.61 ±0.08^{a}	0.67 ±0.16^{ab}
C20:4n6	2.29 ±0.08^{a}	3.16 ±0.20^{b}	3.69 ±0.02^{c}	4.48 ±0.28^{d}	5.89 ±0.46^{e}
C20:3n3	1.00 ±0.02^{b}	1.03 ±0.42^{ab}	0.98 ±0.04^{a}	0.87 ±0.08^{a}	1.03 ±0.13^{ab}
C20:4n3	0.33 ±0.09	0.33 ±0.23	0.26 ±0.03	0.29 ±0.05	0.48 ±0.18
C20:5n3	4.54 ±0.01	4.73 ±0.54	4.58 ±0.02	4.17 ±0.57	4.94 ±0.43
C22:2n6	0.61 ±0.04^{b}	0.85 ±0.14^{c}	0.53 ±0.04^{a}	0.66 ±0.33^{abc}	0.59 ±0.19^{abc}
C22:5n3	0.69 ±0.04^{c}	0.54 ±0.12^{ab}	0.52 ±0.04^{b}	0.54 ±0.09^{b}	0.43 ±0.00^{a}
C22:6n3	3.99 ±0.49^{a}	4.49 ±0.09^{a}	5.22 ±0.44^{b}	4.77 ±0.75^{ab}	5.19 ±0.35^{b}
∑PUFA (≥18:2n)	31.06 ±0.14^{a}	33.22 ±2.12^{ab}	30.65 ±0.86^{a}	30.68 ±3.12^{ab}	34.11 ±1.77^{b}
∑n-3PUFA	16.32 ±0.21	17.07 ±1.46	17.08 ±0.33	16.42 ±1.96	18.35 ±0.59
∑n-6PUFA	13.86 ±0.26^{b}	15.05 ±0.71^{c}	12.73 ±0.69^{a}	13.37 ±1.25^{abc}	14.60 ±1.21^{bc}
n-3/n-6	1.18 ±0.04^{a}	1.13 ±0.04^{a}	1.34 ±0.05^{c}	1.23 ±0.03^{b}	1.26 ±0.06^{bc}
∑HUFA (≥20:3n)	13.81 ±0.11^{a}	14.93 ±1.67^{ab}	15.97 ±0.41^{b}	15.70 ±1.74^{b}	18.61 ±0.69^{c}
DHA/EPA	0.88 ±0.11^{a}	0.95 ±0.09^{a}	1.14 ±0.10^{b}	1.14 ±0.02^{b}	1.06 ±0.16^{ab}
ARA/EPA	0.50 ±0.02^{a}	0.67 ±0.03^{b}	0.81 ±0.00^{c}	1.08 ±0.08^{d}	1.19 ±0.01^{e}

Data are presented as mean ±SD (n=2). Values within a line having different superscript letter are significantly different (P <0.05).

Use of formulated diets is therefore not a feasible option for lipid nutrition study for early stage phyllosoma (Liddy et al., 2005; Smith et al., 2009a,b). This study utilized enriched *Artemia* for this purpose and the results showed that ARA in self-prepared lipid enrichment emulsions was readily absorbed/assimilated by *Artemia*. Therefore, by careful manipulation of ARA levels in lipid emulsions, graded ARA levels could be achieved in enriched *Artemia*, which can then be used to feed the crab larvae to access their dietary ARA requirements. Although similar dose-response experiments using enriched *Artemia* have been used to examine EPA and DHA requirements of crustacean larvae (Suprayudi et al., 2004b; Nghia et al., 2007; Sui et al., 2007), no published literature exists for such a study on ARA requirements. The outcomes of this study suggested that *Artemia* enrichment, based dose-response design, is a suitable approach for investigating the ARA requirements of crustacean larvae.

Table 9.6 The relative retention ratios between phyllosoma body PUFA percentages and their dietary PUFA percentages of newly metamorphosed Stage III *Panulirus ornatus* phyllosoma fed enriched *Artemia* containing different ARA levels. Relative retention ratio = a fatty acid percentage of phyllosoma body / a respectively fatty acid percentage in the diet.

	T1	T2	T3	T4	T5
C18:2n-6	1.08	1.12	0.92	0.81	0.79
C18:3n-3	0.32	0.35	0.31	0.30	0.34
C20:4n-6	3.47	1.22	0.83	0.82	0.70
C20:5n-3	1.08	0.90	0.97	0.93	0.99
C22:6n-3	1.00	0.94	1.14	1.20	1.04

My previous studies have showed that ARA and DHA are preferentially conserved at a high level during the starvation of newly hatched *P. ornatus*, indicating the important physiological functions for these fatty acids during the early ontogeny of this species (Wu et al, unpublished data). The previous study found the lower ARA levels was found on Stage II and Stage III phyllosoma compared to newly hatched phyllosoma when they were fed un-enriched newly hatched *Artemia* only containing 1.18% of ARA in total fatty acids (Wu et al., 2012). However, the current study showed dietary ARA levels did not significantly affect the survival for the early Stage *P. ornatus* phyllosoma for all treatments. Such a result might be explained by the fact that the Stage II and Stage III *P. ornatus* phyllosoma could preferentially absorb and deposit the dietary ARA in their tissue for the growth and development when their diets are lack of ARA. This explanation was further supported by the highest relative retention ratio of ARA was found in the phyllosoma from treatment 1 among the five treatments (Table 9.7). Therefore, Stage III *P. ornatus* phyllosoma containing ca. 2% ARA of total fatty acids still can support their normal survival (Wu et al., 2012). In the present study, the general survival to Stage III (around 70%) is higher than the previous reports for early stage phyllosoma only fed newly

hatched *Artemia* or on-grown *Artemia* (Johnston et al., 2008) (40%); this difference maybe ascribe to the batch variation and/or different culture conditions.

The present results clearly showed that the suitable diet ARA levels could promote the growth and shorten the development period while the dietary excessive ARA would inhibit/depress larval growth and delay the moulting. There are two possible explanations for this phenomenon. Firstly, crustacean growth generally depend on the moulting process and tissue growth, particularly for muscle growth, and the fatty acid composition of tissue membrane is relatively conserved for a specific species (Sheen and Wu, 2002). Inappropriate fatty acid composition in the diet might lead to unbalanced fatty acid composition of tissue membrane, which further resulted in slower growth and longer inter-moulting period (Merican and Shim, 1996b; Sui et al., 2007). Secondly, the nutritional significant of ARA might be explained by the fact that the ing process in crustaceans is regulated by prostaglandin production and their ratios (Koskela et al., 1992; Bransden et al., 2005). It is well known ARA and EPA are the precursors of series-2 (e.g. prostaglandin E_2, PGE_2) and series-3 prostaglandin (e.g. prostaglandin E_3, PGE_3), respectively (Yang et al., 2002). Optimal dietary ARA levels are therefore likely to result in increased PGE_2 synthesis and concentration in crustaceans (Reddy et al., 2004; Meunpol et al., 2010). Indeed, research has shown that brown tiger prawn *Penaeus esculentus* injected with PGE_2, a series-2 prostaglandin derived from ARA, displayed shorter cycles compared to the controls (Koskela et al., 1992). It is therefore possible that when dietary ARA is below an optimal level, increased dietary ARA would lead to increased PGE_2 synthesis and concentration, hence enhancing larval development. However, since there exists a competitive inhibition between conversion of ARA to PGE_2 and EPA to PGE_3 (Bell and Sargent, 2003), further increase of dietary ARA beyond an optimal level may lead to excessive PGE_2 being produced with decreased PGE_3 production, resulting in an imbalanced PGE_2/PGE_3 ratio. The imbalanced PGE_2/PGE_3 ratio could lead to extended development time and slower growth rate in early stage *P. ornatus* phyllosoma. Although some studies have revealed that ARA enriched *Artemia* would promote early stage phyllosoma growth for rock lobsters, including western rock lobster *Panulirus cygnus* (Liddy et al., 2005) and southern rock lobster *Jasus edwardsii* (Phleger et al., 2001), the optimal dietary ARA levels has not been identified for any lobster phyllosoma, even for any crustacean larvae. The present results clearly demonstrated, for the first time, that the optimal dietary ARA content is around 3.7 mg/g DW or 1.9% of total fatty acids (Fig. 3), which is significant higher than that of newly hatched and un-enriched *Artemia* (0.91 mg/g DW) (Wu et al., 2012). This suggested the ARA enrichment for newly hatched *Artemia* is important and essential for the culture of early stage *P. ornatus* phyllosoma when fed on newly hatched *Artemia* or on-grown *Artemia*.

The relative levels and ratios of major fatty acids in Stage III phyllosoma were positively related to the values for the same fatty acid parameter within the enriched *Artemia*, but with some modifications in some fatty acids, such as 16:0, 18:0 and 18:1n9. Compared to the enriched *Artemia*, those modifications of fatty acid composition in Stage III phyllosoma may be reflect their nature for body fatty acid composition. The fatty acid profile of Stage III phyllosoma was similar to our previous reports (Wu et al., 2012). Although there was a positive correlation between ARA levels of enriched *Artemia* and its percentages in Stage III phyllosoma (n=10, r=0.995, $P<0.01$), the relative retention ratio of ARA decreased dramatically from treatment 1 to treatment 5. This suggested early stage *P. ornatus* phyllosoma are able to preferentially assimilate and accumulate ARA from their diets when the dietary ARA level is inadequate. The similar results were found in western rock lobster *Panulirus cygnus* (Liddy et al., 2005) and southern rock lobster *Jasus edwardsii* (Nelson et al., 2004). Consequently, when dietary ARA level is above the optimal level, the relative retention ratio of ARA decreased with the increasing dietary ARA levels. Moreover, the decreasing trend of 18:2n6 levels (%total fatty acids) in treatment 1 - treatment 5 maybe ascribe to the increasing ARA levels. This is because both of them are belong to n-6PUFAs, and excessive tissue ARA percentages probably depress/hinder the assimilation and accumulation of 18:2n6 in *P. ornatus* phyllosoma. Our previous study has identified precocious *Eriocheir sinensis* could accumulate higher 18:2n6 in their gonads and muscle when diets were lack of HUFA (Wu et al., 2010a). Those results indicated that there may be strong interaction between C18-PUFA (such as 18:2n6, LOA) and HUFA for crustacean (González-Félix et al., 2003). Therefore, further research is required to determine the optimal ratios of dietary ARA/LOA and ARA/EPA for the early stage *P. ornatus* phyllosoma, and to gain a better understanding of the interaction between dietary LOA and ARA as well as dietary EPA and ARA. In black tiger prawn *Penaeus monodon* juveniles, excessive dietary ARA has been reported to reduce hepatopancreas palmitic acid (16:0) and total saturated fatty acid (SFA) contents (Glencross et al., 2001). Since 16:0 is one of the most important fatty acids for energy storage in crustaceans, low 16:0 levels have been suggested to have negative effects on moulting and growth of this species (Teshima et al., 1977; Teshima, 1998). However, this effect is not clear in our results. This may be explained by the species-specific differences.

9.5 Conclusion

This study demonstrates the ARA contents in enriched *Artemia* was able to be improved via ARA-rich emulsion oil, and then the dietary ARA would effectively deliver to the early stage *P. ornatus* phyllosoma. The appropriate diet ARA level was able to sustain high survival, short

development time and fast growth, which led to the high individual dry weight and specific growth rate for the Stage III *P. ornatus* phyllosoma larvae. Further analysis suggested that the optimal ARA content was estimated at 3.69 mg/g DW for early stage phyllosoma of *P. ornatus*. The ARA percentage in Stage III phyllosoma increased significantly with the elevated dietary ARA levels while its relative retention ratio decreased dramatically from T1 to T5, which suggested early stage *P. ornatus* phyllosoma are able to preferentially assimilate and accumulate dietary ARA when their dietary ARA levels are inadequate.

Chapter 10

The effects of dietary DHA/EPA ratios on larval survival, growth and fatty acid composition of blue swimmer crab, *Portunus pelagicus*

10.1 Introduction

The blue swimmer crab, *Portunus pelagicus* (Linnaeus, 1758), is a commercially important species that is widely distributed in the tropical and subtropical waters of Indo-Pacific region (Stephenson and Campbell, 1959). Because of their large size and delicate flavor, there is an increasing market demand for this crab species (Wu et al., 2010d). It is believed that wild *P. pelagicus* stocks have been fully exploited, therefore, further increase in *P. pelagicus* production is likely have to come from either aquaculture or stock enhancement programs (Maheswarudu et al., 2008; Dixon and Hooper, 2009), both of them rely on successful hatchery seed production.

Larval development of *P. pelagicus* includes four zoeal instars and one megalopal instar (Josileen and Menon, 2004b; Andres et al., 2010). While larval culture of *P. pelagicus* generally has better survival than other commercially important portunid crabs, such as the mud crab *Scylla* spp. and the blue crab *Callinectes sapidus* (Zmora et al., 2005; Nghia et al., 2007), high incidences of the moulting death syndrome (MDS) during the time zoea IV metamorphose to megalopae are still commonly observed (Soundarapandian et al., 2007; Castine et al., 2008). This has signficantly impacted on the final yield of crab seed production.

Previous research has shown that unbalanced dietary highly unsaturated fatty acids (HUFAs) could lead to low larval survival with high incidences of MDS in larval culture of the mud crab, *S. serrata* (Suprayudi et al., 2004a,b; Dan and Hamasaki, 2011) and the swimming crab, *Portunus trituberculatus* (Takeuchi et al., 1999a,b; Arai et al., 2004). Larval nutrition, particular fatty acid nutrition, has also been suggested as a major contributor to unsuccessful larval rearing of *P. pelagicus* (Maheswarudu et al., 2008). HUFA is defined as fatty acids with carbon chain lengths $\geq$ 20 and containing $\geq$ 3 double bonds (Tocher, 2003) and they are considered as a crucial group of fatty acids to larval survival and growth of many decapod crustaceans (Sheen and Wu, 2002; Glencross, 2009). The important of appropriate dietary HUFA supply to crustaceans is primarily stemmed from the fact that many crustaceans have limited capacity to synthesize HUFAs *de novo* and therefore rely on dietary source for the supply (Kanazawa et al., 1979a).

Generally marine crustaceans have higher HUFA requirements as compared to freshwater crustaceans. This is thought to be reflective of higher HUFA levels presence in marine food chain (D'Abramo, 1997; Wu et al., 2010a). The most biologically significant HUFAs include 20:4n6 (ARA), 20:5n3 (EPA) and 22:6n3 (DHA) and they are considered to have a higher nutritional value for marine crustaceans as compared to 18-carbon poly-unsaturated fatty acids (C18-PUFA) (Xu et al., 1993; Xu et al., 1994a; Merican and Shim, 1996; Glencross and Smith, 2001). Past studies have focused on the nutritional effects of n-3 HUFAs for aquaculture of crustaceans, particularly EPA and DHA (Xu et al., 1993; Rees et al., 1994; Nghia et al., 2007; Sui et al., 2007; Wu et al., 2007c). EPA and DHA play important roles in many important physiological processes of crustaceans, including the important constitute of membrane phospholipids, as the precursors for biologically active eicosanoids and enhancement of neural development (Bell et al., 1986; Mourente and Tocher, 1992; Wouters et al., 2001). Previous studies have demonstrated that larval diets deficient in n-3 HUFA can result in a slow growth rate, high mortality, and prolonged inter period in larvae of many decapod crustaceans (Suprayudi et al., 2004b; Nghia et al., 2007; Wu et al., 2007c). Similarly, despite there are few reports, excess dietary HUFA supply has also been shown to have negative effects on larvae (Takeuchi et al., 1999b; Suprayudi et al., 2004b; Dan and Hamasaki, 2011).

