Towards development of a formulated diet for mud crab (*Scylla serrata*) larvae, with emphasis on lipid nutrition

Thesis submitted by
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Abstract

Crabs of the genus *Scylla*, commonly referred to as mud crabs, are commercially important crabs with an Indo-Pacific distribution. *Scylla serrata* is the most widespread of the four recognized *Scylla* species, and it has traditionally been an important fishery in coastal communities throughout its range. In recent decades it has also become a targeted species for aquaculture, however, due to a lack of low cost formulated diets, mass production of mud crab seed stock is not reliable, and the majority of mud crab farms rely on juveniles caught in the wild. This has lead to a widespread seeds-stock shortage and over-exploitation of wild populations. Development of more effective hatchery techniques and more reliable production of juveniles is therefore considered critically important for sustainable growth of the mud crab farming industry in this region.

Current methods for *Scylla* hatchery production rely on live foods such as rotifers and *Artemia*. These prey species are costly to produce, have an inconsistent nutritional profile and lack certain nutritional components essential for normal growth of marine larvae. Development of a nutritionally optimised diet is therefore considered critically important, and recent research at James Cook University has shown that mud crab larvae readily accept microbound diet (MBD) particles. As the contents of such diets easily can be manipulated, MBD shows enormous potential as a tool for further specification of the nutritional requirement of mud crab larvae.

Lipids are required in crustacean diets as an important source of energy, essential fatty acids, sterols, phospholipids and fat-soluble vitamins. An appropriate supply of these

nutrients is particularly critical during larval development, where a series of important morphological, physiological and biochemical changes take place. On this basis, this thesis was designed to first collect and review all information on the nutritional requirements of *S. serrata* larvae published to date, and highlight areas where more research is required. Experiments were then conducted to fill gaps in our current knowledge on larval dietary lipid requirements. Some of the most important aspects of lipid metabolism; dietary cholesterol requirements, interaction between cholesterol and phospholipids, the optimum balance of dietary *n*-3 and *n*-6 fatty acids, as well as lipid class and fatty acid utilization by developing larvae have been addressed in separate chapters, with the aim to provide information assisting formulation of more appropriate species-specific MBD for *S. serrata* larval culture.

While previous experiments have demonstrated that megalopae of the mud crab can be cultured exclusively on MBD, the potential of MBD as a food source for zoea larvae of the same species has not previously been determined. The first experiment of this thesis was therefore set up to investigate the effect of partial and total replacement of live food with MBD for zoeal larvae, with the aim to determine the best suited larvae stage for further nutritional studies. Zoea III larvae were cultured communally at a density of 25 larvae L^{-1} and were fed either 100% live *Artemia* nauplii, 100% MBD or a 50%:50% combination of MBD and *Artemia* nauplii. Highest survival (66%) and development rate to the zoea IV stage were recorded for larvae fed the 50%:50% combination of MBD and *Artemia* nauplii. Some successful molts were also found among larvae fed exclusively on MBD, while total mortality was observed in the unfed control. The results indicate that the experimental MBD may contain certain beneficial nutrients lacking in *Artemia* nauplii, and that co-feeding the MBD with *Artemia* nauplii enhanced

larval survival and development. However, the findings also suggested that total replacement of live food with the experimental MBD will result in poor survival of zoea III larvae, and based on these data, the megalopa stage was chosen as the most appropriate larval stage for further studies.

The experimental MBD previously used for rearing *S. serrata* megalopa contained a high proportion of dried rotifers (38% of total diet dry weight). Although this work demonstrated that mud crab megalopa readily ingest and assimilate formulated diet particles, it did not reduce the reliance on live food cultures. Fundamental to the success of this study was therefore to replace the rotifer components with a commercially available protein source, and four marine animal meals (rotifer meal, *Artemia* meal, fish meal and squid meal) were evaluated based on larval survival, dry weight and carapace weight of newly molted first stage crabs (C1) and development time from megalopa to C1. Fifteen megalopae were reared individually in 250 mL aquaria, and survival of MBD-fed megalopae to C1 ranged from 46.7% to 60.0% with those fed MBD containing fish meal or squid meal showing higher survival than those fed MBD containing dried *Artemia* or rotifers. Larvae fed live *Artemia* nauplii showed the highest overall survival (80%), while none of the unfed megalopae survived to C1. There were no significant differences ($P > 0.05$) in the average time required for megalopae to reach the first crab stage when fed any of the four MBD, however, shortest development time was recorded for larvae fed live *Artemia* nauplii. The results indicate that squid meal is a suitable protein source for formulation of MBD for *S. serrata* megalopae, and this meal was used in MBD formulated for subsequent experiments.

Cholesterol is an important sterol, serving as a precursor for many physiological compounds such as sex and molting hormones, adrenal corticoids, bile acids and vitamin D. As crustaceans are incapable of *de novo* production of cholesterol, dietary cholesterol is essential for optimum growth and high survival in crustaceans. Chapter 4 of this thesis reports on an experiment assessing the effects of dietary cholesterol levels on growth, development time and survival of mud crab megalopae. Five semi-purified, iso-nitrogenous and iso-energetic MBD containing cholesterol levels from 0.14% to 1.00% diet dry weight were tested, using fifteen individually reared megalopae for each dietary treatment. The results showed that megalopae from all treatments were able to metamorphose to the first crab stage, suggesting that the endogenous level of cholesterol in the basal diet (0.14%) was sufficient to meet dietary requirements. Highest overall survival (74.3%) was recorded for megalopae fed a diet containing 0.80% cholesterol, while the widest mean carapace width (3.53 ± 0.08 mm) and highest mean dry weight (21.11 ± 2.22 mg) were recorded for juveniles molting from megalopae fed live *Artemia*. No megalopae in the unfed control treatment metamorphosed into crabs. The average development time from megalopa to the C1 stage varied among the treatments, with megalopae fed live *Artemia* or MBD containing 0.20%, 0.40% or 0.80% total cholesterol showing relative shorter development time to C1; between 8.0 (± 1.46) days and 9.9 (± 1.37) days, whereas the longest development time was recorded for the megalopae fed diets containing 0.14% or 1.00% total cholesterol; 11 (± 1.45) days and 11 (± 1.24) days, respectively. Based on these findings, 0.80% total dietary cholesterol was suggested as optimum in semi-purified diets for *S. serrata* megalopa.

For some marine crustaceans, an interaction occurs between dietary cholesterol and dietary phospholipid. This has important implications for diet formulation, and to determine if this interaction is present in *S. serrata* larvae, the experiment reported in Chapter 5 was conducted to evaluate the effects of varying dietary levels of lecithin and cholesterol on growth, development and survival of megalopa. Six semi-purified MBD were formulated to be iso-energetic and iso-nitrogenous and to containing three levels of supplemental lecithin (0.0, 2.0 and 4.0% diet dry weight) and two levels of supplemental cholesterol (0.0 and 0.7% diet dry weight). The experiment was designed in the same manner as in Chapter 4, and the results showed a significant interaction between supplemental dietary lecithin and cholesterol for final mean dry weight of newly settled crabs. Highest survival (60%) was recorded for megalopae fed diets containing the highest levels of dietary lecithin regardless of whether diets were supplemented with cholesterol, and this rate of survival was identical to that of megalopae fed live *Artemia* nauplii. The experiment indicated that supplemental dietary cholesterol may not be essential for mud crab megalopae when fed a diet containing fish oil and sufficient levels of supplemental dietary phospholipids.

Most crustacean diets today are formulated to contain a 2:1 fish oil/corn oil ratio based on known requirements of penaeid prawns. As the results from the previous chapters indicated that the nutritional requirements of *S. serrata* larvae are different from those of other marine crustaceans, the experiment in Chapter 6 was designed to examine the optimum fish oil/corn oil ratio in semi-purified diets formulated for mud crab megalopae. Six iso-energetic and iso-nitrogenous MBD containing 6% total lipid were formulated to contain fish oil and corn oil either singly or in various ratios (0:1, 1:2, 2:1, 3:1, 1:0, 1:1), and each dietary treatment consisted of 20 individually reared megalopae.

Survival, growth, development time to C1 and signs of molting death syndrome (MDS) were recorded daily, and carapace width and dry weight of newly molted crabs were measured immediately after molt. Megalopae from all dietary treatments successfully molted to C1; however, best survival (70%) was achieved by megalopae fed MBD containing a fish oil/corn oil ratio of 1:1. Megalopae fed MBD containing ratios of 3:1and 1:0 showed survival of 65%, while survival of megalopae fed either live *Artemia* nauplii or MBD containing a fish oil/corn oil ratio of 2:1 was 60%. Lower survival (55% and 35%) was recorded for megalopae fed MBD with ratios of 1:2 and 0:1, respectively. Significantly greater mean carapace width (3.51 ± 0.03 mm) and significantly higher mean dry weight (2.14 ± 0.14 mg) was recorded for crabs molting from megalopae fed live *Artemia* nauplii compared to those resulting from megalopae fed MBD. Results from this experiment indicate that the optimal fish oil/corn oil ratio is around 1:1 when oil is supplied at a level of 6% of total diet dry weight. The study further showed that complete replacement of fish oil with corn oil in the formulated diet resulted in a high occurrence of MDS-related mortality, indicating an essential dietary requirement for >C18 highly unsaturated fatty acids (HUFA), and a link between *n*-3 HUFA availability and the occurrence of MDS.

In nature the nutritional requirements of crab larvae are satisfied by the diversity and variability of natural prey and their broad nutrient contents. As the study of larvae in the wild is practically impossible, analysis of the biochemical changes occurring in the tissue during larval development in the laboratory has been used as an alternative method for elucidating information on the nutritional requirements of larvae. These principles have been applied successfully to studies of several crustaceans, but a complete record of changes in lipid and fatty acid profile during ontogenetic

development of mud crabs was not available. Comprehensive analysis of changes in dry weight, organic content, total lipid, lipid class and fatty acid composition in developing *S. serrata* larvae reared under standard hatchery conditions is reported in Chapter 7. An increase in mean dry weight (DW) was observed during larval development, from 12.11 ± 0.31 µg for newly hatched zoea I, to 1025.52 ± 87.11 µg for newly molted megalopae. The organic content of larvae ranged between 58.18 ± 2.37% (zoea I) and 70.16 ± 0.68% (zoea III) of sample DW, and statistical analysis indicated significant differences in the percentage organic content between the larval stages. On this basis the results relating to fatty acid composition of larvae were presented on a per unit ash free dry weight (AFDW) basis. The total fatty acid content of larval tissues increased from 22.89 ± 3.72 µg mg^{-1} AFDW in newly hatched zoea I larvae, peaking at 31.38 ± 18.30 µg mg^{-1} AFDW at zoea V before dropping down to 24.92 ± 13.56 µg mg^{-1} AFDW at the megalopa stage. During the first zoea stage, saturated fatty acids (SFA) and highly unsaturated fatty acids (HUFA), dominated the fatty acid profile, while monounsaturated fatty acids (MUFA) were less abundant. As the larval developed the HUFA were depleted, while an increase in MUFA was recorded during the zoea V and megalopa stages, suggesting that HUFA requirements go down during the later part of larval development. The fatty acid composition was relatively stable throughout larval development, dominated by palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1*n*-9), arachidonic acid (20:4-*n*-6, AA), eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA). A substantial increase in linolenic acid (18:3*n*-3, LNA) levels was recorded in zoea V larvae, a result probably linked to the elevated LNA content in enriched *Artemia* meta-nauplii used as food for later stage larvae.

When subjected to starvation, crustaceans go through distinct phases of biomass degradation. This process is useful for identification of key fatty acids, and Chapter 8 reports on the changes in lipid and fatty acid metabolism observed in zoea V and megalopa subjected to feeding and starvation, respectively. The objective was to gain a better understanding of fatty acid utilization, and to identify possible changes in lipid requirements during the two last larval stages, which again may be linked to the high incidence of MDS during these larvae stages in mud crab hatcheries. Larvae were reared following established hatchery protocols, and newly molted zoea V and megalopae were subject to 4 days of feeding or starvation, and larval tissue was sampled for lipid analysis. An additional 6 day starvation treatment was incorporated for the megalopa stage as a response to the longer duration of this larval stage compared to the zoea stages. The results showed that fatty acids had an important role as an energy store in starved larvae, and the fatty acid content of larval tissue was reduced by 71.93% and 72.96% for zoea V and megalopa, respectively, during the 4 day starvation period. Further depletion of stored fatty acids was not, however, observed when megalopae were starved for another 2 days, indicating that other nutrient sources are utilized for energy when larvae are subject to prolonged starvation. Fatty acids from the polar lipid fraction dominated larval tissue, while fatty acids from the neutral lipid fraction were preferentially metabolized for energy during food deprivation. Depletion of both SFA, MUFA and HUFA was observed among unfed larvae, and in accordance with the findings in the previous chapter, depletion of EPA, DHA and AA indicated a possible decreasing requirement of these fatty acids during later larval development. Fed larvae, on the other hand, maintained more stable fatty acid levels, and they were able to sequester a range of fatty acids from live *Artemia.* Comparison of the lipid profile of

starved and fed larvae highlights an inability for *de novo* synthesis of both C18 fatty acids such as LNA, and HUFA like DHA, EPA and AA.

This project utilised integrated methodology to study lipid requirements of mud crab larvae. Growth trials were the most optimal way of specifying nutritional requirement, while biochemical analysis was used to gain a deeper understanding of the underlying mechanisms behind growth promotion. The findings have significantly enhanced our current understanding of dietary lipid requirements and utilization in mud crab larvae, which will have significant implications for the development of a nutritionally appropriate MBD for *S. serrata* larval culture in the future.

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Chapter 1

General Introduction

1.1 Mud crab farming; the past, the present and the future

Various species of mud crabs, *Scylla* spp., occur throughout the tropical to warm temperate zones of the Pacific and Indian oceans. Also known as mangrove crabs, they are commonly associated with mangrove swamps and nearby intertidal and subtidal muddy habitats where they can be caught using simple traps and nets (Keenan, 1999). The crabs remain alive for considerable periods after capture (Gillespic and Burke, 1992), and due to their large size, high meat yield and delicate flavour, they are considered a quality food item everywhere they occur (Rattanachote and Dangwatanakul, 1992). As a result, mud crab farming has become a significant industry throughout the Asia and Indo- Pacific region (Keenan, 1999).

Species identification of mud crab has been controversial, and for many years only one species in the genus *Scylla* was recognized (Fuseya, 1998). Based on genetic and morphological discoveries, however, Keenen et al. (1998) identified four species form the genus *Scylla* and revised their taxonomic nomenclature as *S. serrata, S. olivacea, S. paramamosain* and *S. tranquebarica.* Among the four mud crab species, *S. serrata* has the widest distribution range, and is commonly found from southern Africa to Tahiti, including the northern half of Australia, north to Okinawa, and south to the Bay of

Islands in New Zealand (Keenan, 1999). *S. serrata* is exploited throughout its range, and in Australia it is estimated to accounts for 99.5% of the total mud crab harvest (DPI&F, 2007).

1.1.1 Mud crab farming in the past and at present

Mud crab farming is by no means a new industry, as grow-out of juvenile crabs has been conducted for at least 100 years in China, and for more than 30 years in other Asian countries (Yalin and Qingsheng, 1994). More recently, sea-ranching of hatchery reared mud crab seed has been employed at a small scale in Japan, but due to a lack of reliable hatchery protocols, mass production of crab seed is still not reliable (Genodepa et al., 2006). Consequently, the majority of mud crab farms still rely on juveniles caught in the wild.

Two types of land based aquaculture is common; fattening of 'empty' crabs, or grow-out of juveniles (Keenan, 1999). Fattening of crabs with low meat content employs high densities of crabs at low costs, but due to cannibalism, total production can be low (Mann and Paterson, 2003). Grow-out methods for juveniles to market size, on the other hand, show more variety and the production output can be much higher. The grow-out systems are usually pond-based, with or without mangroves, and are typically classified as either intensive or extensive. Intensive systems employ high stocking rates and high levels of supplemental feeding, while extensive systems utilize lower stocking densities and no supplemental food (Quinitio et al., 2002). If ponds are stocked at appropriate densities, the mud crabs can grow from juvenile to adult size in 4-6 months, dependent on species and water temperature. This provides the potential for two crops per year in tropical regions, and it means that crabs can meet market demand outside the seasonal

catches from mud crab fisheries. Additionally, harvesting from farms can be timed to meet peak demand (Quinitio et al., 2002).

Grow-out facilities can be set up in rural costal communities where local employment opportunities are scarce, using simple, low cost technology. It is suitable for small-scale operations, with low environmental impacts and opportunities for employment of women. Development of the mud crab farming industry will also have spin-offs, as associated with most seafood enterprises, including an increased demand for transport and freight services and more jobs in packaging and processing (if the product is cooked or the meat picked), all of which will provide employment and business development opportunities. In Australia the grow-out of mud crabs by Aboriginal communities is seen as a farming practice that can complement traditional fishing and gathering activities, and in countries such as the Philippines, mud crabs are successfully farmed in polyculture with other species such as prawns (*Penaeus monodon*) and milkfish (*Chanos chanos*) (Agbayani, 2001).

1.1.2 Mud crab farming in the future; constrains and prospects

The major challenge restricting further expansion of mud crab culture is the limited supply of crab seed for stocking enclosures. Even at the current size of the mud crab culture industry, quantities of crab seeds caught by fishermen has not traditionally been sufficient to meet demand (Cowan, 1984; Liong, 1992; Keenan, 1999). Contributing to this is the loss of mangrove forest, overexploitation of wild stocks and recent growth in crab culture operations (Keenan, 1999).

In addition to recent hatchery trials in Japan, commercial production of crablets for farming has also commenced at a small scale in countries such as Vietnam and the Philippines (Baylon et al., 2004). In Australia crabs from hatchery production have been grown out in a commercial prawn farm and government institutions on an experimental basis. Still, low and inconsistent larval survival is a major problem hindering further industry development. High mortality in hatcheries has been linked to a lack of appropriate larval diets, and research into defining larval nutritional requirement and development of hatchery foods for the larval stages has begun (Nghia et al., 2007). Some promising results have been obtained, but the current knowledge lags far behind industry needs, and ongoing research is critically needed before a commercial larval food can be formulated (Genodepa et al., 2004b).

1.1.3 The mud crab market

Market opportunities for the mud crab industry are promising, and the demand for crab meat is rapidly increasing in countries throughout Asia, America and Europe (Brien and Miles, 1994; Globefish, 1995; Austrade, 1996). In addition to the live mud crab trade, there is a market for soft shelled crabs, and niche markets can be developed for 'egg crab' (females with eggs), all male crabs (as they grow faster and larger than females) and for canned crab meat. Special markets also exist for banquet size crabs (over 1 kg) with a peak demand around New Years and Chinese New Year celebrations (Williams, 2002).

The mud crabs are luxury food items and are well-appreciated for their exquisite taste and texture (Angell, 1992). They are low in fat, high in protein, and are rich in vitamin and minerals. As the prawn industry has come under threat of viral diseases in the recent

years, many farmers are facing major losses. As a result, mud crab aquaculture is expanding rapidly throughout its range, and it is widely recognized that it will grown into a major industry in the near future if the problem with seed-stock shortage is addressed (Angell, 1992; Keenan and Blaskshaw, 1999).

1.2 A review of aspects influencing the use of formulated diets in *Scylla serrata* hatcheries, and current knowledge of nutritional requirements for *S. serrata* larvae

1.2.1 Problems associated with use of live food in larval culture

As previously discussed, one of the main challenges for mud crab hatcheries is the lack of appropriate larval diets. Seed production of most aquatic animals, including *Scylla* spp., relies on live foods such as rotifers and *Artemia* nauplii (Brick, 1974; Heasman and Fielder, 1983; Mann et al., 1999; Zeng and Li, 1999; Hamasaki, 2003), as these live foods have several beneficial characteristics; they are slow swimmers, available in different sizes and are hardy enough to be suitable for mass culturing (Verischele, 1989). From a nutritional perspective, however, rotifers and *Artemia* are far from ideal, as they show nutritional inconsistency depending on source, age and culture technique (Sorgeloos et al., 1986; Tucker, 1992). They also lack certain highly unsaturated fatty acids (HUFA) essential for growth and survival of marine larvae (Southgate, 2003), and they are known to be a vector for introduction of pathogens into the larvae culture (Person-Le Ruyet, 1990). To overcome the nutritional deficiency it is common practice to 'enrich' live foods to enhance their levels of important HUFA, particularly decosahexaenoic acid (22:6*n*-3, DHA) and eicosapentaenoic acid (20:5*n*-3, EPA), prior to introduction to the larval rearing tanks (Southgate and Lou, 1995). This practice

results in increased growth and survival of larvae for many species and has been viewed as a solution to the nutritional deficiency problem associated with the live prey. However, recent experiments conducted on the effectiveness of this enrichment method has shown that despite elevated HUFA levels in *Artemia* following enrichment, these fatty acids may not be readily available to crustacean larvae. For example, a study conducted on the larvae of rock lobster, *Panulirus cygnus* showed that DHA in tissue of larvae fed enriched *Artemia* was low compared to the level in *Artemia* itself, indicating that crustacean larvae have a limited ability to absorb DHA directly from *Artemia* (Liddy et al., 2004). This may be linked to *Artemia*'s ability to metabolize dietary DHA for energy (Dhert et al., 1993; Danielsen et al., 1995; Triantaphyllidis et al., 1995) or alternatively, DHA is being convert to EPA in *Artemia* shortly after enrichment (Han et al., 2001).