DHA and EPA may perform different physiological functions in crustacean larvae (Takeuchi et al., 1999b; Suprayudi et al., 2004a,b). For example, in larvae of shrimp *Penaeus monodon* (Merican and Shim, 1997) and the crabs *P. trituberculatus* (Takeuchi et al., 1999a) and *Eurypanopeus depressus* (Levine and Sulkin, 1984), DHA has been reported to affect larval moulting and growth while EPA content has a significant impact on survival. However, there are limited efforts in addressing species-specific quantitative requirement of EPA and DHA (Merican and Shim, 1996; Takeuchi et al., 1999b; Glencross and Smith, 2001; González-Félix et al., 2003a). There are a number of studies on marine crustacean larvae demonstrated that a greater DHA requirement as compared to EPA and it has been suggested that the dietary ratio of DHA to EPA could significantly affect larval survival and growth (Takeuchi et al., 1999b; Sui et al., 2007). However, there are considerable impediments to the efforts to better understand the provision and uptake of dietary DHA to marine crustaceans, especially considering that their preponderance to readily retro-convert DHA to EPA in both live foods, such as *Artemia* (Evjemo et al., 2001; Han et al., 2001), and cultured species such as *Macrobrachium rosenbergii* (D'Abramo and Sheen, 1993) and *P. monodon* (Merican and Shim, 1996a). However, the dietary DHA/EPA ratio is clearly important to marine crustacean larvae (Merican and Shim, 1997; Mourente and Rodríguez, 1997; Sui et al., 2007) as demonstrated in the swimming crab, *P. trituberculatus* (Takeuchi et al., 1999a) and the Chinese mitten crab,

Eriocheir sinensis (Sui et al., 2007), if the ratio is not appropriate, it can negatively impact growth and survival.

The previous studies have showed that DHA was preferentially deposited during larval development of *P. pelagicus*, indicating the important of DHA during the early ontogeny of this species (Chapter 4). However, to date there is no published information on the HUFA requirements of *P. pelagicus* larvae. This can potentially hinder the development of successful hatchery protocols and stifle the formulation of larval feeds for *P. pelagicus*. Currently, the primary food sources for larval culture of *P. pelagicus* are rotifer and *Artemia* nauplii, there is no commercial formulated diet has been developed for their larval culture (Soundarapandian et al., 2007; Andrés et al., 2010). *Artemia* nauplii generally lack of HUFAs, however there are a number of enrichment products that can be used to increase *Artemia* HUFA content (Chapters 4). The present study was carried out to assess the effects of dietary DHA/EPA ratio on larval performance, occurrence of MDS as well as fatty acid composition of the newly moulted *P. pelagicus* megalopae.

10.2 Materials and methods

10.2.1 Broodstock source and maintenance

Broodstock source and husbandry were described in Chapter 5 (Section 4.2.1).

10.2.2 Artemia enrichment

The DHA/EPA ratios and fatty acid compositions of the five oils used for *Artemia* enrichment are shown in Table 10.1. Five oils contained different DHA/EPA ratios, while their SFA, MUFA and HUFA levels are in a close range. The formulations of five emulsion oils are shown in Table 10.2. Macadamia oil was used to provide saturated fatty acids and mono-unsaturated fatty acids. Ten percent of soy lecithin was added as an emulsifier while 2% of α-tocopherol acetate was added as an antioxidant (Estévez et al., 2001; Villalta et al., 2005). Each emulsion oil was then emulsified with equal amounts of distilled water using an Ultra-turrax T25 homogenizer (IKA Works Inc, Wilmington, North Carolina, USA) operating at 10, 000 rpm for 60 seconds. They were then stored in bottles and kept in a refrigerator at 4^{o}C.

To obtain newly hatched *Artemia* nauplii for the experiment, *Artemia* cysts (EG grade, INVE (Thailand) Ltd, Thailand) were hatched daily in 1-µm filtered seawater with a salinity of 25‰ at 28°C. After 24 h, nauplii (size between 430-520µm) were harvested for subsequent enrichment. Six-hour-old *Artemia* nauplii were enriched in 300 mL containers at a density of 200-250 *Artemia*/mL for 24 h with 0.6 g/L of each emulsion oil (Sui et al., 2007). During

enrichment, temperature and salinity were maintained at 28 °C and 25‰, respectively. Enriched *Artemia* were then harvested and rinsed with freshwater to remove traces of the emulsion before being fed to *P. pelagicus* larvae. Any enriched *Artemia* that were not fed to larvae were immediately placed at 4 ℃ in a refrigerator to minimize fatty acid catabolism (Merchie, 1996), they were to be used within 24 h and any leftovers discarded after this period. *Artemia* (ca.1-2 g/sample) enriched with each emulsion were sampled three times over the same period as used for the larval culture for lipid and fatty acid analysis. All samples were stored at -70 ℃ before analysis took place.

Table 10.1 Fatty acid compositions (% total fatty acid) of the five type HUFA oils containing different DHA/EPA ratios.

Fatty acid	Oil 1	Oil 2	Oil 3	Oil 4	Oil 5
14:0	0.57	0.80	0.88	0.58	0.72
16:0	6.15	6.95	6.22	7.45	8.31
18:0	2.96	2.81	3.20	4.11	3.62
∑SFA	**9.68**	**10.56**	**10.31**	**12.14**	**13.65**
16:1n7	4.32	4.71	3.76	3.51	2.86
18:1n-9	20.92	18.81	17.00	17.13	15.15
18:1n-7	--	1.12	1.83	2.16	4.33
20:1n-9	2.16	2.02	1.18	1.36	1.82
20:1n-7	0.56	0.69	0.39	0.56	1.65
∑MUFA	**27.96**	**27.91**	**24.16**	**24.72**	**25.81**
16:2n-4	--	1.03	1.02	--	--
16:3n-4	1.51	0.85	0.52	--	--
18:2n-6	0.91	--	1.14	1.14	--
18:3n-3	1.03	0.87	1.22	1.91	1.42
18:3n-6	3.69	3.84	2.92	1.57	1.43
18:3n-4	1.85	1.03	1.25	1.48	3.28
20:5n-3	35.05	30.51	25.47	16.83	12.32
20:4n-6	3.07	2.06	2.40	1.99	2.03
20:4n-3	0.98	--	--	--	--
22:5n-3	1.17	1.64	2.16	3.00	3.32
22:6n-3	12.07	17.77	24.76	34.00	37.56
∑PUFA(≥ 18:2n)	58.65	58.26	62.50	61.92	58.04
∑HUFA(≥ 20:3n)	49.27	49.92	52.40	53.83	53.20
DHA/EPA	0.34	0.58	0.97	2.02	3.05
Unknown	2.20	1.39	1.51	1.22	2.50

10.2.3 Experimental design and setup

Larval culture condition was described in Chapter 8-Section 8.2.3. There were 5 treatments (Treatment 1 to 5, defined as T1-T5) with larvae fed *Artemia* enriched with 5 self-prepared emulsions, respectively. Every treatment had 6 replicates and among them, one replicate was used exclusively for sampling of zoea IV for measurement of chela length (CHL) and carapace length (CL) to calculate the ratio of chela length (CHL) to carapace length (CL)

(CHL/CL), which has been reported as a good index of morphogenesis at the final zoea stage in portunid crabs (Hamasaki et al., 2002a; Arai et al., 2004). Throughout the experiment, water temperature was maintained at 28±1 ℃ using a water bath. At the beginning of the experiment, three samples of newly hatched zoea I (50 larvae/sample) were collected and briefly rinsed with 0.5 M ammonium bicarbonate to remove trace of salt before being blotted dry on a filter paper. They were then oven-dried at 60 ℃ for 24 h before being weighed to determine the average dry weight of an individual newly hatched zoea I larvae.

Previous research in this laboratory has shown that when cultured in beakers with daily 100% water exchange, newly hatched larvae of *P. pelagicus* fed *Artemia* alone could achieve comparable survival to those fed on rotifers (Josileen and Zeng, unpublished data). As a result, throughout this experiment, the larvae were fed *Artemia* enriched with 5 emulsions (Table 10.2). *Artemia* were fed at a density of 4 ind. mL^{-1} for all treatments. Each morning, 100% water exchange was carried out when surviving larvae in each beaker were counted and staged before being transferred to a new beaker with clean seawater and fresh enriched *Artemia*. Mortality and ing of larvae in each beaker were checked again 12 h after water exchange (i.e. every 12 h). This was done to ensure that the average intermoult period of each larval instar could be estimated more accurately. Since cannibalism typically occurs after larvae metamorphose to megalopae when they develop two chelipeds (Castine et al., 2008), to avoid cannibalism, any newly appeared megalopae were removed from beakers as soon as they were found. The experiment terminated when all larvae had either ed to megalopae or died.

Table 10.2 The formulation (mg/g) of five experimental emulsions oils used for *Artemia* enrichment

	Source	**Emulsion 1**	**Emulsion 2**	**Emulsion 3**	**Emulsion 4**	**Emulsion 5**
Oil 1	A	680				
Oil 2	A		680			
Oil 3	A			680		
Oil 4	A				680	
Oil 5	A					680
Macadamia oil	B	200	200	200	200	200
Soy Lecithin	C	100	100	100	100	100
α-tocopherol acetate	D	20	20	20	20	20

Source of the ingredients:

A. Obsidian Research Ltd, U.K.. The fatty acid composition of oil 1- 5 were showed in Table 1 and these oils contained different DHA/EPA ratios;
B. Macadamias (Aust) Ltd, Gympie, Queensland, Australia;
C. Sigma-Aldrich Pty Ltd, Castle hill, NSW, Australia;
D. Blackmores Ltd, Warriewood, New South Wales, Australia.

10.2.4 Data collection

See section 8.2.4.

10.2.5 Total lipid and fatty acid analysis

See section 4.2.4.

10.2.6 Data analysis and statistics

Homogeneity of variance of all data was first tested with Levene's test. Where necessary, arcsine-square root or logarithmic transformation was performed prior to analysis. One-way ANOVA was used to determine significant differences among treatments for various parameters measured. If a significant difference was detected, Tukey's multiple range test was used as the means separation procedure. Pearson-test was used to determine statistical relationship between dietary DHA/EPA ratio and different biological parameters measured. The relationships between dietary DHA/EPA ratio and different biological parameters were further evaluated with regression analysis. When a normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Kruskal-Wallis H non-parametric test followed by the Games-Howell non-parametric multiple comparison test. $P < 0.05$ was regarded as the statistically significant level. The coefficient of variation was calculated as the percentage of standard deviation divided by the mean. For any treatment, all CHL/CL ratio data were measured from one beaker, the CHL/CL ratio from one beaker was considered as the unreplicated data. Therefore, Pearson Chi-square was performed to test the correlations between zoea IV CHL/CL ratio and dietary DHA/EPA ratios as well as the zoea IV CHL/CL ratio and the percentage of MDS during the metamorphosis moult to megalopa (Hurlbert, 1984). All statistics were performed using SPSS package (version 12.0).

10.3 Results

10.3.1 Total lipid and fatty acid profile of enriched Artemia

The total lipids and fatty acid compositions of *Artemia* enriched with five emulsions of different DHA/EPA ratios are presented in Table 10. 3. *Artemia* enrichment increased total lipid contents and percentages of highly unsaturated fatty acids (HUFAs), particularly EPA (20:5n3) and DHA (22:6n3). Total lipid contents of enriched *Artemia* were similar among all five treatments. All enriched *Artemia* also contained similar levels of saturated fatty acid (SFA) and mono-unsaturated fatty acids (MUFA). For the polyunsaturated fatty acids (PUFA), while all enriched *Artemia* generally had similar contents of 18:2n-6 and 18:3n-3, as expected, a trend of increasing 22:6n-3 with decreasing 20:5n-3 were found in the *Artemia* enriched with 5 emulsions for 1 to 5, which resulted in a significant increase of DHA/EPA ratio of the enriched *Artemia.* Although enriched *Artemia* of 5 treatments differed in DHA/EPA ratio, they contained

close range of total poly-unsaturated fatty acids (PUFA), total HUFA, DHA+EPA (Table 10.3). The five experimental emulsions with elevated DHA/EPA ratio resulted in increasing DHA/EPA ratio of enriched *Artemia* and a significant positive correlation was detected between DHA/EPA ratios of enriched *Artemia* and that of the emulsions (r=0.965, n=5, P <0.01).

Table 10.3 Total lipid contents (% dry weight) and fatty acid compositions (% total fatty acids) of newly hatched *Artemia* enriched with emulsions containing different DHA/EPA ratios

Fatty acid	**Newly hatched *Artemia***	***Artemia* enriched with emulsion 1**	***Artemia* enriched with emulsion 2**	***Artemia* enriched with emulsion 3**	***Artemia* enriched with emulsion 4**	***Artemia* enriched with emulsion 5**
Total lipid	18.61	28.35	29.43	28.67	28.74	29.66
Fatty acid						
C14:0	0.66	0.59	0.61	0.67	0.84	0.70
C15:0	0.25	0.39	0.41	0.39	0.48	0.38
C16:0	16.75	9.54	10.04	10.24	11.19	10.67
C18:0	7.06	4.31	4.76	4.85	5.11	4.72
∑SFA	25.15	14.82	15.82	16.15	17.61	16.47
C14:1n7	0.79	0.22	0.22	0.20	0.21	0.22
C16:1n7	5.03	4.86	4.40	4.34	4.51	4.72
C16:1n5	0.52	0.46	0.49	0.50	0.52	0.48
C18:1n9	18.20	29.26	28.34	28.54	26.01	28.10
C18:1n7	6.61	5.19	5.48	5.54	5.42	5.13
C20:1n	0.66	1.31	1.44	1.61	1.58	1.89
∑MUFA	31.82	41.31	40.33	40.73	38.25	40.55
18:2n6	10.10	8.15	7.92	8.01	7.59	8.26
18:3n3	16.94	6.32	5.88	5.52	5.84	4.72
20:5n3	1.43	16.34	14.36	12.18	8.59	8.87
20:4n6	1.02	1.39	1.28	1.19	1.09	0.99
22:6n3	0.18	3.77	4.88	6.46	11.79	13.08
∑PUFA	37.26	38.24	36.77	36.78	37.84	38.68
∑HUFA	3.90	23.36	22.62	22.35	24.05	25.47
DHA/EPA	0.13	0.23	0.34	0.53	1.36	1.47
DHA+EPA	1.61	20.11	19.24	18.64	20.30	21.95

10.3.2 Larval survival, development and growth

For all treatments, larval survival to the megalopal stage was successfully achieved. The cumulative larval survival was not significantly different among all treatments until zoea IV stage, however, the survival to megalopal stage were significant different (Table 10.4, P < 0.05), suggesting survival at zoea IV were particular impacted. During the zoeal development, it was observed that in all treatments, highest mortalities occurred during zoea IV metamorphosis ing to megalopa (Table 10.4). Larvae in the T3 treatment has the highest overall zoeal survival (i.e. cumulative survival to megalopa = 54.46%), followed by T4, T5, T2 and T1. The overall zoeal survival of T3 treatment was significantly higher than both T1 and T2 treatments (P < 0.05) but

the differences among other treatments was not significant (Table 10.4). Among all treatment, the lowest and second lowest coefficient of variation (CV) of zoeal survival was found for T4 (13.41%) and T3 treatments (28.61%), respectively, and both were substantially lower than that of other treatments (>51%), indicating that survival was most consistent among replicates for these treatments. During the zoeal development, most of mortality occurred during period of the zoea IV ing to megalopa (Table 10.4).

Table 10.4 Cumulative survival (%) to each subsequent larval stage of newly hatched *Portunus pelagicus* larvae fed enriched *Artemia* containing different DHA/EPA ratios

Treatments	Cumulative survival (%)			
	Zoea II	Zoea III	Zoea IV	Megalopa (CV)
T1	93.11±2.32	87.94±6.44	76. 67±10.58	27.94±14.37^{a} (51.43)
T2	96.44±5.69	92.92±10.88	89.01±11.05	29.04±16.35^{a} (56.30)
T3	93.53±7.39	87.01±10.18	80.38±15.61	54.46±15.58^{b} (28.61)
T4	93.89±5.05	91.46±5.23	85.95±3.48	46.22±6.20^{ab} (13.41)
T5	90.32±5.61	85.83±6.51	75.47±7.41	31.92±17.53^{ab} (54.92)

Data are presented as mean±SD (n=5). Values within a same column with different superscript letters are significantly different ($P < 0.05$). CV: coefficient of variation (%) = 100×standard deviation/mean.

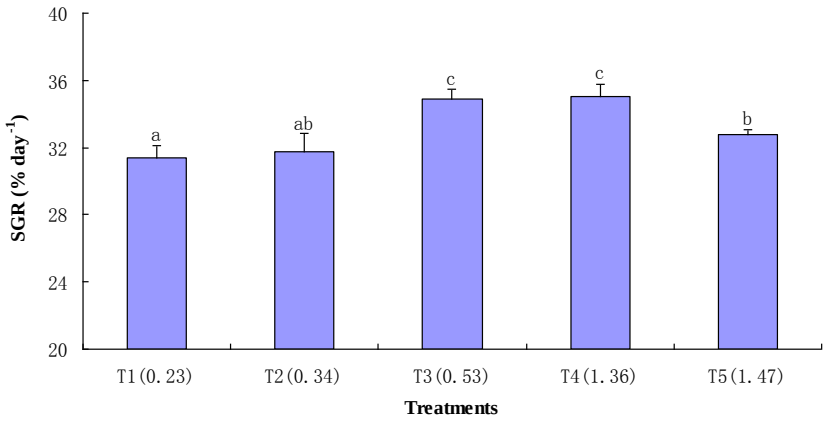

Figure 10.1 The specific growth rates (SGR, % day^{-1}) of *Portunus pelagicus* newly hatched larvae fed enriched *Artemia* with different DHA/EPA ratios. SGRs are calculated based on dry weight of newly ed megalopae. The value in the bracket under the x-axis indicates its dietary DHA/EPA ratio of each treatment. Data are presented as mean±SD (n=4). Bars with different letters on the tops are significantly different ($P < 0.05$).

In contrast to survival, significant differences in larval cumulative development time were detected as early as at the first moult to the zoea II stage (Table 10.5). Until zoea IV stage, larvae of T5 consistently had the shortest development time while larvae of T1 had the longest development time ($P < 0.05$). For the overall zoeal development time, larvae of T4 had the shortest development time (9.1 days), and larvae from T3 and T5 had the second shortest time (9.5 days), which was followed by T2 (9.6 days) and T1 (9.9 days). Overall zoeal development time of T1 was significantly longer than that of larvae in T4 (P <0.05, Table 10.5). For the

specific growth rate (SGR, % day^{-1}) of all treatments, T3 and T4 achieved the highest SGR of dry weight (DW) over zoeal development while T1 showed the lowest SGR (Figure 10.1).

10.3.3 Carapace length and dry weight of newly settled megalopae

The mean carapace length (CL) and dry weight (DW) of newly metamorphosed megalopae from different treatments are shown in Table 10.6. The larvae from T3 had the widest CL and the highest DW among all treatments and the differences were significant when compared to other treatments except T4 treatment (P <0.05). The T4 treatment produced the second widest CL and the second highest DW, which was also higher than other treatments (P <0.05). The lowest CL and DW were recorded for larvae of the T1 treatment, of which larvae were fed *Artemia* with the lowest DHA/EPA ratio (Table 10.6).

Table 10.5 Cumulative development time (days) to each subsequent larval stage of newly hatched *Portunus pelagicus* larvae fed enriched *Artemia* containing different DHA/EPA ratios

Treatments	**Cumulative development period (days)**			
	Zoea II	Zoea III	Zoea IV	Megalopa
T1	2.2 ±0.1^{b}	4.5 ±0.1^{c}	6.8 ±0.2^{c}	9.9 ±0.3^{b}
T2	2.1 ±0.1^{ab}	4.3 ±0.1^{b}	6.3 ±0.2^{b}	9.6 ±0.6^{ab}
T3	2.1 ±0.0^{ab}	4.2 ±0.1^{ab}	6.2 ±0.2^{b}	9.5 ±0.1^{ab}
T4	2.1 ±0.1^{ab}	4.2 ±0.1^{ab}	6.2 ±0.2^{b}	9.1 ±0.1^{a}
T5	2.0 ±0.1^{a}	4.1 ±0.1^{a}	5.9 ±0.3^{a}	9.5 ±0.2^{ab}

Data are presented as mean ±SD (n=5). Values within a same column with different superscript letters are significantly different (P < 0.05).

Table 10.6 Mean carapace length and body dry weight of newly metamorphosed *Portunus pelagicus* megalopae fed enriched *Artemia* containing different DHA/EPA ratios.

Treatments	Carapace length (mm)	Body dry weight (µg.ind.$^{-1}$)
T1	2.18 ±0.03^{a}	292.26 ±20.15^{ab}
T2	2.23 ±0.03^{a}	273.57 ±37.26^{a}
T3	2.34 ±0.05^{c}	354.02 ±18.63^{c}
T4	2.30 ±0.07^{bc}	322.87 ±22.31^{bc}
T5	2.25 ±0.06^{ab}	295.99 ±9.93^{ab}

Data are presented as mean ±SD (n=5). Values within a same column with different superscript letters are significantly different (P <0.05).

10.3.4 Zoea IV CHL/CL ratio and its relationship to MDS

Figure 10.2 shows the ratio of chela length to carapace length (CHL/CL) of zoea IV larvae and the percentage of MDS during metamorphosis moult of different treatments. A trend of increasing CHL/CL ratio was detected in larvae with the increase of DHA/EPA ratio from 0.23 to 1.36 for T1 to T4 treatments. However, CHL/CL ratio decreased significantly for T5 treatment when DHA/EPA ratio further increased to 1.47. Consequently, larvae from T4 treatment had the highest CHL/CL ratio while the lowest CHL/CL ratio was found in the T5

treatment. Larvae of T3 treatment had the lowest occurrence of MDS (29.45%) during the metamorphosis moult to megalopa while the significant higher MDS was observed in T1 (63.75%) and T2 treatment (66.47%). Further analysis demonstrated that the downer parabolic curves could be described for the relationship betwee the ratio of zoea IV CHL/CL and the percentage of MDS as well as the relationship between the dietary DHA/EPA ratio and the ratio of zoea IV CHL/CL (Figrure 10.3).

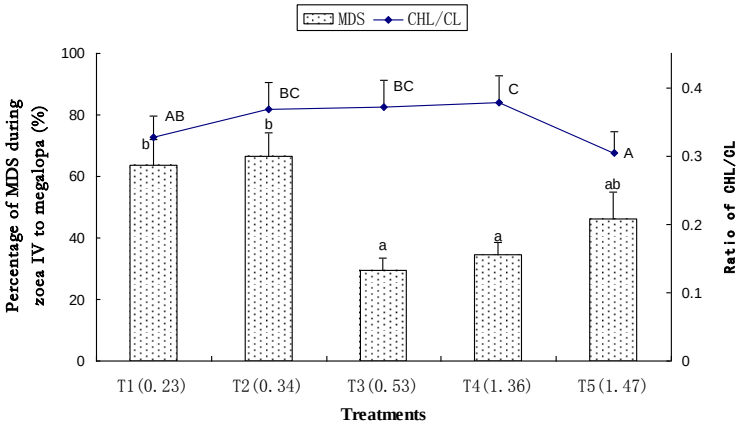

Figure 10.2 The chela length to carapace length (CHL/CL) ratio of zoea IV and percentage of moulting death syndrome (MDS) during metamorphosis of *Portunus pelagicus* newly hatched larvae fed enriched *Artemia* with different DHA/EPA ratios. Values in the bracket of x-axis indicate dietary DHA/EPA ratio. Data are presented as mean ±SD (n=5). Bars or diamonds with different letters on the top are significantly different (P <0.05).

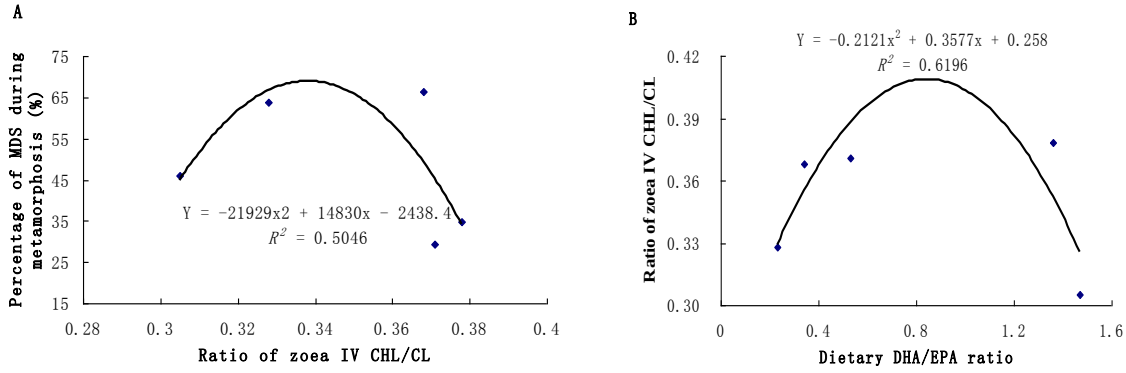

Figure 10.3 Regression between the ratio of zoea IV CHL/CL and the percentage of moulting death syndrome (MDS) during metamorphosis (A); dietary DHA/EPA ratios and the ratio of zoea IV CHL/CL (B) of *Portunus pelagicus* larvae fed enriched *Artemia* containing different DHA/EPA ratios

10.3.5 Fatty acid profile of newly moulted megalopae

The fatty acid profile of newly moulted megalopae showed that there were 7 dominant fatty acids that contributed to more than 5% of the total fatty acids, which were 16:0, 18:0, 16:1n7, 18:1n9, 18:2n6, 20:5n3 (EPA) and 22:6n3 (DHA) (Table 10.7). The percentage of these

dominant fatty acids was generally reflective of larval dietary fatty acid compositions (Table 10.3), particularly for the 16:0, 18:2n6 and DHA. For example, the DHA percentage of newly

Table 10.7 Fatty acid composition (% total fatty acids) of newly moulted *Portunus pelagicus* megalopae fed by enriched *Artemia* containing different DHA/EPA ratios

	T1	T2	T3	T4	T5
Fatty acid					
C14:0	1.31	1.05	0.99	1.31	1.04
C15:0	1.40	1.16	0.89	1.21	1.32
C16:0	19.44	17.26	17.08	15.75	15.75
C18:0	11.60	10.99	10.93	10.25	10.10
SFA	33.76	30.45	29.89	28.51	28.22
C14:1n7	0.87	0.84	0.74	0.81	0.87
C16:1n7	12.89	11.31	11.49	11.01	11.17
C16:1n5	0.70	3.19	1.41	0.92	2.25
C18:1n9	14.70	14.44	15.55	16.24	14.01
C18:1n7	4.24	4.17	4.74	5.39	4.67
C20:1n9	0.44	0.30	0.47	1.07	0.75
C20:1n7	0.63	0.75	0.84	1.36	1.19
MUFA	34.47	35.01	35.23	36.79	34.91
C18:2n6	6.83	6.25	6.79	7.04	6.10
C18:3n6	0.35	0.34	0.37	0.62	0.48
C18:3n3	2.06	2.39	2.48	2.24	1.86
C18:4n3	0.89	0.70	0.66	0.67	0.65
C20:4n6	1.69	1.66	1.86	1.82	1.77
C20:3n3	0.69	0.88	0.99	1.29	0.94
C20:4n3	0.21	0.25	0.27	0.22	0.58
C20:5n3	7.90	8.17	8.53	9.00	8.28
C22:2n6	0.00	0.00	0.00	0.00	0.00
C22:5n3	0.63	0.58	0.63	0.57	0.80
C22:6n3	3.71	4.34	4.67	5.76	6.20
∑PUFA (≥18:2n)	24.95	25.56	27.26	29.23	27.66
∑HUFA (≥20:3n)	14.83	15.88	16.96	18.67	18.57
∑n-3PUFA	16.09	17.31	18.23	19.75	19.30
∑n-6PUFA	8.86	8.25	9.02	9.48	8.35
n-3/n-6	1.82	2.10	2.02	2.08	2.31
DHA/EPA	0.47	0.53	0.55	0.64	0.75
DHA+EPA	11.61	12.51	13.21	14.76	14.48

moulted megalopae increased significantly from T1 (3.71%) to T5 (6.20%). A linear correlation was detected between dietary DHA/EPA ratio and megalopal DHA/EPA ratio (Fig. 10.4A, n=5, $P<0.01$). A similar linear relationship was also shown between dietary DHA percentage (% total fatty acids) and DHA percentage of newly moulted megalopa (Figure 10.4B, n=5, $P<0.01$).

Although dietary EPA decreased substantially in *Artemia* enriched with various emulsions from T1 to T5 (reduced from 16.34% to 8.87%; Table 10.3), there was no concurrent decline in EPA noted in the megalopea. Newly moulted megalopae generally contained lower levels of 18:1n9, 18:3n3 and EPA as well as DHA/EPA ratio, but higher 18:0, 16:1n7 and 20:4n6 when compared to their respective diets.

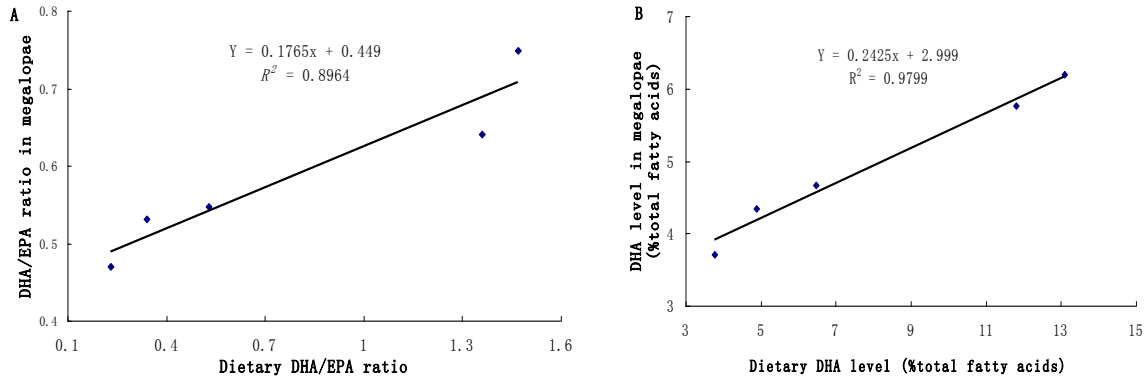

Figure 10.4 Linear regression of A) dietary DHA/EPA ratio and larval DHA/EPA ratio; and B) dietary DHA percentage (% total fatty acids) and DHA percentage of newly moulted *Portunus pelagicus* megalopae.