Live foods production in aquaculture hatcheries is further disadvantaged by the need for specialized personnel, dedicated equipment and facilities, and the need for micro-algae culture as a food source for the live food culture. On this basis, live prey production may account for 50-75% of the total running costs of aquaculture hatcheries (Dainteath and Quin, 1991). Finally, as *Artemia* cysts are collected from the wild environment they are subject to an inconsistent supply, unpredictable prices and varying quality. These factors influence the sustainability of the use of *Artemia* as a food source and may present a serious bottleneck for the global aquaculture industry in the coming years (Lavens and Sorgeloos, 2000).

1.2.2 Development of a formulated diet for hatchery production of Scylla serrata

In response to the problems associated with the use of live foods, research into the development of alternative diets has become increasingly important (Teshima et al., 1982; Kanazawa et al., 1985; Guerden et al., 1995; Jones, 1998). Although total replacement of live prey is routinely accomplished in the laboratory for penaeid prawns (Kanazawa et al., 1982; Jones et al., 1987; Galgani and Aquacop, 1988), this practice has been less successful for caridean and other crustaceans. In prawn hatcheries, it is standard practice to use formulated foods, at least as a supplement or in co-feeding with live foods, but complete replacement with the elimination of algal culture units is still rare (Teshima et al., 2000). The recent focus on development of micro-particulate food particles for crustaceans, fish, and even bivalves (Knauer and Southgate, 1999) has facilitated research into the factors controlling larval ingestion, digestion, and energetic requirements and allowed identification of some major nutritional requirements. As herbivorous larvae are able to digest food that cannot contribute enzymes towards autolysis, energetic studies suggest that the feeding strategy adapted by herbivorous larvae is more efficient than that adopted by carnivorous crustacean larvae (Jones et al., 1993; Kumlu and Jones, 1995b). This may explain the success achieved with formulated diets for the former, and identifies the need for easily digestible high-energy diets for carnivore crustacean species like *S. serrata* (Jones, 1998).

1.2.2.1 Types of food particles

Several different types of formulated diet particles with potential for use with crustacean larvae have been developed. These are better known as microparticulate diets and have commonly been categorized as either microencapsulated diets (MED) or microbound diets (MBD) (Kanazawa, 1986). Although less common, commercial larval foods are

also available as microcoated diets (MCD), flakes, granulated foods and liquid foods (lipid walled capsules) (Fegan, 2004). A shared advantage of these formulated diet particles is that unlike live foods, the size and composition of microparticulate diet particle can be adjusted to suit the exact requirements of the various species and the different larval stages (Southgate and Partridge, 1998). Examples of the use of various microparticulate particles in experiments with crustacean larvae are shown in Table 1.1.

Table 1.1 Formulated diets previously tested as live food replacement for various crustacean species. MBD = microbound diets, MBD = microencapsulated diets, PL = postlarvae, M = mysis, P = protozoea, Z = zoea, ML = megalopa, C1 = first crab stage.

Species	**Type of diet**	**Survival**	**Reference**
Penaeus monodon	MED	3-29% to PL7	(Jones et al., 1987)
P. monodon	MBD	85% to M1	(Galgani and Aquacop, 1988)
P. monodon	MED	51-64% to PL	(Kurmaly et al., 1989b)
P. monodon	Microcapsules + algae	76%	(Jones et al., 1989)
P. monodon	MED	80% to PL1	(Amjad et al., 1992)
P. monodon	MBD	52% VI nauplii to mysis	(Paibulkichakul et al., 1998)
P. japonicus	MED	50% to PL1	(Jones et al., 1979)
P. japonicus	MBD	90% to PL1	(Kanazawa et al., 1982)
P. japonicus	MBD	80% to PL	(Kanazawa et al., 1985)
P. japonicus	MBD	75% to PL1	(Kanazawa, 1990a)
P. japonicus	MED + algae	79.5% to PL1	(Le Vay et al., 1993)
P. indicus	MBD	62% to M1	(Galgani and Aquacop, 1988)
P. indicus	MED	55.25% to M1	(Kumlu and Jones, 1995a)
P. indicus	MBD	100% PL20-PL50	(Immanuel et al., 2003)
P. stylirostris	MED + algae	65% to PL7	(Jones et al., 1987)
P. vannamei	Microcapsules + algae	80% to PL7	(Jones and Kurmaly, 1987)
P. vannamei	MBD	47% to M1	(Galgani and Aquacop, 1988)
P. vannamei	MED	97.3% PIII to mysis	(Pedroza-Islas et al., 2004)
P. vannamei	MED + algae	98.6% PIII to mysis	(Pedroza-Islas et al., 2004)
Macrobrachium rosenbergii	MED	84% from Z4 to PL1	(Deru, 1990)
M. rosenbergii	MBD	77.3% from Z5 to PL1	(Kovalenko et al., 2002)
Palaemon elegans	Micro-granulated diets	49% from Z5 to PL1	(Kumulu and Jones, 1995)
Crangon nigricauda	*Artemia*-microcapsules	None beyond ZII	(Villamar and Brusca, 1987)
Homarus gammarus	MBD	None beyond stage III	(Kurmaly et al., 1990)
Eurypanopeus depressus	Microcapsules + rotifers	83-93% to ML	(Levine and Sulkin, 1984)
Portunus trituberculatus	Microcapsules + rotifers	16.1% to juvenile	(Kanazawa et al., 1983)
Scylla serrata	MBD	90% from ML to C1	(Genodepa et al., 2004b)

Microencapsulated diets are composed of dietary material encapsulated within a capsule wall that separate the bulk of the diet from the water in which the MED is suspended (Teshima et al., 1982) (Fig. 1.1). This limits leaching of the dietary components and it assists in maintain the integrity of the MBD particles until eaten (Meyers, 1979). However, as some leaching is considered important to stimulate feeding activity in crustaceans, MED may be the more appropriate for presenting formulated diets to fish and bivalve larvae than for some crustacean larvae.

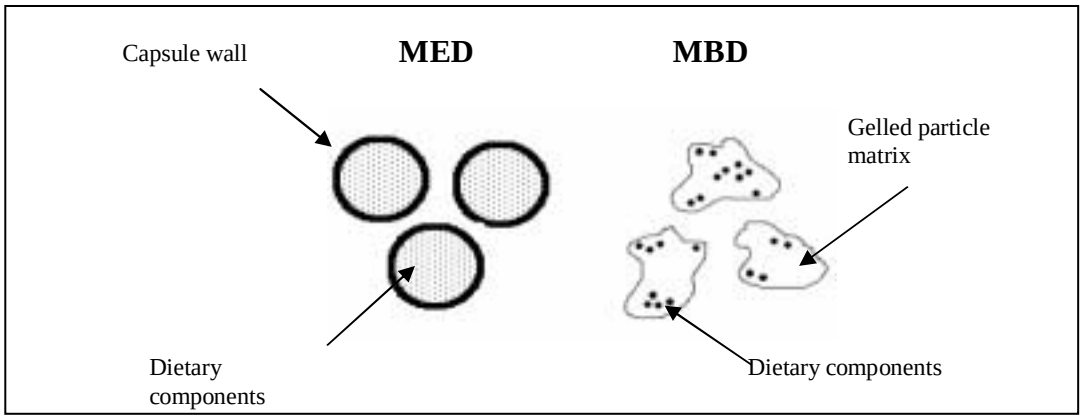

Fig. 1.1 Generalized structure of microencapsulated diet (MED) and microbound diet (MBD) particles.

Microbound diet (MBD) particles on the other hand, have found broad application in nutritional studies with crustacean larvae, and provide an alternative to MED for species like *S. serrata*, which may rely on water-borne nutrients to detect food items (Borner et al., 1986; Kurmaly et al., 1990). MBD particles are produced by mixing diet ingredients with a binder such as alginate, carrageenean, agar, zein or gelatin, before the mixture is oven- or freeze-dried, ground and sieved to obtain particles of an appropriate size range (Knauer and Southgate, 1999; Kumulu, 1999). MBD particles are inexpensive to

produce, offer “off-the-shelf” convenience and allow for short to medium term food storage (Southgate and Partridge, 1998). Several hatcheries and laboratories around the world have reported successful use of MBD (Kanazawa et al., 1982; Galgani and Aquacop, 1988; Liao et al., 1988; Kanazawa, 1990a); however, as this diet shows relatively poor stability in water, there are potential problems relating to water quality and bacterial proliferation, as well as nutrient deficiency resulting from leaching (Amjad et al., 1992).

1.2.2.2 Binding agents

Most diets formulated to suit the larvae of carnivorous species have high animal protein content. These ingredients contain few natural binding agents, and incorporation of an ‘external’ binder is therefore important (Melcion, 2001). Binder type and binder strength are likely to play important roles in determining the attractiveness and digestibility of MBD, and many different binders have been used in the formulation of aquaculture feeds (Partridge and Southgate, 1999). Some of these binders, such as wheat gluten or pregelatinised starches, can have a nutritive value for the animals, while other are inert raw materials used purely as gelling agents or thickeners in the diet (Melcion, 2001). Synthetic binding agents are also available, but although these are efficient, they may be harmful to the larvae or the consumers. This applies particularly to binders like formaldehyde (Melcion, 2001). Even though a few successful commercially produced MBD are available, the search for the best binding agent in crustacean diets is ongoing. Kanazawa (1985) conducted experiments with kuruma prawn, *Penaeus japonicus*, larvae using a diet bound with carrageenan at 5% of total diet dry weight. Paibulkichakul et al. (1998) used the same binder in experiments with tiger prawn, *Penaeus monodon,* larvae and both authors reported stable diet particles

that were suitable for nutritional studies with crustacean larvae. In studies with *S. serrata* larvae, Genodepa et al. (2006) used ^{14}C-labeled MBD particles to determine the ingestion rate of diet particles prepared with either zein, gelatin, agar, alginate or carrageen as the binding agent. Ingestion rates of the various particle types by the different larval stages were not significantly different, indicating that any of the tested binders could successfully be used in production of MBD particles for this species. From a nutritional perspective, however, given that mud crab larvae are carnivorous, a protein binder (e.g. zein or gelatin) may be more appropriate than a polysaccharide binder (e.g. agar, alginate or carrageen) (Genodepa et al., 2006).

1.2.2.3 Particle size

An important consideration when presenting formulated diets to crustaceans is particle size. In order to maximize ingestion, particle size should be appropriate for the size of the larval mouth, and should gradually be increased as the larval grow (Knauer and Southgate, 1999). In general terms, the size of dried food particles should be smaller than that of the live pray (rotifers and *Artemia* nauplii) which they replace, as formulated food particles are harder, and therefore degrade more slowly in the mouth and oesophagus of larvae. In addition, MBD particles are prone to swelling once immersed in water (Genodepa et al., 2004a).

Studies with penaeid prawn larvae have shown that optimum diet particle size varies with species, and that the preferred particle size increases as the larvae grow. Within the genus Penaeus, for example, *P. monodon, P. japonicus* and *P. indicus* larvae feed on small particle sizes (5-25 µm), while larvae of *P. stylirostris, P. kerathurus* and *P. vannamei* are capable of feeding on larger particles (5-50 µm) (Galgani and Aquacop,

1988). Jones (1998) further showed that first feeding *P. monodon* larvae only ingested particles similar in size to that of algal cells (3-30 µm), while second stage zoea larvae were capable of capturing food items up to 100 µm in size.

The feeding behavior of *S. serrata* larva is yet to be described in detail, but the ability of zoea I larvae to feed on both rotifers and *Artemia* (Heasman and Fielder, 1983; Zeng and Li, 1999) indicates that the feeding method is raptorial, and that the larvae are able to ingest a wide size range of food particles. Genodepa et al. (2004b) examined particle size acceptance of the various larval stages of *S. serrata*, and suggested a preferred particle size of <150 µm for zoea I, 150-250 µm for zoea III, 250-400 µm for zoea V and 400-600 µm for the megalopa stage. However, the 400-600 µm particle size range did not facilitate significantly greater ingestion than the 600-800 µm particles for megalopae, and the acceptance of particles larger than 800µm was not tested. Research on larvae of other crustaceans, including the American lobster, *Homarus americanus*, has shown that particles in the size range of 800-1200 µm are preferred during late larval development when claws are present (Fiore and Tlusty, 2005). Based on this it might be useful to test the acceptance for equally larger food particles sizes for *S. serrata* megalopae in order to minimize the surface area of suspended particles, which again can significantly reduce nutrient leaching and loss of important dietary components.

1.2.2.4 Considerations in development of formulated larval diets

Successful development of a formulated diet for larval stages of crustaceans requires an understanding of the behavioral, mechanical and physiological processes of feeding in the target animal. Mud crab larvae, unlike those of penaeid prawns, are carnivorous and

feed on zooplankton from the first feeding stage (Genodepa et al., 2004b). However, experiments conducted on ingestion and retention rates of food particles by *S. serrata* suggests that feeding behavior varies both from larval to adult form, and between the various larval stages; megalopae seem to feed continuously, while newly hatched zoea I larvae feed less frequently. This may indicate that young larvae still possess and metabolize maternally-inherited energy reserves (Genodepa et al., 2004b). Other studies suggest that early larval stages (zoea I-V) are passive in their feeding behavior catching food by chance, while the more developed megalopae actively pursue food items (Zeng, 1998; Zeng and Li, 1999). This resulted in significantly higher ingestion rates at the megalopa stage, and it implies that replacement of live foods with MBD may be more successful at zoea V and megalopa stages. From a cost saving perspective, the long duration of the megalopa stage (8-10 days) compared to that of the zoeal stages (3-5 days) indicate that development of an MBD for the older larval stages will be most cost-effective; however, complete or partial replacement at all larval stages would clearly be beneficial, and this aspect warrants further study.

Water quality may become an issue when substituting live food with a formulated diet. Over-feeding is generally not a problem while using rotifers and *Artemia* that remain alive and in suspension until consumed, but formulated diet particles are negatively buoyant and may accumulate at the bottom of the tank shortly after feeding. This makes the food particles less available to larvae, and nutrient leaching from the uneaten food may negatively affect water quality (Genodepa et al., 2004a). Feeding a ration as close as possible to the required rates is therefore important to minimize particles settlement and to optimize water quality, and the practice of feeding many small rations throughout the day may be advantageous.

1.2.2.5 Digestibility and enzyme activity in *Scylla serrata* larvae

A formulated diet can be well balanced and contain all the dietary essential nutrients, but still not produce good survival and growth if the various nutrients are not readily available to the larvae. The true nutritive value of a formulated food therefore ultimately depends on the bioavailability of the ingredients, and not purely on diet composition (Lee and Lawrence, 1997). Animals rely on a functional digestive system to efficiently utilize the nutrients present in the food (Anderson and De Silva, 2003), and the morphology of the digestive tract, the physiological conditions of the larvae and the rearing environment all play major roles in determining the digestibility of a feedstuff (Lee and Lawrence, 1997).

Crustacean larvae are generally small, poorly developed and lack a fully functional digestive system. For many fish species it has been shown that underdeveloped larvae lack the enzymes required for efficient breakdown of food particles, and therefore rely on the enzymes present in live food organisms (such as rotifers and *Artemia*) to assist digestion (Bromage and Roberts, 1995). This means that total replacement of live food is often not a viable option, especially during the early larval stages. Following ingestion, the enzymes in the live prey are transferred to the digestive tract of crustacean larvae by autolysis or as zymogens, which again activates endogenous digestive enzymes within the larval gut (Kumulu and Jones, 1995). Some work has been done on the inclusion of digestive enzymes (particularly proteases) in formulated diets for fish, and results have shown that it is possible to improve nutrient assimilation and larval growth by up to 30% (Jones et al., 1997b). For crustaceans, inclusion of digestive enzymes in larval diets is not common, but supplementation of formulated diets with algal extracts has been demonstrated to enhance tryptic activity in penaeid protozoea

(Kumlu and Jones, 1995c). This resulted in enhanced digestion of the formulated diet, which again promoted growth and survival. These results suggest that addition of digestive enzymes and chemoattractants to crustacean diets can reduce reliance on live food in mud crab zoeal culture, a topic that warrants further study.

The natural level of enzyme activity in the digestive tract has been studied in some species, and despite a common belief that most crustacean larvae lack pepsin and stomach acid in early larval stages (Conklin, 1980; Fair et al., 1980; Lee et al., 1984; Lee and Lawrence, 1985; Dall et al., 1990), Jones et al. (1991) reported that enzyme activity in penaeid larvae can be at high levels immediately after hatch. These authors showed that a correlation exists between the level of enzyme present and gastro-evacuation rate, and that the highest enzyme activity coincided with the shortest gut evacuation time. Similar work was conducted on *S. serrata* larvae, where radiotracer studies showed that gut residence time was shortest in poorly developed early zoea stages, increasing substantially as the larvae got more developed (Genodepa et al., 2006). The specific enzyme activity in *S. serrata* larvae was further investigated by Tang et al. (1995), who identified four digestive enzymes in the larval gut; protease, alpha-amylase, cellulose and lipase. These authors further reported that enzymes activity varied between the different developmental stages, and was strongly influenced by feeding conditions and energy demands.

As the ability of crustacean larvae to produce enzymes is closely related to the development of the digest tract, an understanding of the morphological and physiological changes associated with the different larval stages is important and should be clearly defined. For *S. serrata,* major changes have been observed as the larvae molt

from zoea III to zoea IV, where the number of abdominal segments increase from five to six, the gastric mill of the digestive system starts to develop (Ong, 1964), and the hepatopancreas ecomes more functional (Li and Li, 1998). Quinitio et al. (1999) conducted a study to examine the potential for total or partial replacement of live food fed to zoea I of mud crabs and reported on lower growth rates and a high incidence of deformities for co-fed larvae compared to the controls fed live food only. Furthermore, zoea I larvae did not survive to the zoea III stage when fed a formulated prawn diet alone, although this diet had a fatty acid composition (particularly the EPA and DHA) closer to that of crab zoea than live food (rotifers and *Artemia*). In the same experiment, larvae from the co-fed treatment were able to molt to the megalopa stage, while best overall survival was recorded for larvae fed a full ration of live food.

Similar work has been done with *S. serrata* zoea III, where larvae were fed either 100% *Artemia*, 100% experimental MBD or a 50%:50% combination of *Artemia* and MBD (Holme et al., 2006). Highest survival and fastest development to the zoea IV stage were recorded for the larvae fed the 50%:50% combination of the two foods, while some successful molts were found among larvae fed MBD exclusively. These results may indicated that the experimental MBD used in this experiment contained a favorable HUFA profile compared to that of live food, but due to the lack of a fully functional digestive system, zoea III larvae still relied on the enzymes present in the live food to be able to utilize the beneficial nutrients in the MBD. However, experiments by Genodepa et al. (2004b) with *S. serrata* megalopa showed that 100% replacement of live food with MBD is possible at this larvae stage, as survival of MBD-fed megalopa was identical to that of megalopa fed live *Artemia*. These encouraging results suggest that *S. serrata* megalopae have a full complement of digestive enzymes that efficiently catalyze the

breakdown of foods, which again implies significant potential for the use of MBD in future experiments to investigate larval nutritional requirements.

Although larvae of some crustaceans are able to fully digest formulated diets, the diet particles may not be recognized by the larvae as food. Weaning onto microbound diets may therefore only be possible after larvae have been reared with live food for some time (Teshima et al., 2000). Experiments with mud crab megalopa have shown that high mortality was associated with 100% MBD diets during the first days after introduction of the MBD; however, daily survival increased significantly as larvae became used to the MBD (Genodepa et al., 2004b). Similarly, less synchronous molting of megalopae fed MBD alone reflected variation in the ability of larvae to adjust from *Artemia* to a MBD diet, and highest survival was found where the larvae were fed a combination of MBD and *Artemia* (Genodepa et al., 2004b). Stress associated with diet weaning process may therefore be reduced if an appropriate mixture of the two foods is provided.