When relative retention ratio of a fatty acid is defined as the percentage of a specific fatty acid incorporated in the body of megalopae was divided by the percentage of the same fatty acid incorporated in their diet (enriched *Artemia*), DHA showed a decreasing trend with increased dietary DHA level while relative retention ratio of EPA and ARA general showed an increasing trend. Among the five PUFAs, 18:3n-3 had the lowest relative retention ratio of between 0.33 to 0.45, while the highest relative retention ratio was found for ARA (> 1.20 for all treatments), signifying relative preservation of ARA in larval *P. pelegicus* at concentrations that were greater than it presented in their respective diets (Table 10.8).

Table 10.8 The relative retention ratios (% of a specific fatty acid incorporated in megalopae/ % of the same fatty acid incorporated in their enriched *Artemia* diet) of major PUFAs in newly moulted *Portunus pelagicus* megalopae fed with enriched *Artemia* containing different DHA/EPA ratios from newly hatched larvae.

PUFA	T1	T2	T3	T4	T5
C18:2n6	0.84	0.79	0.85	0.93	0.74
C18:3n3	0.33	0.41	0.45	0.38	0.39
C20:4n6	1.21	1.30	1.57	1.67	1.79
C20:5n3	0.48	0.57	0.70	1.05	0.93
C22:6n3	0.99	0.89	0.72	0.49	0.47

10.4 Discussion

No previous study has used a dose-response design to examine the effect of dietary DHA/EPA ratio on the occurrence of MDS and larval survival, development and growth of a

portunid crab. Microbound or microencapsulated diets are generally inadequate for supporting good survival and normal development through larval ontogeny of portunid crabs, particularly for early zoeal stages (Jones, 1998; Holme et al., 2006). Use of formulated diets is therefore not a feasible option for lipid nutrition studies for zoeal larvae of portunid crabs (Holme et al., 2006). Consequently, the present study utilized enriched *Artemia* for the study and the results showed that during enrichment, DHA and EPA of self-prepared lipid emulsions was readily absorbed/assimilated by the *Artemia*. Therefore, by careful manipulation of DHA/EPA ratio in lipid emulsions, graded DHA/EPA ratio could be achieved in *Artemia* enriched with different emulsions. These enriched *Aretmia* with different DHA/EPA ratios can then being used to feed crab larvae to access their dietary DHA/EPA requirement. Although both DHA and EPA in enrichment emulsions were readily incorporated into enriched *Artemia*, the EPA retention was higher than that of DHA in enriched *Artemia*. This led to comparatively lower DHA/EPA ratio of enriched *Artemia* than that of the corresponding emulsion used to enrich them. The lower DHA/EPA ratio in the enriched *Artemia* is likely due to metabolic conversion of DHA to EPA by the *Artemia* (Han et al., 2001). Overall, the results of the present study demonstrated that *Artemia* enrichment based on a dose-response design is an appropriate approach for investigating effects of dietary DHA/EPA ratio on performance of crustacean larvae.

Survival data showed that for all treatments, mass mortality (30-50%) occurred during metamorphosis from zoea IV to megalopae and a high percentage of such mortalities could clearly be attributed to moulting death syndrome (MDS), i.e., mortality due to inability of the larvae to completely shed their old exoskeleton (Hamasaki et al., 2002a; Wu et al., 2007c). MDS is a common phenomenon found during metamorphosis of brachyuran crab larvae and mass mortalities due to MDS have been reported for various portunid crabs targeted for aquaculture, including the mud crabs, *Scylla serrata* (Hamasaki et al., 2002a), *S. paramamosain* (Takano et al., 2004), *S. tranquebarica* (Baylon, 2009) and the swimming crab, *P. trituberculatus* (Arai et al., 2004). More recent studies have revealed that an imbalance of dietary fatty acids as a major contributor to MDS in larval mud crab (Hamasaki et al., 2002b; Suprayudi et al., 2004a). The present results further demonstrated that dietary DHA/EPA ratio is clearly linked to the occurrence of MDS for *P. pelagicus* larvae.

In the mud crab *S. serrata*, excessive dietary n-3HUFA (DHA and EPA) has been linked to the appearance of abnormal advanced morphological features in the last zoeal stage (zoea V), i.e. showing morphological features of megalopae, such as large rudimentary chelipeds and plumose setae on pleopods (Hamasaki et al., 2002b; Suprayudi et al., 2004a). Hamasaki et al. (2002a) defined such phenomenon as ‘hyper-morphogenesis’ and reported that *S. serrata* zoea

V larvae with hyper-morphogenesis were often led to MDS. These authors have proposed that the ratio of chela length to carapace length (CHL/CL) in zoea V larvae of *S. serrata* can be used as a criterion for indicating the degree of hyper-morphogenesis. In their experiments, a positive correlation between dietary n-3 HUFA (particularly DHA) level and the occurrence of MDS was identified while both dietary n-3 HUFA level and the occurrence of MDS were positively correlated to the ratio of CHL/CL of zoea V larvae, suggesting that CHL/CL ratio is a good indicator for MDS (Hamasaki et al., 2002a,b; Suprayudi et al., 2002; Suprayudi et al., 2004a; Dan and Hamasaki, 2011). It was subsequently reported that for *P. trituberculatus*, if the CHL/CL ratio of a final stage zoeal larva was higher than 0.37, MDS was most likely to occur during metamorphosis (Arai et al., 2004; Dan and Hamasaki, 2011). Interestingly, in the current study, different results were shown because, in general, both dietary DHA/EPA ratio higher or lower than 1.36, resulted in decreases (someimtes significantly) CHL/CL ratios of *P. pelagicus* zoea IV larvae. Moreover, in contrast to what was reported for *S. serrata* and *P. trituberculatus* (Hamasaki et al., 2002a,b; Arai et al., 2004; Dan and Hamasaki, 2011), lower CHL/CL ratios did not result in signficantly reduced MDS, but they generally led to signficant increase in MDS. Understandably, dietary DHA deficiency is likely to affect larval growth and hence lead to lower CHL/CL ratio in zoea IV larvae. Our results suggested that excessive dietary DHA could also depress larval growth and lead to a reduced ratio of CHL/CL; and in both cases, increased incidences of MDS as the result. In fact, an overall significant negative correlation was identified between the CHL/CL ratio and the percentage of MDS for *P. pelagicus* zoea IV, which contradict the previous results reported for *S. serrata* and *P. trituberculatus* (Hamasaki et al., 2002a; Arai et al., 2004; Dan and Hamasaki, 2011). Such obvious discrepancy is difficult to explain, but possible indicating species-specific differences.

The present results clearly showed that an elevated dietary DHA/EPA ratio could accelerate larval development and shorten intermoult period for *P. pelagicus* larvae. This phenomenon is similar to marine crustacean larvae (Xu et al., 1993; Xu et al., 1994b; Suprayudi et al., 2004a,b; Sui et al., 2007). The internal mechanism maybe explained by the ecdysis modulation. Moulting of crustaceans and insects is regulated by ecdysteroids (Huberman, 2000; Subramoniam, 2000), which is secreted by Y-organ in the anterior branchial chamber of decapod crustacean and released into the haemolymph (Huberman, 2000). Ecdysteroids circulating in the haemolymph are converted to 20-hydroxyecdysone (20-E) or related compounds by peripheral tissues, such as the hindgut, ganglia, eyestalk and hepatopancreas (Mykles, 2011). However, in the tick *Ornithodoros moubata*, ecdyson was metabolized into three different compounds in embryos and larvae, and one of them conjugated with a C-22 fatty acid, which suggests that a C-22 fatty acids like DHA may possibly regulate moulting by the

modulation of ecdysteroid metabolism for insects (Dotson et al., 1993). Since both crustacean and insects are from the arthropod family and ecdysteroid metabolism is involved in their moulting regulation. Therefore, similar conjugation mechanisms may exist in the modulation of ecdysteroid metabolism.

Previous studies have demonstrated that DHA has a superior nutritional value when compared to EPA in terms of crustacean larval and juvenile growth (Xu et al., 1993; Merican and Shim, 1996; Sui et al., 2007). In vertebrates DHA is indispensable in the development of neural tissues, such as brain and eye retina (Bell et al., 1995; Innis, 2007), while marine crustacean species generally contain very high DHA levels in their tissue membranes (Teshima, 1998; Sheen and Wu, 2002) for the maintenance of cell membrane permeability, fluidity and resistance to osmotic shock (Palacios et al., 2004; Sui et al., 2007). Hence marine crustaceans generally have higher DHA requirements when compared to brackish or freshwater crustacean species (D'Abramo and Sheen, 1993; Sui et al., 2007). Although several previous reports have identified the importance of dietary DHA for the larvae of other crab species (Suprayudi et al., 2004a; Nghia et al., 2007; Sui et al., 2007), there is no published information on the dietary DHA/EPA ratio requirement for *P. pelagicus* larvae. While this study demonstrated that dietary DHA has very important role in the development, moulting and growth for *P. pelagicus* larvae, the function of EPA is unclear from the current study. This is because the lowest dietary EPA level was 8.59% of total fatty acids in this study, together with the potential retro-conversion of DHA to EPA in enriched *Artemia* and crustacean larvae (Merican and Shim, 1997b; Han et al., 2001; Sui et al., 2007). Dietary EPA input into larval crustaceans had an impact on larval survival and growth for the mud crab *S. serrata* (Suprayudi et al., 2004a), and influenced larval survival in the swimming crab *P. trituberculatus* (Takeuchi et al., 1999b).

The percentages and ratios of the major fatty acids in the newly moulted megalopae were positively related to the same fatty acid parameter within the enriched *Artemia*, especially for 16:0, 18:2n6 and DHA. The highly relative retention ratio of DHA was found in the larvae from treatment 1 to 3, which suggested *P. pelagicus* larvae are able to preferentially assimilate and accumulate DHA from their diets when the dietary DHA content is low and perhaps inadequate. Consequently, when dietary DHA level is above the optimal level, the relative retention ratio of DHA decrease with the increasing dietary DHA. However some of this lipid biochemistry may be explained the reduced inclusion of EPA stimulating retro-conversion of DHA to EPA to maintain a baseline concentration, as occurred in this study (Kanazawa et al., 1979a; Merican and Shim, 1997). Among the five PUFAs, 18:3n3 had the lower relative retention ratio, which suggested *P. pelagicus* larvae had low 18:3n3 requirements. As the requirement of dietary

18:3n3 is dependent on the dietary levels of DHA and EPA (Merican and Shim, 1997; Glencross and Smith, 1999; González-Félix et al., 2003), the low requirement and relative retention ratio of 18:3n3 may indicate the dietary n-3 HUFAs is nearly sufficient for the survival and growth of *P. pelagicus* larvae.

The fatty acid composition of newly hatched larvae or embryo from wild females can generally provide valuable information on the fatty acid requirement of crustacean larvae (Phleger et al., 2001; Limbourn and Nichols, 2009). Compared to fatty acid composition of the embryos of wild *P. pelagicus* (ARA=2.97%, EPA=9.94%, DHA=7.41%), megalopae from treatments 4 and 5 contained similar levels of DHA and EPA , but lower ARA content, which indicated the dietary ARA level should be improved in the future for hatchery culture of *P. pelagicus.* Future studies to improve the fatty acid composition of emulsions, enriched *Artemia* and formulated diets, will assist in the provision of an optimized HUFA profile for *P. pelagicus* larvae, whicha may lead to the improvement of seed production of *P. pelagicus*.

10.5 Conclusion

The present results suggested that dietary DHA/EPA ratio has significant effects on survival, development, growth, occurrence of MDS and fatty acid composition of newly ed *P. pelagicus* megalopae. The results indicate that both low and high dietary DHA/EPA ratios in larval diets could negatively affect larval performance. The optimal dietary DHA/EPA ratio was identified to be around 0.53 for *P. pelagicus* zoeal larvae. The results highlight the importance of dietary DHA/EPA ratio for larval survival and development of *P. pelagicus* and provide knowledge basis for the development of formulated diets for seed production of *P. pelagicus* in the future. The mechanisms underlying observed effects of dietary DHA/EPA ratio on larval performance of *P. pelagicus* larvae warrant further research.

Chapter 11

The effects of dietary DHA/EPA ratios on survival, growth and fatty acid composition of early stage phyllosoma of ornate rock lobster, *Panulirus ornatus*

11.1 Introduction

Highly unsaturated fatty acids (HUFA) with carbon chain lengths of ≥ 20 containing ≥ 3 double bonds (Tocher, 2003) are considered to be an important fatty acid family for the survival and growth of many crustacean species (Glencross, 2009). This is primarily because many crustaceans have limited capacity to synthesize HUFAs *de novo* and therefore require a dietary source (Kanazawa et al., 1979a). Generally, freshwater crustaceans have a lower HUFA requirement compared to marine crustaceans, this is thought to be reflective of a low HUFA presence in freshwater systems; similar to terrestrial environments, significant terrestrial input into freshwater bodies and the adaptation of freshwater crustaceans to this environment (D'Abramo and Sheen, 1993; D'Abramo, 1998). The most biologically significant HUFAs include 20:4n6 (ARA), 20:5n3 (EPA) and 22:6n3 (DHA); they are considered to have a higher nutritional value for marine crustaceans when compared to 18-carbon poly-unsaturated fatty acids (C18-PUFA) (Xu et al., 1993; Xu et al., 1994a; Merican and Shim, 1996; Glencross and Smith, 2001). To date, many studies have focused on the nutritional effects of n-3 HUFAs for aquaculture crustacean species, particularly concentrating on the fatty acids EPA and DHA (Xu et al., 1993; Rees et al., 1994; Nghia et al., 2007; Sui et al., 2007; Wu et al., 2007c). These particular fatty acids are involved in many important physiological roles for crustaceans, such as the structural integrity of membrane phospholipids, precursors of biologically active eicosanoids and enhancement of neural development (Bell et al., 1986; Mourente and Tocher, 1992; Wouters et al., 2001). Many marine crustacean studies have demonstrated that diets deficient n-3 HUFA can result in slow growth rate, induce high mortality, prolonged inter period and reduced swimming activity, while similar results are also demonstrated with excess dietary HUFA (Takeuchi et al., 1999b; Suprayudi et al., 2004b; Dan and Hamasaki, 2011).

It is known that DHA and EPA perform different physiological functions in crustacean larvae. For the shrimp *Penaeus monodon* (Merican and Shim, 1997) and the crabs *Portunus trituberculatus* (Takeuchi et al., 1999a) and *Eurypanopeus depressus* (Levine and Sulkin, 1984) DHA is implicated in larval moulting and growth, while EPA content has an impact on survival. However, there have only been limited efforts made to address species specific EPA and DHA

quantitative requirements (Merican and Shim, 1996; Takeuchi et al., 1999b; Glencross and Smith, 2001; González-Félix et al., 2003). There are a number of marine crustacean larval studies that have demonstrated a greater DHA requirement compared to EPA, it is likely that the proportions of DHA and EPA have a significant impact on larval survival and growth (Takeuchi et al., 1999b; Sui et al., 2007). There is a considerable impediment to the provision and uptake of dietary DHA to marine crustaceans especially their preponderance to readily retro-convert DHA to EPA in both live feeds, such as *Artemia* (Evjemo et al., 2001; Han et al., 2001), and cultured species such as *Macrobrachium rosenbergii* (D'Abramo and Sheen, 1993) and *P. monodon* (Merican and Shim, 1996a). Therefore the dietary DHA/EPA ratio that is delivered to marine crustaceans is critical (Merican and Shim, 1997b; Mourente and Rodríguez, 1997; Sui et al., 2007) because if inappropriate, as demonstrated in the black tiger prawn, *P. monodon* (Glencross et al., 2002) and the Chinese mitten crab, *Eriocheir sinensis* (Sui et al., 2007), it can result in negative lipid physiology impacting growth and survival.