1.2.2.6 The use of formulated diets to investigate nutritional requirements

The dependence of larvae upon live food has made the study of their nutritional requirements difficult (Jones et al., 1997a). Dietary requirements have traditionally been investigated by examining the nutritional composition of live prey, and as a result, gross biochemical analysis of both phyto- and zooplankton in terms of protein, lipid and carbohydrate levels are well documented (e.g. Raymont, 1983). Unfortunately, the results obtained from cultured larvae do not appear to directly relate to analysis conducted on wild caught larvae (Rodriguez et al., 1994), suggesting that nutrient content of *Artemia* and rotifers vary significantly from that of the natural diet in the wild. Therefore, although providing some basic information on the array of nutrients

consumed, analysis of cultured live prey cannot reveal the precise nutritional requirements of the larvae (Jones et al., 1997a). The alternative approach is to use formulated diets (Villamar and Langdon, 1993), and once a preferred particle size and optimum ration for each larval stage have been determined, the ingredients can easily be adjusted and the optimum level of each nutrient established through experimental growth trials. Over the last 20 years this technique has proven useful in the development of appropriate diets for both fish (Kanazawa, 1993b; Kanazawa and Tago, 1998; Tago et al., 1999) and crustaceans (Kanazawa et al., 1985; Kanazawa, 1993b; Teshima, 1998). For *S. serrata* larvae, the search for an optimized diet has started, but due to difficulties in larval rearing and problems associated with larval reliance on live food, studies of the nutritional requirements have been limited. However, as the recent breakthrough in use of MBD as a nutrient source for megalopae has made studies of the nutritional requirements at this larval stage possible, enormous potential lies in the use of MBD as a means for development of optimized mud crab hatchery diets in the near future.

1.2.3 The nutritional requirements of Scylla serrata larvae

The following sections outline the known information on nutritional requirements of *S. serrata* larvae and juveniles. As the focus of this thesis is on lipids, the main emphasis will be on dietary lipid requirements, but for the purpose of completeness, requirements for other nutrients are also briefly discusses. As limited research has been conducted on dietary requirements of *S. serrata* larvae, known nutritional requirements for larvae of other crustacean species have been included for comparison when necessary.

1.2.3.1 Protein

Proteins are composed of amino acids, the key group of essential nutrients required by all animals for growth (Glencross, 2006). Protein is also the largest and most expensive component of aquaculture diets, and nutritionists therefore aim to formulate diets in which the energy required by the animal is provided by non-protein sources, sparing protein for growth (Anderson and De Silva, 2003). The ability to spare protein differs between species, and an imbalance in the ratio of dietary protein to other energy sources leads to either wasted protein, or the production of lower-value 'fatty' animals (D'Abramo, 1998). A good understanding of the protein requirements is therefore a key factor in development of a well balanced, cost effective formulated diet.

Total protein requirement

The optimal protein level in larval diets varies between species, development stages (Durruty et al., 2002) and protein sources, and it is strongly influenced by the digestibility and amino acid composition of the protein components (Le Vay et al., 1993). A summary of the protein levels used in diet for larval crustaceans is shown in Table 1.2. Most diets formulated for larvae and juvenile crustacean contain between 30% and 50% protein (Conklin et al., 1980; Briggs et al., 1988; Thongrod and Boonyaratpulin, 1998; Sheen, 2000), although higher dietary protein levels have been used successfully for larvae of *P. monodon* (Paibulkichakul et al., 1998) and *P. japonicus* (Moe et al., 2004). For adult crustaceans it has been reported that the protein requirement is higher for carnivores species such as *P. japonicus* compared to that of herbivorous species such as *P. vannamei* (Kanazawa, 1990b), differences that have been attributed to evolution and adoption to specific feeding habitats (Guillaume, 1997). This theory was strengthened by the work of Chuang (1990) who proved that high protein

requirements correlated with high proteolytic activity in the digestive tract of carnivorous species. Based on this information it can be assumed that *S. serrata* larvae, which are carnivorous and display high protease activity from hatch (Hong et al., 1995), have a high protein requirement similar to that of other carnivorous crustacean larvae.

Table 1.2 Examples of dietary protein levels previously used in formulated diets for crustacean juveniles and larvae.

Species	**Dietary protein**	**Reference**
Larvae		
Penaeus monodon	44%	(Kurmaly et al., 1989a)
P. monodon	30%	(Khannapa, 1979)
P. monodon	55%	(Paibulkichakul et al., 1998)
P. monodon	48-52%	(Kurmaly et al., 1989a)
P. japonicus	50%	(Kanazawa et al., 1985)
P. japonicus	44%	(Besbes, 1987)
P. japonicus	45-55%	(Kanazawa, 1990a)
P. japonicus	56%	(Moe et al., 2004)
P. indicus	40.0-40.8%	(Immanuel et al., 2003)
P. vannamei	52.7%	(Pedroza-Islas et al., 2004)
P. setiferus	52.7%	(Gallardo et al., 2002)
Macrobrachium rosenbergii	46.1%	(Kovalenko et al., 2002)
M. rosenbergii	56.9-57.6%	(Kamarudin and Roustaian, 2002)
Homarus americanus	57%	(Fiore and Tlusty, 2005)
Scylla serrata (megalopa)	79.4%	(Genodepa et al., 2004b)
Juvenile		
P. monodon	46%	(Lee, 1971)
P. monodon	35-40%	(Lin et al., 1982)
P. monodon	40%	(Alava and Lim, 1983)
P. monodon	40-50%	(Bautista, 1986)
P. monodon	40-44%	(Shiau et al., 1991)
P. monodon	36-40%	(Shiau and Chou, 1991)
P. monodon	42.9%	(Merican and Shim, 1996)
P. setiferus	50%	(Brito et al., 2000)
Macrobrachium rosenbergii	47.91-50.76%	(Briggs et al., 1988)
Homarus americanus	50%	(Conklin et al., 1980)
H. americanus	50%	(Kean et al., 1985)
Panulirus ornatus	30-55%	(Smith et al., 2003)
Cherax quadricarinatus	50%	(Thompson et al., 2003)
Eriocheir sinensis	39-42.5%	(Mu et al., 1998)
Scylla serrata	50%	(Sheen and Wu, 1999)
S. serrata	50%	(Sheen, 2000)
S. serrata	34.2-51.8%	(Catacutan, 2002)

Although protein requirements are well documented for larvae and juveniles of several prawn and lobster species with aquaculture potential (Guillaume, 1997), limited information is available on the protein requirements of the various crab species. One study has been conducted on juveniles of the Chinese mitten crab, *Eriocheir sinensis*, and an optimum dietary protein level of between 39.0% and 42.5% was reported (Mu et al., 1998). For mud crab larvae, however, no such experiment has been conducted, and as a result all experimental diets used to date have been formulated on the basis of known protein requirements for other crustacean larvae. Sheen and Wu (1999) and Sheen (2000) reported on good growth and survival when mud crab juveniles were fed a MBD containing 50% lipid-free casein, and Genodepa et al. (2004a) showed that a MBD containing 39.7% squid meal and 39.7% rotifer meal provide good growth and survival in feeding trails at the megalopa stage. These findings indicating that *S. serrata* larvae, like penaeid larvae (Rodriguez et al., 1994), may have a dietary protein requirement that is higher than that reported for juvenile and adult stages. Nevertheless, a significant research effort is still needed to specify the optimal level and source of dietary protein for the various larval stages of mud crabs.

Essential amino acids

An effective dietary protein source must satisfy an animal's requirement for both essential and non-essential amino acids (Guillaume, 1997). The essential amino acids of crustaceans are well known from studies on adults of several species, including the Dungeness crab, *Cancer magister* (Lasser and Allen, 1976), the common prawn, *Palaemon serratus* (Cowey and Forster, 1971), the brown prawn, *Penaeus aztecus* (Shewbart et al., 1972), the kuruma prawn, *P. japonicus* (Kanazawa et al., 1981), the giant tiger prawn, *P. monodon* (Coloso and Cruz, 1980), the giant freshwater prawn,

Macrobrachium rosenbergii (Watanabe, 1975), the Ohio prawn, *Macrobrachium ohione* (Miyajima et al., 1977), the European crayfish *Astacus astacus* (Zendee, 1966) and the American lobster, *Homarus americanus* (Gallagher and Brown, 1975). These studies are all in agreement, indicating that arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are essential in the crustacean diet. Although not strictly required, tyrosine and cystine should be considered semi-essential as their presence in a diet reduces the requirement of phenylalanine and methionine, respectively (Guillaume, 1997). However, the qualitative requirement varies at different life stages, and as the traditional method for determining qualitative essential amino acid requirements are long, complex and unsuitable for small marine larvae (Bengtson, 1993), very little research has been conducted on the precise amino acid requirements of developing crustacean larvae.

From a dietary formulation perspective, it should be noted that some responses to dietary protein sources seem to be independent of their amino acid profile. Squid protein, for example, has a biological value that is higher than would be expected from its amino acid profile (Deshiramu and Shigheno, 1972; Fenucci and Zein-Eldin, 1976), and Cruz and Guillaume (1983) suggested that an unknown growth factor is present in squid protein. Incorporation of purified amino acids into diets may improve growth performances through their qualities of attraction and stimulation of food intake, and protein sources or amino acids may therefore be incorporated into crustacean diets for a reason other than provision of nutrients (Guillaume, 1997).

1.2.3.2 Lipids and sterols

Dietary lipids are known to play an important role in animal nutrition as they provide energy, maintain the structural integrity of biological membranes and function as precursors for important steroids (Corraze, 2001). The optimum level of dietary lipid for crustaceans juveniles and adults generally range from 2% to 10% (Deshiramu et al., 1979; Davis and Robinson, 1986; Sheen and D'Abramo, 1991a; Sheen, 1997) (Table 1.3), but studies indicate that lipid requirements vary not only between species, but also between larvae and adult stages, making detailed and specialized studies necessary. Diets with deficient levels of lipids generally result in lower weight gain and reduced molting frequency (Sheen and Wu, 1999), while animals fed diets with excessive levels of lipid show reduce growth due to insufficient lipid utilization. This is particularly common when other energy sources are available, and it results in lipid accumulation in the tissue and lower meat quality (Castell and Covey, 1976; Ponat and Adelung, 1980). The requirement for dietary lipid is furthermore influenced by the digestibility of the lipids, which again is controlled by the total lipid level and the overall fatty acid composition in the diet. In experiments with adult *P. monodon,* for example, lipid levels under 4.5% and over 10% reduced digestibility significantly (Glencross et al., 2002). For mud crabs the effect of dietary lipid levels on growth of juveniles were investigated by Sheen and Wu (1999), and the results suggested that dietary lipid level between 5.3% to 13.8% meet the dietary needs of the crabs. Interestingly, 13.8% lipid was the highest level tested, and no significant reduction in survival or growth was observed in this treatment compared to other treatments. Furthermore, no lipid accumulation was observed in the tissue of these crab juveniles, suggesting that *S. serrata* more effectively utilize higher dietary lipid levels than most other marine crustaceans studied to date. From a commercial standpoint these results are promising, as high lipid diets have a

protein-sparing effect and allow for production of low cost diets (Anderson and De Silva, 2003).

Table 1.3 Summary of dietary lipid levels used in previous nutritional studies with crustacean larvae.

Species	Dietary level	Reference
Larvae		
Penaeus monodon	8%	(Paibulkichakul et al., 1998)
P. monodon	4.3%	(Kurmaly et al., 1989b)
P. monodon	18.2%	(Kurmaly et al., 1989a)
P. monodon	8%	(Paibulkichakul et al., 1998)
P. japonicus	8-16.6%	(Kanazawa et al., 1985)
P. japonicus	8.7%	(Moe et al., 2004)
P. japonicus	5.5%	(Kanazawa, 1990a)
P. indicus	8.9-13.3%	(Immanuel et al., 2003)
P. vannamei	11.5%	(Pedroza-Islas et al., 2004)
Macrobrachium rosenbergii	37.4%	(Kovalenko et al., 2002)
M. rosenbergii	12.3-12.6%	(Kamarudin and Roustaian, 2002)
Homarus americanus	12-19%	(Fiore and Tlusty, 2005)
Scylla serrata (megalopa)	6%	(Genodepa et al., 2004b)
Juvenile		
Penaeus monodon	7.4%	(Merican and Shim, 1996)
P. indicus	9-12%	(Shivaram and Raj, 1997)
P. vannamei	7.88%	(Gong et al., 2000b)
P. vannamei	8.78-9.99%	(Gong et al., 2000a)
P. vannamei	8.6%	(Pascuala et al., 2002)
Macrobrachium rosenbergii	8.46-11.95%	(Briggs et al., 1988)
M. rosenbergii	2-10%	(Sheen and D'Abramo, 1991b)
Homarus americanus	6%	(Conklin et al., 1980)
H. americanus	10-17%	(Kean et al., 1985)
Cherax quadricarinatus	6%	(Thompson et al., 2003)
Scylla serrata	1.7-13.8%	(Sheen and Wu, 1999)
S. serrata	8-9.5%	(Sheen, 2000)
S. serrata	6 and 12%	(Catacutan, 2002)

Essential and non-essential fatty acids

Studies have shown that *S. serrata*, like many other marine crustaceans, lack the enzymes needed for *de novo* synthesis of long chained highly unsaturated fatty acids (HUFA) (Sheen and Wu, 2003). These fatty acids are therefore considered essential dietary components, and deficiency in supply of the *n*-3 HUFA eicosapentaenoic acid

(20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA), for example, has been identified as a major cause of low survival, longer intermolt period and narrower carapace width of newly molted *S. serrata* larvae (Suprayudi et al., 2004b) and juveniles (Sheen and Wu, 1999). Current literature suggests that fatty acids also affect the reproductive response in crustaceans, as EPA affects the egg lipid content and fecundity, and DHA affects egg hatchability (Kobayashi et al., 2000; Wen et al., 2002). Kobayashi et al. (2000) further suggested that EPA is important for survival in *Scylla tranquebarica* larvae, while DHA is important in promoting growth. The different actions of EPA and DHA may be explained by the different tissues affected by these fatty acids, as DHA is exclusively found in neural tissue, brain and eye-stalk, and EPA is distributed evenly throughout the animal (Bell et al., 1995; Masuda et al., 1999).

Lack of dietary supply of HUFA has also been recognized as a possible cause of molting death syndrome (MDS) (Hamasaki et al., 2002), a phenomenon characterized by the inability of crustacean larvae to completely shed the old carapace before the new one hardens (Fielder and Heasman, 1999; Thompson et al., 2003). MDS is commonly observed when mud crab larvae molt from the zoea V to the megalopa stage, or from megalopa to the first crab stage (C1). The affected larvae display various degrees of deformities, which again result in mass mortalities (Hamasaki et al., 2002). Molting in crustaceans is regulated by ecdysteroids (Subramoniam, 2000) which are secreted by the Y-organ located at the eye-stalk (Naya and Ikeda, 1993) and are released into the haemolymph from where they directly control molting (Subramoniam, 2000). Experiments on the tick, *Ornithodoros moubata,* have shown that ecdyson is metabolized in conjugation with C22 fatty acids (Doston et al., 1993), and based on this it has been assumed that C22 fatty acids, such as DHA, associated with molting

regulation in crabs. This again can be related to DHA's important role in accelerating the intermolt period and production of wider carapace widths in the first crab stage (Takeuchi et al., 1999), and the possible link between incorrect supplementation of DHA in the diets and the occurrence of MDS in larvae cultures (Suprayudi et al., 2004a).

Determining the accurate requirement for individual fatty acids is complicated as aquatic animals require very small amounts of essential fatty acids, and the likelihood of other ingredients used in test diets containing HUFA of unknown composition is high. It is therefore extremely difficult to be certain that a known amount of HUFA is being fed (Anderson and De Silva, 2003). To overcome this problem, common practice in formulation of crustacean diets is to use a 2:1 mixture of marine oils (rich in *n*-3 HUFA) and terrestrial oils (rich in *n*-6 HUFA) (Sheen and Wu, 1999), a mixture that contains a large range of different fatty acids.

Cholesterol

Cholesterol is an important steroid that occurs free or chemically bound to fatty acids in all cells and blood, and in crustaceans it serves as a precursor of numerous compounds such as sex hormones, molting hormones, adrenal corticoids, bile acids and vitamin D (Sheen, 2000). Most animals can synthesize sterols from acetate, but crustaceans, like other arthropods, have been found to be incapable of synthesizing sterols *de novo* (Teshima and Kanazawa, 1971; Sheen et al., 1994). Dietary cholesterol is therefore considered essential for good growth and survival in crustaceans (Sheen, 2000).

The quantitative and qualitative requirements for an exogenous source of cholesterol in formulated diets for crustaceans juvenile have been studied since the early nineteen-

seventies and a wide range of estimated cholesterol requirement have been reported (Table 1.4). Levels required by juvenile *P. japonicus* range from 0.2% (Shudo et al., 1971) to 2.1% (Deshiramu and Kuroki, 1974b), while requirements for marine lobster (*Homarus* sp.) range from 0% for adults (Castell and Covey, 1976) to between 0.12% and 0.5% for juveniles (Castell et al., 1975; D'Abramo et al., 1984). Similarly, white prawn, *P. vannamei*, juveniles showed better growth when fed a diet containing 0.23-0.42% cholesterol compared to any other dietary treatment (Duerr and Walsh, 1996). In experiments with *S. serrata* juveniles, significantly higher weight gain has been observed for crabs fed diets containing 0.50% and 0.79% dietary cholesterol (Sheen, 2000). The same study showed that no crabs fed the diet without dietary cholesterol survived, and that dietary cholesterol levels higher than 1.12% had an adverse effect on mud crab growth. Little information is available on the cholesterol requirements of larval stages of crustaceans, but a study on *P. monodon* postlarvae by Paibulkichakul et al. (1998) showed that 1% cholesterol gave best results among the three cholesterol levels tested (0, 0.5 and 1%), and larvae *P. japonicus* (Kanazawa et al., 1985) and *P. vannamei* (Pedroza-Islas et al., 2004) have both shown good growth on a diet containing 0.5% cholesterol. Similarly, preliminary work on development of a formulated diet for mud crab larvae showed good survival among megalopae fed a diet containing 1% cholesterol (Genodepa et al., 2004b).

Cholesterol is an important metabolic precursor for ecdysone biosynthesis in crustaceans, and studies have shown that Y-organ uptake of cholesterol is greatly improved at the time the molting sequence is initiated (Spazani and Kater, 1973; Watson and Spaziani, 1982). Based on this, the reduced molting rate commonly observed in crustacean larvae fed a cholesterol-free diet is believed to be linked to low

biosynthesis of ecdysone (Watson and Spaziani, 1982). Too much dietary cholesterol, however, has shown to adversely reduce growth and survival of crustacean (Sheen, 2000), and although not yet fully understood, this is believed to be a nutrient-response characteristic rather than toxicity (Mercer, 1982). Either way, the inclusion level of cholesterol in the diet has proven to be of great importance, and further investigation into the optimum inclusion level and the specific role of dietary cholesterol should be conducted on a species specific level.

Table 1.4 Examples of cholesterol levels previously used in nutritional studies with crustacean juveniles and larvae.

Species	**Cholesterol level**	**Reference**
Larvae		
Penaeus monodon	1%	(Paibulkichakul et al., 1998)
P. japonicus	1%	(Teshima et al., 1983)
P. japonicus	0.5%	(Kanazawa et al., 1985)
P. vannamei	0.5%	(Pedroza-Islas et al., 2004)
Scylla serrata (megalopa)	1%	(Genodepa et al., 2004b)
Juvenile		
P. monodon	0.5%	(Chen, 1993)
P. monodon	0.2-0.8%	(Sheen et al., 1994)
P. monodon	0.5%	(Merican and Shim, 1996)
P. japonicus	0.5%	(Kanazawa et al., 1971)
P. japonicus	0.2%	(Shudo et al., 1971)
P. japonicus	2.1%	(Deshiramu and Kuroki, 1974b)
P. vannamei	0.23-0.42%	(Duerr and Walsh, 1996)
P. vannamei	0.5%	(Emery, 1987)
P. vannamei	0-0.5%	(Gong et al., 2000b)
P. vannamei	0.6%	(Pascuala et al., 2002)
Macrobrachium rosenbergii	0.5-1%	(Briggs et al., 1988)
Homarus americanus	0.5%	(Conklin et al., 1980)
H. americanus	0.19-0.59%	(D'Abramo et al., 1984)
H. americanus	0-1%	(Kean et al., 1985)
Panulirus penicillatus	0.5%	(Chen and Jenn, 1991)
Cherax quadricarinatus	1%	(Thompson et al., 2003)
Carcinus maenas	1.5%	(Ponat and Adelung, 1983)
Scylla serrata	1%	(Sheen and Wu, 1999)
S. serrata	0.51%	(Sheen, 2000)

It should be noted that the digestion and assimilation of cholesterol is strongly affected by the total dietary level of lipids and the presence of dietary phospholipids (Teshima et al., 1983; D'Abramo et al., 1985). For species like *P. monodon* (Paibulkichakul et al., 1998) *P. japonicus* (Shudo et al., 1971), *P. vannamei* (Duerr and Walsh, 1996) and *S. serrata* (Sheen, 2000), reduced absorption of cholesterol has been observed when crustacean larvae were fed lipid-free diets, while addition of dietary phospholipids has been found to enhance digestibility of sterols (Conklin et al., 1980; Paibulkichakul et al., 1998; Thongrod and Boonyaratpulin, 1998). On this basis it can be assumed that the effective dietary level of cholesterol is very much a function of other dietary factors.