The spiny rock lobster, *Panulirus ornatus*, is widely distributed throughout the Indo-West Pacific region, particularly in the tropical and subtropical waters of the region, including the Torres straits and Northeastern Australia (Ye, 2008; Ye and Dennis, 2009). Although global demand for *P. ornatus* product is increasing at 15% per annum (Jones et al., 2007; Smith et al., 2009a), the fishery for wild *P. ornatus* is fully exploited with landing yields plateauing during the past 10 years despite increased fishing effort (FAO, 2005; Hung and Tuan, 2009). Of all the spiny lobster aquaculture candidate, *P. ornatus* is recognized as the prime species due to its short planktonic phyllosoma phase (< 6 months), rapid growth (attaining 1 kg after 18 months post-settlement) and delicate flavor (Johnston et al., 2008; Smith et al., 2009a). It is likely that sustainable production of this species will only be possible through closed-life cycle aquaculture production and the development of commercially robust hatchery technologies (Priyambodo and Sarifin, 2009; Smith et al., 2009a).

The larval cycle of *P. ornatus*, is complex, consisting of eleven distinct morphological pelagic planktonic phyllosoma stages, numerous intermediate instars, and a transitional planktonic post-larval puerulus stage (Smith et al., 2009a). The current bottleneck in the aquaculture production for all Palinurid lobster species is the inability to rear large quantities of larvae from egg through the multiple planktonic phyllosoma stages to puerulus and the benthic juvenile stages. Successful larval production has been achieved for *P. ornatus* in Australia, but only at small scale (Smith et al., 2009a; O'Sullivan, 2010). High mortalities during culture of the phyllosoma stages to puerulus, and the first instar juvenile have been linked to a number of culture inadequacies, including poor larval health, a lack of understanding of phyllosoma

physiological requirements and insufficient larval nutrition (Bourne et al., 2007; Johnston et al., 2008; O'Sullivan, 2010). Larval nutrition, particular fatty acid nutrition, has also been suggested as one of major contributor to incidents of unsuccessful survival and metamorphosis of this species (Wu et al., 2012). Previous research has shown that dietary DHA could promote phyllosoma growth for spiny lobsters *Panulirus cygnus* (Liddy et al., 2005), and *Jasus edwardsii* (Nelson et al., 2004), the benefits of dietary DHA supplementation for *P. ornatus* is unknown.

The previous chapter has showed that DHA is preferentially conserved during starvation of newly hatched *P. ornatus* phyllosoma, indicating the important physiological functions of DHA during the early ontogeny of this species (Chapter 7). However, to date there is no published information on the optimal DHA/EPA ratio for *P. ornatus* phyllosoma. This will potentially hinder the development of hatchery protocols by not optimizing larval condition and stifle the cost-effective formulation of larval feeds for *P. ornatus*. Currently, the primary food source for early-stage phyllosoma rearing is *Artemia* nauplii and instar 2 nauplii, there is no cost effective diet that supports comparative survival at this stage of development (Johnston et al., 2008; Wu et al., 2012). *Artemia* nauplii generally lack HUFAs, however there are a number of enrichment products that can be used to increase the *Artemia* HUFA content, and subsequently the HUFA content of phyllosoma (Ritar et al., 2004; Liddy et al., 2005). This current study was designed and carried out to assess the effects of dietary DHA/EPA ratios on survival, development, growth and fatty acid composition for the early-stage *P. ornatus* phyllosoma.

11.2 Materials and methods

11.2.1 Broodstock source and maintenance

Broodstock source and husbandry were described in Chapter 5 (Section5.2.1).

11.2.2 Artemia enrichment

Five groups of instar 2 *Artemia* were enriched with different oil emulsions from a range of triacyglycerol-rich (TAG) oil sources to provide a graduated range of DHA/EPA. The fatty acid composition and respective DHA/EPA ratios of the oils are shown in Table 10.1. The preparation of each oil emulsion was conducted using the direct method (Suprayudi et al., 2004a). Briefly, 0.5 g of each oil was emulsified with 0.1 g of raw egg yolk and 100 ml distilled water using an Ultra-turrax T25 homogenizer (IKA Works Inc, Wilmington, North Carolina, USA) operating at 10, 000 rpm for 2 min. They were then stored in bottles and kept in a

refrigerator at 4°C. Specific *Artemia* cysts (GSL strain), containing very low HUFA (EPA: 0.7% of total fatty acids, DHA: 0%), was provided by the Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Belgium. To obtain newly hatched *Artemia* nauplii for the experiment, *Artemia* cysts were hatched daily in a 2 L conical plastic bottle at 28 ±1 ℃ in filtered seawater (33-36‰). Under these environmental conditions *Artemia* hatch after 18-20 h, routine harvesting occurred at 24 h, followed by a disinfection treatment (400mg L^{-1} formalin bath for 1 h), rinse with freshwater and allocation into 400 mL vessels in seawater at a density of 200-250 *Artemia* mL^{-1}. Post disinfection sufficient time had passed for *Artemia* to develop to instar 2; their first exogenous feeding stage. The emulsified oil solution was added once only at the commencement of the enrichment period at a rate of 0.6 g oil emulsion per liter of enrichment medium (Sui et al., 2007). During enrichment, temperature and salinity were maintained at 28 °C and 33‰, respectively. After 24h enrichment the *Artemia* were then harvested and rinsed with UV treated freshwater to remove all external traces of the emulsion before suspension in 200ml of sterilised seawater and some of enriched *Artemia* were dispensed to *P. ornatus* phyllosoma immediately. The remaining enriched *Artemia* were immediately stored at 4℃ in a refrigerator to minimize fatty acid catabolism (Merchie, 1996), and were dispensed to the phyllosoma in a second feed 14 hr later. Enriched *Artemia* (ca.1-2 g/sample) in each treatment were sampled three times during the larval culture period for total lipids and fatty acid analysis. All samples were stored at -70℃ before analysis.

11.2.3 Experimental design and setup

On the day of hatching active photopositive phyllosoma were removed from the surface of the hatch vessel and transferred into a 20 L container. Phyllosoma required for stocking into culture or experimental vessels were treated with 25mg L^{-1} formalin for 30 min to reduce the transfer of bacterial pathogens, they were rinsed with clean sterilised seawater (ozonated) and individually counted and stocked into the experimental vessels. The experimental culture system was comprised of 20 x 250 ml plankton kriesels, they were stocked at 25 phyllosoma per vessel (quadruplicate). All experimental vessels were supplied with flow-through water equivalent to 300% water exchanges per hour. Water was introduced on the top and against the curved side of the vessel, causing a circumferential water flow in the vessel, this provided minor levels of turbulence that was sufficient to maintain phyllosoma and feed in suspension. Seawater was pumped from an offshore seawater line into two storage dams where it was settled for 1- 4 days followed by sequential passage through bag filters (100, 50 and 10 µm), foam fractionation, ozonation (700-750 mV), UV (254 nm) and particulate/carbon filtration to 1µm. Seawater within this system was delivered to the point of usage within 20 minutes of processing. Salinity and water temperature were maintained at 32‰ and 28ºC, respectively, during the

period of the experiment. Light was provided by fluorescent tubes providing 420nm wavelength light with a photoperiod of 14 h light: 10 h dark and light intensity of 0.2 μE $s^{-1}m^{-2}$.

The culture experiment was conducted using 5 enrichment treatments designated 1-5 (Treatment 1 to 5, T1-T5), each treatment with four replicates, utilizing 25 phyllosoma per replicate. Prior to stocking the experiment three samples of newly hatched Stage I phyllosoma (50 phyllosoma/sample) were collected, rinsed with 0.5 M ammonium bicarbonate to remove trace of salt before being blotted dry on a filter paper. They were then oven-dried at 60 ℃ for 24 h before being weighed to determine the average dry weight of individual newly hatched Stage I phyllosoma. Phyllosoma stocked in the experiment were individually counted and randomly allocated to each of the treatments. *Artemia* fed to the phyllosoma were enriched with five different DHA/EPA oils (Table 10.1) that provided a graduated DHA/EPA ratio in the enriched *Artemia* from 0.19-1.64 (Table 11.1). *Artemia* were fed to the phyllosoma culture vessels at a rate of 2 ind. mL^{-1} twice daily for all treatments. Each morning, surviving phyllosoma were counted and staged before being transferred to a new culture vessel, while old *Artemia* were removed. Fresh enriched *Artemia* were then fed to each vessel. Mortality and ing of larvae in each vessel were checked again 12 h later (i.e. every 12 h). By doing this, the average development time (intermoult period) of larval instars could be estimated more accurately. When any newly moulted Stage III phyllosoma (Smith et al., 2009) were found, it was removed for the later measurement and lipid analysis. The experiment terminated when all larvae either moulted to Stage III phyllosoma or died.

11.2.4 Data collection

Phyllosoma survival, development and specific growth rate (SGR)

Phyllosoma survival was expressed as cumulative survival to a particular larval stage, which was calculated by dividing the total number of phyllosoma that ed successfully to a particular stage with the initial number of phyllosoma stocked into each culture vessel (i.e. 25). Similarly, phyllosoma development was expressed as the mean cumulative development time required to reach a particular stage from newly hatched Stage I animals. The specific growth rate (SGR, % day^{-1}) was calculated for each replicate using the following formulae:

$SGR = 100 \times (\ln Wt - \ln Wo)/D$

where Wo is the mean dry weight of newly hatched Stage I phyllosoma while Wt is the mean dry weight of newly moulted Stage III instar 1 phyllosoma; D is the cumulative development time (in days) from Stage I to Stage III instar 1 phyllosoma.

Total body length and dry weight of newly moulted stage III phyllosoma

The body length (BL) and dry weight (DW) of each newly moulted Stage III instar 1 phyllosoma were measured. Phyllosoma BL was measured on a Leica MZ16A microscope (precision=0.01mm, Leica Microsystems, Wetzlar, Germany). BL was defined as the distance between from the anterior margin of the cephalic shield to the posterior edge of the pleon (Smith et al., 2009). The phyllosoma was then briefly rinsed with 0.5 M ammonium bicarbonate to remove traces of salts, and blotted dry on a filter paper. A total number of 3-5 individual Stage III instar 1 phyllosoma were randomly sampled from each replicate and then oven dried at 60 ℃ for 24 h. The phyllosoma dry weight was subsequently weighed using a Cahn C-33 micro-balance (precision=1μg, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). Individual mean DW of Stage III instar 1 phyllosoma was calculated by the division of phyllosoma number. The remaining samples were kept in a -70 ℃ freezer for the later fatty acid analysis.

11.2.5 Total lipids and fatty acid analysis

See section 4.2.4.

11.2.6 Statistical analysis

Homogeneity of variance of all data was first tested with Levene's test. Where necessary, arcsine-square root or logarithmic transformation was performed prior to analysis. One-way ANOVA was used to determine significant differences for various parameters among treatments. If any significant difference was detected, Tukey's multiple range test was used as the means separation procedure. Pearson-test was used to determine statistically significant relationship between dietary DHA/EPA ratio (or DHA level) and different biological parameters measured. The relationships between dietary DHA/EPA ratio (or DHA level) and different biological parameters were further evaluated with regression analysis. When a normal distribution and/or homogeneity of the variances were not achieved, data were subjected to the Kruskal-Wallis H non-parametric test followed by the Games-Howell non-parametric multiple comparison test. $P < 0.05$ was regarded as the statistically significant level. All statistics were performed using SPSS package (version 12.0).

11.3 Results

11.3.1 Total lipid and fatty acid profile of enriched Artemia

The total lipid and fatty acid compositions of newly hatched *Artemia* and *Artemia* enriched with five emulsion oils containing different DHA/EPA ratios are presented in Table 11.1. After

24 h enrichment, all groups of enriched *Artemia* contained higher content of total lipids and highly unsaturated fatty acids (HUFAs) compared to newly hatched *Artemia*. *Artemia*

Table 11.1 Total lipid content (% dry weight) and fatty acid composition (% total fatty acids) of newly hatched *Artemia* nauplii enriched with emulsion containing different DHA/EPA ratios.

	Newly hatched *Artemia*	*Artemia* enriched with oil 1	*Artemia* enriched with oil 2	*Artemia* enriched with oil 3	*Artemia* enriched with oil 4	*Artemia* enriched with oil 5
Total lipid	15.82	20.48	20.11	19.91	19.65	20.57
Fatty acid						
C14:0	0.50	1.01	1.09	0.94	0.87	1.11
C15:0	0.67	0.84	0.96	0.74	0.74	0.85
C16:0	9.90	10.98	10.76	11.02	10.99	11.67
C18:0	4.76	5.14	5.08	4.89	6.47	5.63
SFA	16.29	17.97	17.88	17.59	19.07	19.25
C14:1n7	1.06	0.40	0.47	0.42	0.47	0.38
C16:1n7	2.75	3.68	4.08	3.69	3.03	2.93
C16:1n5	0.82	0.99	1.01	0.89	0.97	1.07
C18:1n9	16.48	25.96	25.15	25.01	23.93	23.67
C18:1n7	5.21	7.57	7.46	6.66	6.72	7.76
C20:1n9	0.57	1.00	0.84	0.48	0.53	0.50
C20:1n7	0.55	1.19	1.02	1.03	1.03	1.31
MUFA	27.44	40.80	40.04	38.18	36.67	37.62
C16:2n4	1.25	1.26	1.41	1.22	0.97	1.05
C16:3n4	0.70	0.76	0.78	0.72	0.70	0.80
C18:2n6	6.22	6.79	6.35	6.40	6.37	6.55
C18:3n6	0.84	0.25	0.27	0.25	0.38	0.25
C18:3n3	31.42	12.38	12.35	12.13	12.99	12.00
C18:4n3	7.58	2.50	1.67	2.32	2.76	2.57
C20:4n6	0.16	1.04	0.81	0.98	1.04	0.83
C20:3n3	1.42	0.70	0.42	0.51	0.83	0.54
C20:4n3	1.40	0.69	0.35	0.47	0.77	0.51
C20:5n3	0.71	9.08	7.46	6.17	5.30	4.24
C22:5n3	0.00	0.15	0.34	0.71	0.80	0.89
C22:6n3	0.00	1.74	3.07	4.05	5.59	6.94
∑PUFA (≥18:2n)	49.72	35.31	33.09	33.99	36.83	35.32
∑HUFA (≥20:3n)	3.68	13.39	12.44	12.89	14.34	13.95
∑n-3PUFA	42.52	27.23	25.66	26.36	29.04	27.69
∑n-6PUFA	7.21	8.08	7.42	7.63	7.79	7.63
n-3/n-6	5.90	3.38	3.46	3.46	3.73	3.63
DHA/EPA	**--**	**0.19**	**0.41**	**0.66**	**1.06**	**1.64**
DHA+EPA	0.71	10.82	10.52	10.22	10.89	11.18

enrichment increased total lipid contents and percentages of highly unsaturated fatty acids (HUFAs), particularly for EPA (20:5n-3) and DHA (22:6n-3). Total quantitative lipid content of

enriched *Artemia* was similar for all five treatments. While all enriched *Artemia* contained similar levels of saturated fatty acid (SFA), a decreasing trend was found on 18:1n-9 and total monounsaturated fatty acid (MUFA) of *Artemia* enriched from the oil 1 to oil 5 (Table 11.1). For the polyunsaturated fatty acids (PUFA), all enriched *Artemia* generally had similar contents of 18:2n-6, 18:3n-3 and 20:4n-6 (ARA). As expected, the increasing 22:6n-3 percentage and decreasing 20:5n-3 percentage were found in the *Artemia* enriched from oil 1 to 5, which lead to a significant increase of DHA/EPA ratio in the T5 enriched *Artemia.* Although the five types of enriched *Artemia* differed in DHA/EPA ratios, they contained similar quantitative amounts of total poly-unsaturated fatty acids (PUFA), total HUFA, DHA+EPA. The five experimental emulsions oils with elevated DHA/EPA ratio resulted in increasing DHA/EPA ratio of enriched *Artemia* and a significant positive correlation was detected between DHA/EPA ratios of enriched *Artemia* and DHA/EPA ratio of the emulsion oils (r=0.998, P <0.01).