Phospholipids

Phospholipids is in itself an essential dietary component, and has important roles as structural components of cell walls, and as surfactants for efficient emulsification of ingested lipid which assist the uptake of sterols from the gut (Teshima, 1997). Phospholipids are also a component of high density lipoproteins which aid in the principle transport of lipid from the hepatopancreas and into the haemolymph (Lee and Puppione, 1978; D'Abramo et al., 1985; Gong et al., 2001), which makes it essential for transport of cholesterol to target tissue during the molting process (Conklin et al., 1983). Because of these important roles, supplementation of dietary phospholipids has shown to improve growth and survival, enhance rates of metamorphosis (Anderson and De Silva, 2003) and reduce the occurrence of molt death syndrome for most crustaceans, including juvenile *H. americanus* (Conklin et al., 1980; D'Abramo et al., 1985), juvenile *Penaeus merguiensis* (Thongrod and Boonyaratpulin, 1998), juvenile *P. vannamei* (Gong et al., 2001) and larvae and postlarvae of *P. monodon* (Paibulkichakul et al., 1998). The most commonly used phospholipid in aquatic diets is soybean derived

lecithin, and an overview of dietary lecithin levels included in experimental diets in previous studies with crustacean larvae is presented in Table 1.5.

Table 1.5 Examples of phospholipid levels previously used in nutritional studies with crustacean juveniles and larvae.

Species	Phospholipid level	Reference
Larvae		
Penaeus monodon	1-1.5%	(Paibulkichakul et al., 1998)
P. japonicus	3%	(Kanazawa, 1990a)
P. japonicus	3.5-6%	(Kanazawa et al., 1985)
P. japonicus	3%	(Teshima et al., 1986a)
P. vannamei	1%	(Pedroza-Islas et al., 2004)
Macrobrachium rosenbergii	1%	(Kamarudin and Roustaian, 2002)
Scylla serrata (megalopa)	3%	(Genodepa et al., 2004b)
Juvenile		
P. monodon	1.25%	(Chen, 1993)
P. monodon	2%	(Merican and Shim, 1996)
P. japonicus	1%	(Kanazawa et al., 1971)
P. japonicus	3%	(Teshima et al., 1986b)
P. merguiensis	1-2%	(Thongrod and Boonyaratpulin, 1998)
P. chinensis	2%	(Kanazawa, 1993a)
P. vannamei	0-5%	(Gong et al., 2001)
P. vannamei	3%	(Pascuala et al., 2002)
Macrobrachium rosenbergii	0%	(Hilton et al., 1984)
M. rosenbergii	5%	(Briggs et al., 1988)
Homarus americanus	0-8%	(Conklin et al., 1980)
H. americanus	0-6%	(Kean et al., 1985)
Panulirus penicillatus	1.25%	(Chen and Jenn, 1991)
Cherax quadricarinatus	0-2%	(Thompson et al., 2003)

For most crustacean species examined, the estimated phospholipid requirement of larvae are in the range of 1-3% (Coutteau et al., 1997), however, levels both lower and higher than this have been reported as optimal (Table 1.5). Soybean lecithin is a mixture of neutral and polar phospholipids, and it contains phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid which may vary in purity and composition. Phosphatidylcholine has traditionally been considered the active component for enhancing growth in crustaceans, but more recent studies of the physically and chemically active components of lecithin indicate that the active component is still not found (Thompson et al., 2003). The problems with linking

the effect of lecithin to a singular chemical or physical factor prompted researchers like Kanazawa et al. (1979) to suggest that dietary lecithin plays multiple roles in the body, and that the molecular structure of lecithin may be influential. Whether phospholipid has single or multiple roles, specification of the active characteristics of soy lecithin will lead to a better defined diet whereby qualitative and quantitative nutritional requirements may be more precisely identified.

1.2.3.3 Carbohydrates

Due to poor utilization of carbohydrates in aquatic animals, few studies have been conducted on carbohydrate nutrition of crustaceans, and information is particularly limited for larval crustaceans. As can be seen from the overview of carbohydrate levels used in nutritional studies with larvae and juveniles presented in Table 1.6, most work to date has been done with the penaeid prawns, although a few studies have been conducted on juvenile lobsters and crabs.

Table 1.6 Dietary carbohydrate levels previously used in experimental formulated diets for crustacean larvae and juveniles.

Species	**Dietary level**	**Reference**
Larvae		
Penaeus monodon	24-33%	(Kurmaly et al., 1989a)
P. monodon	7.5-17.8%	(Kurmaly et al., 1989a)
P. japonicus	15-25%	(Kanazawa, 1990a)
P. japonicus	6.10%	(Moe et al., 2004)
P. indicus	24.5-27.9%	(Immanuel et al., 2003)
P. vannamei	13.5%	(Pedroza-Islas et al., 2004)
Homarus americanus	12.0-15.7%	(Fiore and Tlusty, 2005)
Juvenile		
P. monodon	3-35%	(Catacutan, 1991)
P. monodon	20%	(Alava and Pascual, 1986)
P. setiferus	12%	(Brito et al., 2000)
P. setiferus	17.22%	(Gallardo et al., 2002)
Macrobrachium rosenbergii	35.7-37.0%	(Briggs et al., 1988)
M. rosenbergii	29.6-37.0%	(Brito et al., 2000)
J. edwardsii	27%	(Johnston, 2003)

The utilization of carbohydrates by aquatic species varies and seems to be less efficient than that of terrestrial domesticated animals (Shiau et al., 1991). Although studies suggest that most crustaceans do not have a specific requirement for dietary carbohydrates (Fegan, 2004), it is believed that carbohydrate plays an important role in balancing the utilization of protein and lipids for energy production (Johnston, 2003). Studies on adult crustaceans like the prawn, *Crangon crangon* (Regnault, 1981), and *P. vannamei* (Stuck et al., 1996) have shown that carbohydrates are used for short-term energy requirements by marine crustaceans, and they are therefore the first to be exhausted during imposed starvation, followed by lipid then protein. However, no difference in growth or digestibility of crude protein and lipid were found in juvenile *P. monodon* fed iso-nitrogenous diets containing graded levels (5-35%) of gelatinized wheat flour as the carbohydrate source (Catacutan, 1991). Similarly, growth of post larvae of *P. monodon* fed diets containing varied levels of corn starch (10-40%) was not affected by starch content (Bages and Sloae, 1981).

Simple carbohydrates are considered to be inferior to complex carbohydrates in promoting growth in many crustaceans (Chen, 1993). This was shown by Shiau and Pong (1991) in experiments with adult *P. monodon*, where a protein sparing effect was more obvious when the dietary protein level was reduced from 40 to 30% by increasing corn starch level from 20 to 30%. Similar results were reported by Andrews et al. (1972), where addition of glucose to diets fed to adult *Penaeus setiferus* resulted in depressed growth, while supplemental starch did not influence weight gain. The more efficient utilization of starch compared to glucose in crustaceans may be linked to the binding quality (New, 1976), or to the low glycemic index rating of the former (Deng et al., 2001).

The dietary requirement and utilization of carbohydrates by mud crabs are largely unknown. However, one study by Catacutan et al. (2003) with *S. serrata* larvae showed that larvae are able to digest a wide range of carbohydrates, including plant sources like soybean meal, corn meal and rice bran. From a food developer's perspective these findings are very encouraging, as the use of plant ingredients will significantly reduce the cost of MBD production. The few dietary studies conducted with *S. serrata* juveniles to date have used corn starch as a carbohydrate source, usually within a range of 13.5% to 27% of diet dry weight (Sheen and Wu, 1999; Sheen, 2000); inclusion levels that are primarily based on known requirements from other crustacean species. Examination of digestive enzyme profiles of the spiny rock lobster, *Jasus edwardsii,* by Johnston (2003) indicated that carbohydrates are more important for small juveniles compared to larger ones, results that have been linked to a shift in dietary preferences as the animal grows. A study on *S. serrata* gave similar results, showing that juvenile mud crabs (<6 cm carapace length) had higher cellulase activity than those expressed in adult mud crabs (Rutledge, 1999). Based on this it is clear that the ability to utilize dietary carbohydrates for energy varies not only between species, but also with the size and development stage of the animal. Further research into this aspect of mud crab nutrition would therefore be beneficial.

Interestingly, it has been suggested that dietary carbohydrates have roles in crustacean nutrition other than as an energy source. The crustacean carapace is composed mainly of chitin, a polymer of N-acetyl-glucosamine, and some studies have noted a beneficial effect of adding glucosamine to the diet (Akiyama et al., 1992). This indicates that although this carbohydrate can be synthesized by most crustacean species, it appears to be semi-essential. This may explain the observed value of dietary prawn meal, as chitin

is partly digestible (Guzon and Guillaume, 1999). This theory is strengthened by the observation that re-ingestion of exoskeleton after molt is more frequent among crustaceans fed poorly-balanced diets than among those fed good quality feeds (Guzon and Guillaume, 1999).

1.2.3.4 Vitamins and minerals

Vitamins are classified as a micro-nutrient, and are organic molecules that act as cofactors in metabolic reactions or as an important structural component in cells (Reddy and Kumar, 1996). In comparison to terrestrial animals, limited information is available on vitamin requirements of crustaceans, and for those where optimum dietary levels have been demonstrated, information may be derived from only one dietary trial (Conklin, 1997).