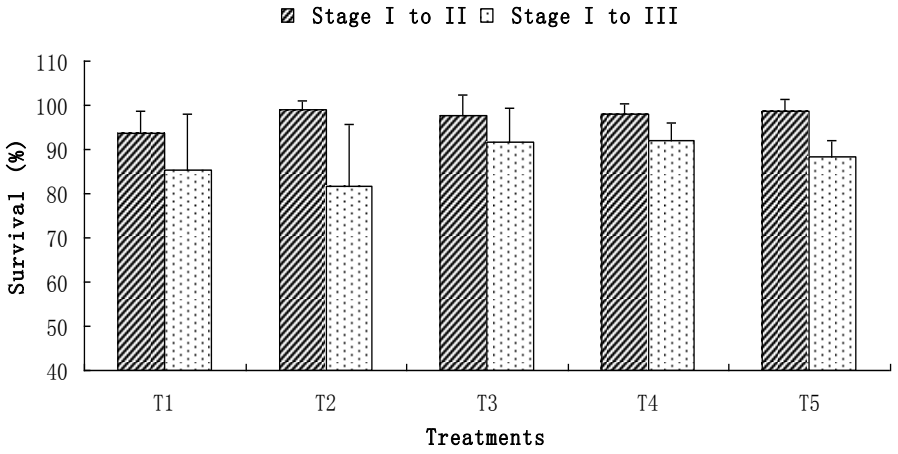

Figure 11.1 Cumulative survival (%) of *Panulirus ornatus* phyllosoma from Stage I to II and Stage I to III fed enriched *Artemia* containing different DHA/EPA ratios. Data are presented as mean ±SD (n=4).

11.3.2 Survival, development and growth

For all treatments there were no significant differences in survivals from Stage I to Stage II (>93%) or from Stage I to III instar 1 (low of 81.2% in T2 to high of 92.1% in T4) (P > 0.05, Figure 11.1). Significant differences in larval development time were detected for both Stage I to II and Stage I to III instar 1. In the first development phase (Stage I to II), phyllosoma in treatments T2 to T5 consistently had the shortest development time (P < 0.05) while T1 had the longest development time. When the total development duration was examined (Stage I to III instar 1), phyllosoma from T5 had the shortest development time, however not significantly less than in T2-T4 (P <0.05, Table 11.2). Among treatments, T5 achieved the highest specific

growth rate (SGR, % day^{-1}) of dry weight (DW) over the period of Stage I to III instar 1, while T1 showed the lowest SGR (Figure 11.2). There was a positive correlation between the dietary DHA/EPA ratio of enriched *Artemia* and SGR, resulting in a significant non-linear regression equation (Figure 11.3, n=20, P <0.01).

Table 11.2 Development time (days) from Stage I to II and Stage I to III in *Panulirus ornatus* phyllosoma fed enriched *Artemia* containing different DHA/EPA ratios.

Treatments	Stage I to II (days)	Stage I to III instar 1 (days)
T1	8.10±0.10^{c}	15.96±0.58^{b}
T2	7.72±0.09^{a}	15.76±0.71^{ab}
T3	7.75±0.13^{a}	15.35±0.26^{ab}
T4	7.73±0.06^{a}	15.39±0.07^{ab}
T5	7.91±0.10^{b}	15.16±0.14^{a}

Data are presented as mean±SD (n=4). Values within a column having different superscript letter are significantly different (P <0.05).

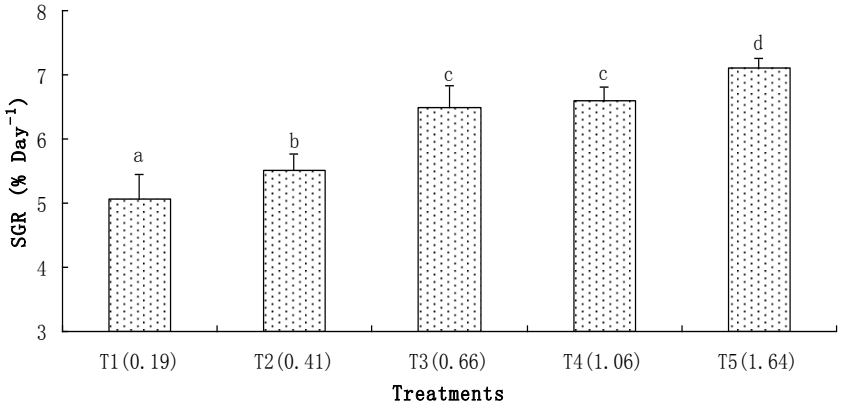

Figure 11.2 The specific growth rates (SGR, % day^{-1}) of *Panulirus ornatus* phyllosoma from Stage I to III fed enriched *Artemia* containing different DHA/EPA ratios. SGRs are calculated based on dry weight of newly moulted Stage III instar 1 phyllosoma. The value in the bracket under the x-axis indicates its dietary DHA/EPA ratio for each treatment. Data are presented as mean±SD (n=4). Bars with different letters on the top are significantly different (P < 0.05).

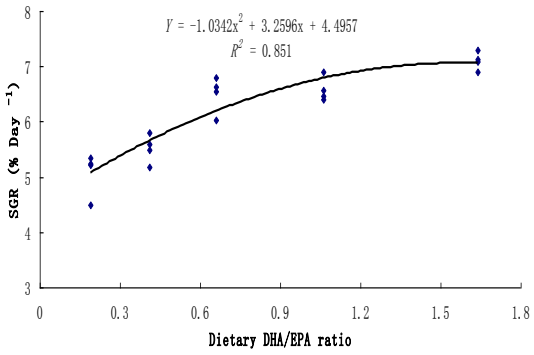

Figure 11.3 Regression analysis between dietary DHA/EPA ratio and specific growth rate (SGR) of *Panulirus ornatus* phyllosoma from Stage I to III.

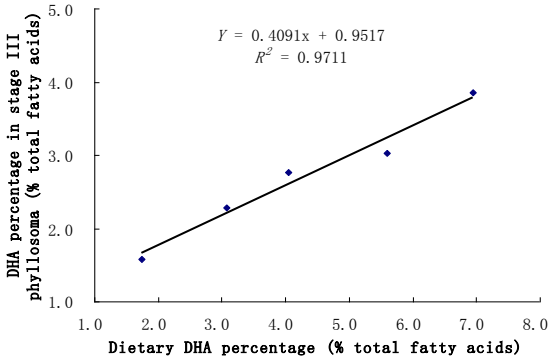

Figure 11.4 Regression analysis between dietary DHA percentage (% total fatty acids) and larval DHA percentage (% total fatty acids) of newly moulted Stage III *Panulirus ornatus* phyllosoma.

Table 11.3 Total body length and body dry weight of newly metamorphosed Stage III *Panulirus ornatus* phyllosoma fed enriched *Artemia* containing different DHA/EPA ratios during the period from Stage I to Stage III.

Treatments	Body length (mm)	Body dry weight (μg.ind.$^{-1}$)
T1	2.26 ±0.05^{a}	102.83 ±3.53^{a}
T2	2.27 ±0.06^{a}	109.14 ±3.30^{b}
T3	2.36 ±0.01^{b}	124.33 ±5.46^{c}
T4	2.36 ±0.03^{b}	126.28 ±3.84^{c}
T5	2.42 ±0.02^{b}	134.54 ±4.34^{d}

Data are presented as mean ±SD (n=4). Values within a column having different superscript letter are significantly different (P <0.05).

11.3.3 Body length and dry weight of newly moulted stage III phyllosoma

There were significant differences in the BL and DW of newly metamorphosed Stage III instar 1 phyllosoma (Table 11.3, P <0.05). Phyllosoma from T3, T4 and T5 had significantly higher BL than those from T1 and T2, while the DW of T5 was significantly greater than from phyllosoma in T3 and T4, in turn these phyllosoma had significantly greater DW than those from T2, while those from T2 were significantly greater than phyllosoma from T1 (Table 11.3, P <0.05).

11.3.4 Fatty acid profile of newly moulted stage III phyllosoma

The qualitative fatty acid profile of newly moulted Stage III instar 1 phyllosoma contained 6 fatty acids that contributed more than 5% to the total fatty acid profile, these included 16:0, 18:0, 16:1n7, 18:1n9, 18:3n3 and 20:5n3(EPA). The percentage of dominant fatty acids was generally reflected by the dietary fatty acid composition, particularly the incorporation of 16:0, 18:0 and DHA. The DHA percentage in the phyllosoma larvae increased significantly from T1 (1.58%) to T5 (3.86%). A positive linear correlation was detected between dietary DHA percentage and larval DHA percentage (% total fatty acids) of newly moulted Stage III instar 1 phyllosoma (Figure 11.4, n=5, P <0.01). Although dietary EPA inclusion decreased significantly in *Artemia* enriched with oil emulsion across the diets from T1 to T5 (9.08-4.24%), there was no concurrent decline noted in the incorporation in phyllosoma in those treatments. Stage III instar 1 phyllosoma contained lower concentrations of 18:1n9, 18:2n6 and 18:3n3, but higher 18:0 and 20:4n6 incorporation compared to their respective feeds. Generally, DHA has decreasing retention ratios with increased dietary incorporation while relative retention ratios of EPA increased significantly. Among the five PUFAs, 18:3n-3 had the lowest relative retention ratio with the values ranged from 0.42 to 0.52, while the highest relative retention ratio was found on ARA with the values of more than 1.30 for all treatments, signifying relative retention

and preservation of EPA at concentrations greater than present in their respective feed sources (Table 11.5).

Table 11.4 Fatty acid composition (%total fatty acids) of newly moulted Stage III *Panulirus ornatus* phyllosoma fed enriched *Artemia* containing different DHA/EPA ratios

Fatty acid	**T1**	**T2**	**T3**	**T4**	**T5**
C14:0	0.81	0.97	0.69	0.65	0.71
C15:0	0.79	0.99	0.72	0.60	0.52
C16:0	12.19	13.59	11.52	12.06	10.96
C18:0	7.89	10.19	9.85	7.24	8.69
SFA	21.68	25.74	22.78	20.55	20.88
C14:1n7	0.69	0.78	0.65	0.47	0.49
C16:1n7	8.63	7.95	6.01	8.60	6.35
C16:1n5	1.68	1.04	1.27	1.12	1.11
C18:1n9	19.24	20.76	19.81	18.79	19.39
C18:1n7	3.79	4.68	4.77	4.23	4.73
C20:1n9	0.40	0.43	0.21	0.46	0.47
C20:1n7	0.66	0.58	0.37	0.98	1.12
MUFA	35.09	36.22	33.09	34.65	33.66
C16:2n4	0.57	0.71	0.62	0.55	0.63
C16:3n4	0.35	0.39	0.39	0.46	0.40
C18:2n6	4.21	4.44	3.93	3.63	3.87
C18:3n6	1.61	0.33	1.15	1.01	1.41
C18:3n3	6.42	6.22	5.53	5.44	5.58
C18:4n3	2.48	1.38	2.27	2.46	2.29
C20:4n6	1.41	1.73	1.62	1.35	1.52
C20:3n3	0.85	0.36	1.27	1.08	1.19
C20:4n3	0.16	0.32	0.23	0.26	0.27
C20:5n3	6.72	5.81	7.05	6.04	5.69
C22:5n3	0.54	0.29	0.31	0.69	0.37
C22:6n3	1.58	2.28	2.76	3.03	3.86
∑PUFA (≥18:2n)	25.98	23.16	26.12	24.99	26.05
∑HUFA (≥20:3n)	11.26	10.79	13.24	12.45	12.90
∑n-3PUFA	18.75	16.66	19.42	19.00	19.25
∑n-6PUFA	7.23	6.50	6.70	5.99	6.80
n-3/n-6	2.50	2.56	2.90	3.17	2.83
DHA/EPA	**0.24**	**0.39**	**0.39**	**0.50**	**0.68**
DHA+EPA	7.60	8.09	9.81	9.07	9.55

Table 11.5 The relative retention ratios between Stage III *Panulirus ornatus* phyllosoma phyllosoma body PUFA percentages and their dietary PUFA percentages of newly metamorphosed *Panulirus ornatus* phyllosoma fed enriched *Artemia* containing different DHA/EPA ratios. Relative retention ratio = the % of a specific fatty acid incorporated in the phyllosoma body / the % of the same specific fatty acid incorporated in the diet.

	T1	T2	T3	T4	T5
C18:2n-6	0.62	0.70	0.61	0.57	0.59
C18:3n-3	0.52	0.50	0.46	0.42	0.46
C20:4n-6	1.36	2.14	1.65	1.30	1.83
C20:5n-3	0.74	0.78	1.14	1.14	1.34
C22:6n-3	0.91	0.74	0.68	0.54	0.56

11.4 Discussion

As previously reported for *Artemia* live feeds enrichment, total lipids and HUFA composition were enhanced by the enrichment (Han et al., 2001; Sui et al., 2007). Although both DHA and EPA emulsion oils were readily incorporated to body of enriched *Artemia*, the EPA retention ratio of enriched *Artemia* was higher than the DHA retention ratio. This led to lower DHA/EPA ratio in the enriched *Artemia* than its emulsion oil. The lower DHA/EPA ratio in the enriched *Artemia* may be ascribed to the metabolic conversion of DHA to EPA by the *Artemia* nauplii during the enrichment process (Han et al., 2001), similar results have been observed in other studies (Han et al., 2001; Nelson et al., 2004; Sui et al., 2007). Generally the results demonstrated that it was possible to enrich *Artemia* to obtain a graduated range of DHA/EPA ratios that translated into an effect on growth and provided an understanding of early stage phyllosoma lipid metabolism. There were issues noted with low retention of DHA while EPA was retained at high concentrations, thus preventing examination of an equivalent or a doubling of the dietary DHA/EPA ratios on phyllosoma survival, growth and fatty acid composition.

During this study, the range of dietary DHA/EPA ratios studied did not have a significant effect on *P. ornatus* phyllosoma survival up to Stage III. With the retention ratios attained the impact on survival is similar to previous studies on other spiny rock lobsters, including *Jasus edwardsii* (Nelson et al., 2004) and *Panulirus cygnus* (Liddy et al., 2005). However, for mud crab *Scylla serrata* larvae, low dietary EPA, or high EPA and DHA contents in the enriched *Artemia*, had significant effects on survival from zoea I to the first crab stage (Suprayudi et al., 2004a). It is likely that the relatively high initial total HUFA content in the newly hatched Stage I phyllosoma; in spite of the low amount of DHA derived from some treatments (i.e. T1 and T2), was sufficient to sustain high survival for early stage phyllosoma. Furthermore, it is likely that a continuation of low DHA supplementation in mid-late stage phyllosoma may have a greater

impact upon survival (Wu et al., 2012). Survival of Stage III *P. ornatus* phyllosoma was higher than that of previous studies of the spiny rock lobsters *J. edwardsii* (Nelson et al., 2004) and *P. cygnus* (Liddy et al., 2005), which may indicate that the culture condition utilized in our experiment was suitable for the survival and development of early stage of *P. ornatus*. There may also be species specific influences on survival across the different spiny rock lobster species.

Although the dietary DHA/EPA ratio did not significantly affect phyllosoma survival of early stage *P. ornatus* phyllosoma, significant differences were observed in the development duration, individual body dry weight and specific growth rate. This is similar to the phyllosoma of other spiny lobsters as well as the larvae of brachyuran crabs, where studies have shown nutrition influences development and growth more directly than survival (Anger et al., 1981; Nelson et al., 2004; Liddy et al., 2005). For marine crustacean larvae, dietary DHA can generally accelerate larval development and shorten inter- period (Xu et al., 1993; Xu et al., 1994d; Suprayudi et al., 2004a,b; Sui et al., 2007). Moulting of crustacean is modulated by ecdysteroids (Huberman, 2000; Subramoniam, 2000), which is secreted by Y-organ in the anterior branchial chamber of decapod crustacean and released into the haemolymph (Huberman, 2000). Ecdysteroids circulating in the haemolymph are converted to 20-hydroxyecdysone (20-E) or related compounds by peripheral tissues, such as the hindgut, ganglia, eyestalk and hepatopancreas (Mykles, 2011). In the tick *Ornithodoros moubata*, ecdysone was metabolized into three different compounds in embryos and larvae, and one of them conjugated with a C-22 fatty acid, which suggests that a C-22 fatty acids like DHA may possibly regulate moulting by the modulation of ecdysteroid metabolism for insects (Dotson et al., 1993). Since both crustacean and insects belong to the arthropod family and ecdysteroid metabolism is involved in their moulting process, similar conjugation mechanisms may exist in the ecdysteroid metabolism of crustacean.

The results demonstrated that an elevated dietary DHA/EPA ratio was associated with a significant increase in body length and individual dry weight of early stage *P. ornatus* phyllosoma larvae, similar to found in other crustacean larvae, such as the swimming crab, *P. trituberculatus*, mud crab *S. serrata* and the Chinese mitten crab, *E. sinensis* (Takeuchi et al., 1999b; Suprayudi et al., 2004b; Sui et al., 2007). Previous studies have demonstrated that DHA has a superior nutritional value when compared to EPA in terms of crustacean larval and juvenile growth (Xu et al., 1993; Merican and Shim, 1996; Sui et al., 2007). In vertebrates DHA is indispensable in the development of neural tissues, such as brain and eye retina (Bell et al., 1995; Innis, 2007), while marine crustacean species generally contain very high DHA levels in

their tissue membranes (Teshima, 1998; Sheen and Wu, 2002) increasing cell membrane permeability, fluidity and resistance to osmotic shock (Palacios et al., 2004; Sui et al., 2007). Hence marine crustaceans generally have higher DHA requirements when compared to brackish or freshwater crustacean species (D'Abramo and Sheen, 1993; Sui et al., 2007). Capture of wild spiny rock lobster phyllosoma, puerulus and juvenile samples combined with results from feed experiments have demonstrated an elevated presence of DHA (Phleger et al., 2001; Nelson et al., 2003; 2004; Liddy et al., 2005). These observations are supported by a recent ship-board feeding trail where wild-caught *P. cygnus* phyllosoma demonstrated a high preference for chaetognath prey items, this was previously demonstrated in a 2008 plankton collection cruise in the Coral sea by AIMS staff whereby captive reared Stage VI –VIII *P. ornatus* phyllosoma preferentially consumed wild caught chaetognaths over all other captured planktonic prey items (Smith pers. comm.). The fatty acid profile of chaetognaths is extremely high in DHA (ca 21.9% total fatty acids) (Saunders et al., 2012). Although several previous reports have identified the importance of dietary DHA for phyllosoma larvae of other spiny lobster species (Liddy et al., 2004a; Nelson et al., 2004), there is no published information on the dietary fatty acid requirement for early stage *P. ornatus* phyllosoma. While the present study demonstrated that dietary DHA has very important role in the development, moulting and growth for early stage *P. ornatus* phyllosoma, the requirement for EPA is unclear, especially when fed in combination with high concentrations of DHA, due to the sparing of EPA and the potential for retro-conversion of DHA to EPA (Han et al., 2001; Sui et al., 2007). Dietary EPA input into larval crustaceans has had an impact on larval survival and growth for the mud crab *S. serrata* (Suprayudi et al., 2004a), and influenced larval survival in the swimming crab *P. trituberculatus* (Takeuchi et al., 1999b).

The qualitative concentrations and ratios of the major fatty acids in Stage III phyllosoma were positively related to the same fatty acid parameter within the enriched *Artemia*, especially for 16:0, 18:0, 18:1n-9 and DHA. This was despite a reduction in the relative retention ratio of DHA from treatment 1 to 5. This suggested early stage *P. ornatus* phyllosoma are able to preferentially assimilate and accumulate DHA from their diets when the dietary DHA content is low and perhaps inadequate. Similar results were found in the spiny rock lobsters *P. cygnus* (Liddy et al., 2005) and *J. edwardsii* (Nelson et al., 2004). Consequently, when dietary DHA level is above the optimal level, the relative retention ratio of DHA decreased with the increasing dietary DHA, however some of this lipid biochemistry may be explained the reduced inclusion of EPA stimulating retro-conversion of DHA to EPA to maintain a baseline concentration, as occurred in this study (Smith et al., 2003; Liddy et al., 2004a). Previous studies also indicated DHA in enriched *Artemia* maybe not completely digested/assimilated by

some spiny rock lobster species, this is likely to be due to DHA being provided in the form of triacylglycerol (TAG) (Phleger et al., 2001; Liddy et al., 2005). Compared to HUFAs, 18:2n6 and 18:3n3 had the lower relative retention ratio, which suggested early stage *P. ornatus* phyllosoma had low C18-PUFAs requirements, and C18-PUFAs had inferior value to the growth and development of early stage *P. ornatus* phyllosoma. This is similar to what is found in other marine crustacean larvae (Levine and Sulkin, 1984; Xu et al., 1993; Suprayudi et al., 2004a).

The fatty acid composition of newly hatched or wild caught larvae can generally provide valuable information on the fatty acid requirements of crustacean larvae (Phleger et al., 2001; Limbourn and Nichols, 2009). However, to date, the changes in fatty acid composition of wild *P. ornatus* phyllosoma has not been documented. Therefore, laboratory cultured larvae have been used as the alternative to assess the ontogenetic changes of fatty acid composition for *P. ornatus* phyllosoma (Wu et al., 2012). Compared to fatty acid composition of newly hatched phyllosoma (ARA=3.19%, EPA=14.33%, DHA=11.68%), Stage III *P. ornatus* phyllosoma contained a lower HUFA content and lower DHA/EPA ratio. This difference indicates that *Artemia* are not the ideal live food for *P. ornatus* phyllosoma to maintain a high DHA/EPA ratio and HUFA content, and the development of a stable artificial feed would assist in the determination of optimal concentrations of lipid ingredients. The alternative is to utilize live prey that naturally contain high HUFA content with DHA/EPA ratios favoring DHA (Nelson et al., 2003; 2004), species with suitable lipid profiles may include chaetognaths and salps, however, both are difficult to culture using current knowledge and techniques (Takeuchi and Murakami, 2007). At present enriched *Artemia* are readily available, have established culture protocols and have a low cost to produce, therefore they still must be considered as the most feasible food source for the culture of early stage lobster phyllosoma larvae (Takeuchi and Murakami, 2007; Wu et al., 2012). Future studies to improve the fatty acid composition of emulsions; such as provision in microcapsules, HUFA supplied as phospholipids will assist in the provision of an optimized HUFA profile for early stage spiny rock lobster phyllosoma.

11.5 Conclusion

This study demonstrates that the DHA/EPA ratio in *Artemia* was able to be improved via enrichment with an oil emulsion containing different DHA/EPA ratios, and subsequently there was uptake by early stage *P. ornatus* phyllosoma. Provision of a diet with an elevated DHA/EPA ratio could shorten development time, accelerate growth and increase body length and individual dry weight for the early stage *P. ornatus* phyllosoma larvae. The DHA percentage in Stage III phyllosoma increased significantly with the elevated dietary DHA levels

while its relative retention ratio decreased dramatically with lower EPA and higher DHA inputs, respectively. Further comparison of fatty acid composition revealed the incorporation and transfer of DHA decreased from enrichment oil, to enriched *Artemia*, and finally to phyllosoma. Further research should be focused on the improvement of DHA content in diets and maximizing uptake and retention by early stage *P. ornatus* phyllosoma.

Chapter 12

General discussion and conclusion

12.1 Significance of the present study

The blue swimming crab, *Portunus pelagicus* and the ornate rock lobster, *Panulirus ornatus* are two important target aquaculture species in the tropical regions of the Indo-Pacific (Smith et al., 2009a; Wu et al., 2010d). However, mass production of artificial seed is one of the key for the future development of the aquaculture for both species (Soundarapandian et al., 2007; Soundarapandian and Dominic Arul Raja, 2008; O'Sullivan, 2010). Lipid nutrition, particularly fatty acid composition, is one of the important factors to affect larval survival, growth and development time for many crustaceans (Xu et al., 1993; Merican and Shim, 1996b; Nghia et al., 2007; Wu et al., 2007c). Lack of basic information in lipid nutrition is one of the major obstacles for the improvement of hatchery technology for both species (Soundarapandian et al., 2007; Wu et al., 2012). Therefore, the broad objective of this thesis is to expand our understanding of lipid nutrition for early life stage *P. pelagicus* and *P. ornatus,* with emphasis on highly unsaturated fatty acids. The specific aims were to identify which highly unsaturated fatty acids play crucial roles on the embryonic development, larval survival, growth and development during the embryonic development and larval culture of *P. pelagicus* and *P. ornatus*; and then to determine the optimal levels for those important fatty acids in larval diets.

Table12.1 Schematic overview of the results and implications of this study

Experiment	**Main results and recommendation**
Chapter 2 Changes in volume, biomass and lipid composition during the embryonic development of the blue swimmer crab, *Portunus pelagicus*	● TAG and PL were two dominant lipid fractions, while PL was the major energy source in the total lipids; ● Increasing percentage of ARA and EPA suggested they are highly conserved; ● Decreasing DHA/EPA ratio indicated early stage larvae need low DHA level, but high EPA level in their diets.
Chapter 3 Changes of lipid class and fatty acid composition during the embryonic development of the of the ornate rock lobster, *Panulirus ornatus*	● 64% of PL was utilized, which accounted for 76% of total lipid depletion during the embryonic development; ● The relative conservation of EPA and ARA, but preferential utilization of DHA implicated the excessive of egg DHA were oxidated for energy supply during the embryonic development; ● Pre-hatching egg of *P. ornatus* contained higher DHA/EPA ratio and lower ARA level (%total fatty acids) than that of *P. pelagicus;* ● Those differences indicated their different HUFA requirements for early stage larvae

Table12.1 Schematic overview of the results and implications of this study (Continued)

Experiment	Main results and recommendation
Chapter 4 Ontogenetic patterns of growth and lipid composition changes of blue swimmer crab, *Portunus pelagicus* larvae: insights into larval biology and lipid nutrition	● High mortality accompanied with high percentage increment and SGR of dry weight; ● The increases of ARA and DHA in megalopae indicated they are important for later larvae.
Chapter 5 Ontogenetic patterns of growth and lipid composition deposition during early to mid-Stages of development in *Panulirus ornatus* phyllosoma	● The lipid class profile was dominated by PL for all early-mid Stage phyllosoma; ● Early stage phyllosoma preferentially sequestered and accumulated a higher proportion of EPA and DHA compared to that present in their diets, indicating higher DHA and EPA requirements for these stages.
Chapter 6 Effects of starvation on survival, biomass and lipid composition of newly hatched larvae of the blue swimmer crab, *Portunus pelagicus*	● More than 50% of PL was utilized, indicating the oxidation of membrane structural lipids during starvation; ● Higher reductions were found on DHA and EPA than that of ARA, emphasized the importance of ARA for early stage larvae.
Chapter 7 Effects of starvation on survival, biomass and lipid composition of newly hatched phyllosoma of the ornate rock lobster, *Panulirus ornatus*	● Newly hatched phyllosoma contained higher total lipids, which indicated newly hatched *P. ornatus* had the higher starvation resistance than the newly hatched *P. pelagicus*; ● DHA and ARA were relatively conserved, indicating their importance.
Chapter 8 The effects of dietary arachidonic acid levels on larval survival, growth and occurrence of moulting death syndrome in the blue swimmer crab, *Portunus pelagicus*	● Dietary ARA significantly affected the occurrence of ing death syndrome (MDS), larval survival, development and growth; ● Optimal dietary ARA level was suggested to be ca. 2.97% of total fatty acids; ● Significant negative correlation was detected between the chela length/carapace length ratio and the occurrence of MDS.
Chapter 9 The effects of dietary arachidonic acid levels on survival, growth and fatty acid composition of early stage phyllosoma of ornate rock lobster, *Panulirus ornatus*	● Dietary ARA only significantly affected development time and growth for early stage phyllosoma; ● Optimal dietary ARA level was estimated at 1.90% of total fatty acids.
Chapter 10 The effects of dietary DHA/EPA ratios on larval survival, growth and fatty acid composition of blue swimmer crab, *Portunus pelagicus*	● Dietary DHA/EPA ratios significantly affected survival, development time and growth; ● Optimal dietary DHA/EPA ratio was estimated at 0.53, with the highest survival, body size, and shortest development time.
Chapter 11 The effects of dietary DHA/EPA ratios on survival, growth and fatty acid composition of early stage phyllosoma of ornate rock lobster, *Panulirus ornatus*	● Dietary DHA/EPA ratios significantly affected development time and growth; ● Optimal dietary DHA/EPA ratio was estimated more than 1.64, clearly suggesting higher DHA and DHA/EPA ratio requirements of early stage *P. ornatus* than larval *P. pelagicus*.

On this basis, this comprehensive study on the lipid nutrition of early life stages of *P. pelagicus* and *P. ornatus* consisted of a total of 10 experiments (Chapter 2 to Chapter 11), with

emphasis on the importance and requirements of highly unsaturated fatty acids for larval stage of both species. This study fill the gap of lipid nutrition for the early life stage *P. pelagicus* and *P. ornatus*, which would be an important step towards to cost-effective and reliable hatchery production of *P. pelagicus* and *P. ornatus*. The findings provided within this thesis not only have significantly enhanced the understanding of fatty acid nutrition for the larvae of both species, but also have important implications for the further improvement of hatchery technology and formulated diets for the larval culture. The major outcomes are summarized in Table 12.1, while the broader context, major results as well as common issues are discussed in the following.

12.2 Ontogenetic changes of lipid composition

The examination of lipid dynamics during embryonic development not only can provide valuable information on lipid utilization during organogenesis, but also contribute to our understanding of lipid nutrition for both broodstock and early stage of crustacean larvae (Rosa et al., 2003; Rosa et al., 2005; Rosa et al., 2007; Torres et al., 2008). This study is the first one to comprehensively investigate the changes of egg volume, egg biomass, total lipids, lipid class profiles and fatty acid contents during the embryonic development of *P. pelagicus* and *P. ornatus* (Chapter 2 and 3). The depletion of lipids during the embryonic development suggested the importance of lipids as energy source to sustain the organogenesis and embryonic metabolism (Kattner et al., 2003; Anger et al., 2004). Declining contents (microgram/egg) and percentage of phospholipids signified the phospholipid is a major lipid class for the energy supply of both species during the embryonic development. The high conservation of EPA and ARA was observed for both species, which suggested their importance for the developing embryos and newly hatched larvae. Moreover, decreasing DHA percentage during embryonic development of *P. ornatus* strongly implicated the excessive of egg DHA were oxidated for energy supply during the egg development. Generally, pre-hatching egg and newly hatched phyllosoma of *P. ornatus* contained higher DHA/EPA ratio and lower ARA level (%total fatty acids) than that of *P. pelagicus*, which indicated their different HUFA requirements for early stage larvae of both species. These differences may suggest early stage *P. pelagicus* larvae need low DHA level, but high EPA and ARA levels in their diets while newly hatched *P. ornatus* phyllosoma require high DHA level, but low ARA level in their diets.

Analysis of the ontogenetic changes in lipid composition during larval development has been used as a method to understand the lipid requirements and the relative importance of specific fatty acids during larval development (Phleger et al., 2001; Limbourn and Nichols, 2009). Wild crustacean larvae with different development stages are the ideal choice for these

analyses (Saunders et al., 2012). However, collection and identification of marine larvae from plankton samples are technically difficult, tedious and costly, particularly for species with a number of larval development stages and extended larval duration (Smith et al., 2009a). For example, *P. pelagicus* undergoes four pelagic zoeal stages and a postlarval megalopal stage before settling as benthic juvenile, which are assumed to disperse and live in different parts of ocean (Josileen and Menon, 2004). Therefore, use of laboratory cultured larvae has become an alternative mean in the understanding of larval nutrition of various crustaceans (Mourente *et al*., 1995; Roustaian *et al*., 2001; Holme et al., 2009b; Copeman et al., 2012). Under the laboratory conditions for larval *P. pelagicus*, high mortality was accompanied with high percentage of body increments and SGR of dry weight, which suggested the high energy demand during the process of moulting and tissue proliferation (Roustaian et al., 2001). Compared to their diets, significant increases of ARA and DHA in megalopae indicated they are very important HUFAs for later stage larvae, particularly in zoea IV larvae. Zoea III onward larvae were fed enriched *Artemia*, only containing ca. 1% of ARA in total fatty acids, but megalopae had 2.16% of ARA in total fatty acids. This indicated megalopae had higher ARA requirement than early larvae of *P. pelagicus*.

As for ontogenetic patterns of lipid composition changes during early to mid-Stages of *P. ornatus* phyllosoma, the decreasing trends of SGR was detected from early stage to mid stage. This is consistent with the declined metabolism, ammonia excretion, specific oxygen consumption rates and extended development period between the consecutive stages during larval development of *P. ornatus* (Smith et al., 2009a; Ikeda et al., 2011). Furthermore, early stage phyllosoma could preferentially sequestered and accumulated the higher proportions of EPA and DHA compared to that present in their diets, indicating higher DHA and EPA requirements for these stages. These results suggested larval *P. pelagicus* and *P. ornatus* have an ability to preferentially accumulate and modify their dietary fatty acids to support normal growth and development.

12.3 Effects of starvation on lipid composition

In Chapters 6 and Chapter 7, two separate starvation experiments were conducted to assess the fatty acid utilization patterns and preferential conservation of important fatty acids for the newly hatched larvae of *P. pelagicus* and *P. ornatus* during the starvation. The newly hatched *P. ornatus* phyllosoma had the stronger starvation resistance than the newly hatched *P. pelagicus* larvae, which is linked to the high individual total lipids and dry weight in newly hatched Stage I phyllosoma (Anger, 1995; Abrunhosa and Kittaka, 1997; Smith et al., 2010). During the starvation, more than 50% of phospholipids were utilized for the larvae of both

species, indicating the oxidation of membrane structural lipids (Ritar et al., 2003). The higher retention of ARA was found than that of EPA and DHA during the starvation of *P. pelagicus* larvae while both DHA and ARA were highly conserved for newly hatched *P. ornatus* phyllosoma. These results not only indicated the importance of those HUFAs for larval growth and development of both species, but also suggested HUFA requirement of larval *P. pelagicus* is lower and different to early stage *P. ornatus* phyllosoma. Therefore, the major findings of Chapter 2 - Chapter 7 have provided the important baselines and insights for the design of following feeding experiments.

12.4 Effects of dietary highly unsaturated fatty acids on larval performance

Based on the previous experiments (Chapter 2 - Chapter 7), ARA and DHA/EPA ratios are two important factors for the newly hatched larvae of *P. pelagicus* and *P. ornatus*, which suggested the dietary ARA levels and DHA/EPA ratios may have significant effects on the growth and survival of *P. pelagicus* and *P. ornatus* larvae. In addition, many marine crustaceans generally have limited capacity to synthesize HUFAs *de novo* and therefore require certain amount of HUFAs, such as DHA, EPA and ARA, in their diet (Kanazawa et al., 1979a). Therefore, Chapter 8 - Chapter 11 were designed to identify the optimal dietary ARA levels as well as DHA/EPA ratios for early-stage *P. ornatus* phyllosoma and *P. pelagicus* larvae based on enriched *Artemia*. For the evaluation of larval growth of crustacean feeding experiments, previous studies often used final body weight, carapace size and development time as the criteria (Suprayudi et al., 2004a,b; Holme et al., 2006; Sui et al., 2007). The present study found the specific growth rate (SGR; % day^{-1}) was more sensitive than the measurements of body weight, carapace size of larvae because both body weight increase and duration are taken into the calculation (Wu et al., 2012). SGR is very important parameter 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 (Guillaume, 2001). As carapace growth in crustaceans only occurs immediately following when crustaceans are able to increase their body size while the exoskeleton is still soft (Freeman et al., 1983; Freeman et al., 1987), all experiments were conducted over a duration required for larvae 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 different trials possible.

The current results clearly showed that dietary ARA levels significantly affected development and growth of the larvae for both species. Both the lower and higher dietary ARA appeared to negatively impact on larval performance, often leading to longer development time, lower DW and SGR, which maybe ascribe to the imbalance ratios of different prostaglandins. It

is well known ARA and EPA are the precursors of series-2 (e.g. prostaglandin E_2, PGE_2) and series-3 prostaglandin (e.g. prostaglandin E_3, PGE_3), respectively (Yang et al., 2002). There exists a competitive inhibition between conversion of ARA to PGE_2 and EPA to PGE_3 (Bell and Sargent, 2003); when dietary ARA is below an optimal level, increased dietary ARA would lead to increased PGE_2 synthesis and concentration, hence enhancing larval development while further increase of dietary ARA level beyond an optimal level may lead to excessive PGE_2 being produced with decreased PGE_3 production, resulting in an imbalanced PGE_2/PGE_3 ratio. Therefore, optimal dietary ARA levels are therefore likely to result in balanced PGE_2/PGE_3 ratio in crustaceans (Reddy et al., 2004; Meunpol et al., 2010), leading to the highest survival, shortest development time, fast growth and the lowest occurrence of moulting death syndrome (MDS) for *P. pelagicus* larvae. MDS is a common phenomenon found in brachyuran crab larvae during metamorphosis, and mass mortalities due to MDS have been reported for various portunid crabs (Hamasaki et al., 2002a; Arai et al., 2004; Takano et al., 2004; Baylon, 2009). More recent studies have revealed that an imbalance of dietary omega-3 fatty acids is one of the major contributors to MDS in larval mud crabs (Hamasaki et al., 2002b; Suprayudi et al., 2004a). These results demonstrated, for the first time, that dietary ARA level also had a significant effect on the occurrence of MDS for *P. pelagicus* larvae, resulting in different survival from zoea IV to megalopa.

The present results have shown dietary optimal ARA level of larval *P. pelagicus* is higher than that of early-stage *P. ornatus* phyllosoma. This may indicate their different feeding habits in wild sea. Natural diets of *P. pelagicus* larvae, particularly for megalopae stage, may contain relatively higher ARA levels while natural preys of early stage *P. ornatus* phyllosoma have low ARA levels. Sargent and Whittle (1981) had suggested that ARA, derived from the benthic algae (Sargent and Whittle, 1981) and therefore more accessible to post-larvae and juvenile crustaceans such as megalopae. Therefore, pelagic crustacean larvae may have a lower ARA requirement than post-larval or early juvenile crustacean (Ritar et al., 2003). The dietary DHA/EPA ratios did also significantly affect development time and growth for both species. However, optimal dietary DHA/EPA ratio of early stage *P. ornatus* is significant higher than that of larval *P. pelagicus*. This difference indicated early stage *P. ornatus* have higher DHA/EPA ratio and DHA requirement than larval *P. pelagicus*. However, dietary DHA/EPA ratios and ARA levels did not significantly affect the survival of early stage *P. ornatus* phyllosoma. It is likely that the relatively high initial total DHA and ARA levels in the newly hatched Stage I phyllosoma was sufficient to sustain high survival for early stage phyllosoma. Therefore, dietary DHA/EPA ratios and ARA levels only affected growth and development of

early stage *P. ornatus* phyllosoma. In conclusion, optimization the fatty acid composition of hatchery food will be an effective means to improve seed quality and quantity for both species.

12.5 Future directions

Although the present study has made significant contributions to the understanding of lipid nutrition for early life stage *P. pelagicus* and *P. ornatus*, providing the valuable information for the further improvement of hatchery technology and formulated diets for the hatchery culture of both species, the intrinsic mechanism of fatty acid requirement and their interaction largely remains unknown for their modulation on larval growth, development and survival for both species. As there are many complexities in the identification of quality and quantity of essential fatty acids (EFAs), investigation of fatty acid interactions and requirements as well as recognition of their metabolism, the scope of future research is quite extensive and task is very tough. However, the following directions seems should be the priority for the fatty acid nutrition of *P. pelagicus* and *P. ornatus* larvae in the future research, which is emerging from my thesis.

Firstly, wild broodstock females with mature ovary, wild larvae of different developmental stages and with similar stage of moulting cycle, and their natural foods should be sampled for the lipid analysis to get the accurate information for their natural conditions of lipid nutrition. Although lipid analysis has been conducted on wild *P. pelagicus* females with mature ovaries and early stage embryos (Zhou et al., 2011), no available information could be found on the lipid composition of wild *P. pelagicus* larvae. For both *P. pelagicus* and *P. ornatus*, their larval cycles are complicated, consisting of multiple-pelagic planktonic stages, transitional planktonic post-larvae (megalopa for *P. pelagicus*; puerulus for *P. ornatus*) and benthic juveniles (Josileen and Menon, 2004a; Smith et al., 2009a). Previous studies have indicated wild *Jasus edwardsii* phyllosoma undergo several dietary shifts during the pelagic planktonic stages (Jeffs, 2007). The similar phenomena maybe existed in *P. ornatus* phyllosoma. For *P. pelagicus*, generally, adult females leave inshore estuarine areas and move to offshore for spawning and hatching while megalopa immigrate into shallow estuaries for settlement (Meagher, 1971). During the larval phase, the larvae may drift as far as 80 km out to sea before returning to settle in shallow inshore waters (Bryars, 1997). The different habitats and dietary shifts suggested there maybe existing stage differences of fatty acid nutrition, particularly in ARA, DHA and EPA. Therefore, more stages of wild larvae and their natural preys should be sampled and analyzed to provide important cues and insights to fatty acid nutrition of *P. pelagicus* and *P. ornatus* larvae. The stage differences should be taken into consideration in future feeding experiments and hatchery practice.

Although this study have demonstrated the importance of ARA, DHA and EPA, unfortunately, up to date, the numbers of essential fatty acids (EFAs) have not been identified for *P. pelagicus* and *P. ornatus* larvae. As reviewed in Chapter 1, animals including crustacean lack Δ12 (ω6) and Δ15 (ω3) desaturases and so they can not form 18:2n-6 and 18:3n-3 from 18:1n-9 (D'Abramo, 1997; Merican and Shim, 1997). Then, 18:2n-6 and 18:3n-3 are EFAs in the diets of crustaceans (D'Abramo, 1997). However, some crustaceans, particularly freshwater or estuary species, have a certain ability of conversion from 18:3n3 to 20:5n3 and then to 22:6n3 as well as 18:2n6 to 20:4n6 (Reigh and Stickney, 1989b; Mourente, 1996). Therefore, the quality and quantity of EFAs may vary among different crustacean species. For larval *P. pelagicus* and *P. ornatus*, it is quiet difficult to identify the EFAs because larval culture of both species, particularly early larval stages, largely rely on live foods, such as rotifers and *Artemia*. However, live foods (rotifers and *Artemia)* generally contained a certain levels of 18:3n3 and 18:2n6, and they also have strong fatty acid metabolism in their bodies (Han et al., 2001; Sui et al., 2007). The mentioned reasons suggested it is inaccurate to use live foods to investigate the quality and quantity of EFAs for *P. pelagicus* and *P. ornatus*. The purified diets, deleted a particular fatty acid but with the other important PUFAs (deletion experiment) or added a particular fatty acid but without other important PUFAs (presence experiment), are necessary and classical method for the identification of EFAs for animals (Xu et al., 1994b; Merican and Shim, 1996). Recent studies have shown formulated diets, such as microbound diets and moisture diet, can totally replace live foods during the megalopal stage of *P. pelagicus* and later phyllosoma stage of *P. ornatus* (Castine et al., 2008; Johnston et al., 2008). For the research purpose, the quality and quantity of EFAs should be indentified for the megalopal stage of *P. pelagicus* and later *P. ornatus* phyllosoma based on the purified diets using pure fatty acids as fatty acid source. From the practical view, rotifers and *Artemia* are still considered as the most feasible food source for the culture of early stage *P. pelagicus* and *P. ornatus* larvae, and they are unlikely to be replaced in near future (Takeuchi and Murakami, 2007; Holme et al., 2009a; Wu et al., 2012). Therefore, future studies also should be done to improve the fatty acid composition of emulsions and then live foods, such as enrichment methodology, comparison of fatty acid forms in triglyceride, methyl ester and phospholipids, and fatty acids providing in microcapsules to reduce their metabolism.

Last but not least, the current results have shown dietary ARA levels and DHA/EPA ratios significantly affect larval performance of *P. pelagicus* and *P. ornatus*, but the intrinsic mechanism is largely unknown. Ecdysone and different prostaglandins have been suggested to be involved in those regulation processes (Koskela et al., 1992; Suprayudi et al., 2004). Therefore, more concern should be put on the modulation of prostaglandins and ecdysone on

growth and moulting as well as their relationships with dietary and tissue fatty acid composition, particularly for ARA, EPA and DHA. However, due to the small number of larvae reached to megalopal stage of *P. pelagicus* or stage III phyllosoma for *P. ornatus* from the current experiments, together with complicated determination methodology of those hormones, the actual concentration of different prostaglandins and ecdysone were not able to determine for the larvae in this thesis. Clearly, it is ugent and important to investigate the physiological mechanism between dietary HUFA composition and larval performance of *P. pelagicus* and *P. ornatus* in future, which would provide the valuable information for the improvement of hatchery technology of both species.

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