The major challenge in defining vitamin requirements of crustaceans is linked to the problem of delivering water-soluble nutrients to aquatic animals (Conklin, 1997), and because decapod crustaceans are slow feeders, leaching of water-soluble nutrients is another significant problem (Goldblatt et al., 1980). To overcome this, replete vitamin premixes are commonly used in formulated diets (Kanazawa, 1990a). For juvenile and adult crustaceans, dietary requirements for the following vitamins have been identified; thiamin (Deshiramu and Kuroki, 1979), riboflavin, niacin (Cheng and Hwang, 1993), vitamin B_6 (Deshiramu and Kuroki, 1979), pantothenic acid (Akiyama et al., 1992), biotin (Akiyama et al., 1992), folate (Chen, 1993), vitamin B_{12} (Shiau and Lung, 1993), Choline (D'Abramo and Baum, 1981), myo-insositol (Deshiramu and Kuroki, 1976), vitamin C (Deshiramu and Kuroki, 1976), vitamin A (Akiyama et al., 1992), vitamin E (He and Lawrence, 1993), vitamin D (Shiau and Hwang, 1994), vitamin K (Akiyama et

al., 1992). For larvae crustacean, however, the only known vitamin requirements are those reported by Kanazawa (1990a) who worked with adult penaeids and recommended dietary inclusion of carotene, thiamine, riboflavin, pyridoxine, nicotinic acid, folic acid, biotin, cyanocobalamin, choline, insositol, ascorbic cid, vitamin D and vitamin E. More recently, a few studies have also been conducted on vitamin C or L-ascorbic acid (AsA) requirements, and it has been shown to be an essential nutrient for larval *P. japonicus* (Moe et al., 2004), *P. vannamei* (Kontara et al., 1997) and *M. rosenbergii* (Merchie et al., 1997). Furthermore, research has shown that larval *P. japonicus* fed AsA levels of between 43 and 71 mg/kg displayed increased survival, higher numbers of larvae metamorphosing to the postlarval stage, higher body weight and increased stress resistance (Moe et al., 2004). In the absence of such information for *S. serrata* larvae, these levels reported for *P. japonicus* might function as a useful guide when developing mud crab hatchery foods.

Minerals are another group of important micro nutrients, and they have an important metabolizing role in all animals where they act as co-factors and enzyme activators (Davis and Lawrence, 1997). A variety of studies have been conducted on the inorganic component of both semi-purified and practical feeds, and it has been shown, for example, that mineral-rich diets (as high as 19.5% ash) produced the best growth in *P. japonicus* adults (Deshiramu and Kuroki, 1974a). Similarly, Castille and Lawrence (1989) found that growth rates of juvenile *P. vannamei* were significantly reduced when fed a practical feed without added minerals, work that suggests that practical diets without mineral supplements, even in the presence of natural productivity, may not meet the mineral requirements of crustaceans (Davis and Lawrence, 1997).

Dietary requirements of minerals are commonly determined by feeding graded levels of the mineral of interest, and the physical response of the test animal is measured. However, unlike terrestrial animals which are completely reliant on a dietary supply of minerals, aquatic animals may be able to utilize minerals dissolved in the water (Davis and Lawrence, 1997). This was shown in experiments with adult *P. vannamei*, where exoskeleton, hepatopancreas, muscle and serum of the prawns reared in low salinity water showed inhibited tissue mineralization compared to prawns reared in mineral-rich seawater (Cheng et al., 2006). Consequently, the determination of quantitative dietary requirements is difficult (Lall, 1989), and few clearly defined mineral requirements have been reported for marine crustaceans (Davis and Lawrence, 1997). The only published information is based on experiments with adult and juvenile penaeid prawns, and this work has documented dietary requirements for the following minerals; calcium (Kanazawa et al., 1984), phosphorus (Deshiramu and Yone, 1978), potassium (Deshiramu and Yone, 1978), magnesium (Kanazawa et al., 1984), copper (Davis et al., 1993a), iron (Davis et al., 1992) and zinc (Davis et al., 1993b). Due to this limited information, most pelleted diets formulated for larvae, juveniles and adult crustaceans contain various types of mineral premixes (Zimmermann et al., 1994). However, as practical diets generally contain a substantial amount of endogenous minerals, a complete mineral premix is not always necessary. As excessive supplementation of minerals increases the cost of feed, enhances phosphorus pollution and may reduce the bioavailability of other minerals (Davis and Lawrence, 1997), further work on mineral requirements is therefore important for development of a nutritionally optimized, cost-effective and environmentally friendly crustacean diet.

1.2.4 Conclusion

Despite the progress in recent years, knowledge of nutritional requirements of *S. serrata* larvae still lags behind industry needs and further research is necessary. Recent progress has shown that replacement of live food with formulated diets is possible from the megalopa stage, and this opens the possibility for the use of MBD as a tool for further nutritional requirement studies. This work will provide a basis for development of nutritionally optimised diets for use in mud crab hatcheries, which would be a major step towards more cost-effective and reliable hatchery production of mud crabs.

1.3 Aims of this study

The major objective of this study was to expand our understanding of the nutritional requirements of *Scylla serrata* larva, with special emphasis on lipid nutrition. A range of feeding experiments and tissue analyses were carried out to provide information needed for further development of a cost effective and nutritionally optimized practical diet for use in mud crab hatcheries.

The specific aims of this study were:

1. to collect and review all information on *Scylla serrata* larvae nutrition published to date, and to highlight areas where further research is required (Chapter 1),
2. to determine the possibility for partial or total replacement of live food during the zoea III stage, with the aim of identifying the most appropriate larval stage to be used in further dietary trials (Chapter 3),
3. to assess the possibility of replacing the live food component of an existing experimental MBD, and to specify the best suited protein source for the

experimental MBD to be used in the planned feeding experiments with mud crab megalopa (Chapter 3),

4. to establish the dietary cholesterol requirement for megalopae fed formulated, semi-purified MBD (Chapter 4),
5. to determine the effect of supplemental dietary lecithin on growth, development time and survival of megalopae, and to investigate a possible interaction between dietary phospholipids and cholesterol (Chapter 5),
6. to evaluated the possibility of reduced use of fish oil in MBD formulated for megalopae by determining the effects of various ratios of dietary fish oil and corn oil on survival, growth and development time (Chapter 6),
7. to develop a better understanding of ontogenetic changes in lipid metabolism, from newly hatched zoea I to megalopa through tissue analysis of lipid class composition and fatty acid profiles (Chapter 7), and
8. to assess the affect of feeding and starvation on fatty acid composition and lipid utilization during the last two stages of larval development, and to describe shifts in dietary lipid requirements associated with metamorphosis from zoea V to the megalopa stage (Chapter 8).

Chapter 2

General Materials and Methods

The methods for larval production used in this study were based on procedures previously developed at James Cook University (Genodepa, 2004). Practical adjustments were made to suit the requirements of specific experiments.

2.1 Collection and conditioning of broodstock

Mature *Scylla serrata* females of at least 14 cm in carapace width were collected in baited traps in estuarine areas around Townsville, North Queensland, Australia, under the DPI license number PRM39339H. The crabs were transported to the Marine and Aquaculture Research Facility Unit (MARFU) at James Cook University and were disinfected in 100 mg L^{-1} formalin in seawater for 6 h (Mann et al., 1999). Following quarantine the crabs were transferred to 1000 L outdoor polyvinyl chloride (PVC) tanks provided with sand and shelter. The tanks were fitted to a recirculation systems supplied with UV irradiated and 1 µm filtered seawater. The salinity and water temperature were kept at 30-36‰ and 25-30°C, respectively, and the broodstock were fed a diet of prawns, mussels and squid once daily at a rate of 5-8% body weight. As mud crabs are known to be nocturnal eaters (Catacutan et al., 2003) feeding took place in late afternoons, and leftover food was removed every morning to maintain healthy water quality in tanks. Mature females were kept in the outdoor tanks until they spawned.

Berried crabs were disinfected using 50-60 µL L^{-1} formalin solution in seawater for 6 h, before being transferred to 300 L indoor tanks for egg incubation and hatching. The tanks were fitted to a re-circulating system with UV irradiated and 1 µm filtered seawater, where salinity and water temperature were kept at 32-36‰ and 26-29°C, respectively. The females were not fed during the 11-13 day long egg incubation period, and the tanks were siphoned every morning to remove feces and discarded eggs.

2.2 Eye-stalk ablation

The eye-stalk of most crustaceans contains an X-organ, the presumptive site of production and release of many protein and peptide hormones into the haemolymph (Gross and Knowlton, 2002). Reproduction is controlled by the sinus gland and the associated centers on the X-organ, where a spawn-inhibiting hormone is produced (Southgate and Lucas, 2003). Eye-stalk ablation deprives the animal of these hormones (Gross and Knowlton, 2002), and this method was utilized to induce spawning during periods of low spawning activity (May to August).

Females with mature ovaries were selected, and eye-stalks were incised at the base of the stalks using sterile scissors (Fig. 2.1). Newly ablated crabs were placed in a 50 L tanks with aeration, and when the release of haemolymph ceased the female was disinfected using 50-60 µL L^{-1} formalin solution in seawater for 6 h. The females were then transferred to 300 L re-circulating indoor spawning tanks supplied with sand-trays and aeration. A diet of prawns, mussels and squid was supplied on a daily basis until spawn.

Fig. 2.1 Eye-stalk ablation of female mud crab a) female prior to operation b) female with eye-stalks removed c) ablated eye-stalks.

2.3 Larval rearing

Following fertilization, berried female crabs carry the eggs attached to the abdomen for a period of 10-50 days depending on water temperature (Heasman and Fielder, 1983). In this study, berried crabs were kept at temperatures of approximately 25-30ºC, resulting in an egg incubation period of between 12 and 14 days (Fig. 2.2). Once hatched, the larvae go through five distinct zoea stages (each lasting 2 to 5 days), before molting into a sub-benthic megalopa stage. After 8-10 days, megalopa metamorphose into benthic first stage juveniles (C1) (Fig. 2.3).

Newly hatched larvae are highly photopositive, and zoea I were attracted to the surface using a strong light source immediately after hatch. Actively swimming larvae were collected using plastic bowls and transferred to flat bottomed 300 L indoor tanks at a density of 100-120 larvae L^{-1}. The culture water was treated with antibiotics (10-15 mg L^{-1} Streptomycin) (Sigma-Aldrich, S6501) (Mann et al., 1999) once only during initial stocking, and salinity and water temperature were kept at 20-22‰ and 28-30°C, respectively. As larvae grew the salinity was gradually increased to 25-28‰ at the megalopa stage (Table 2.1).

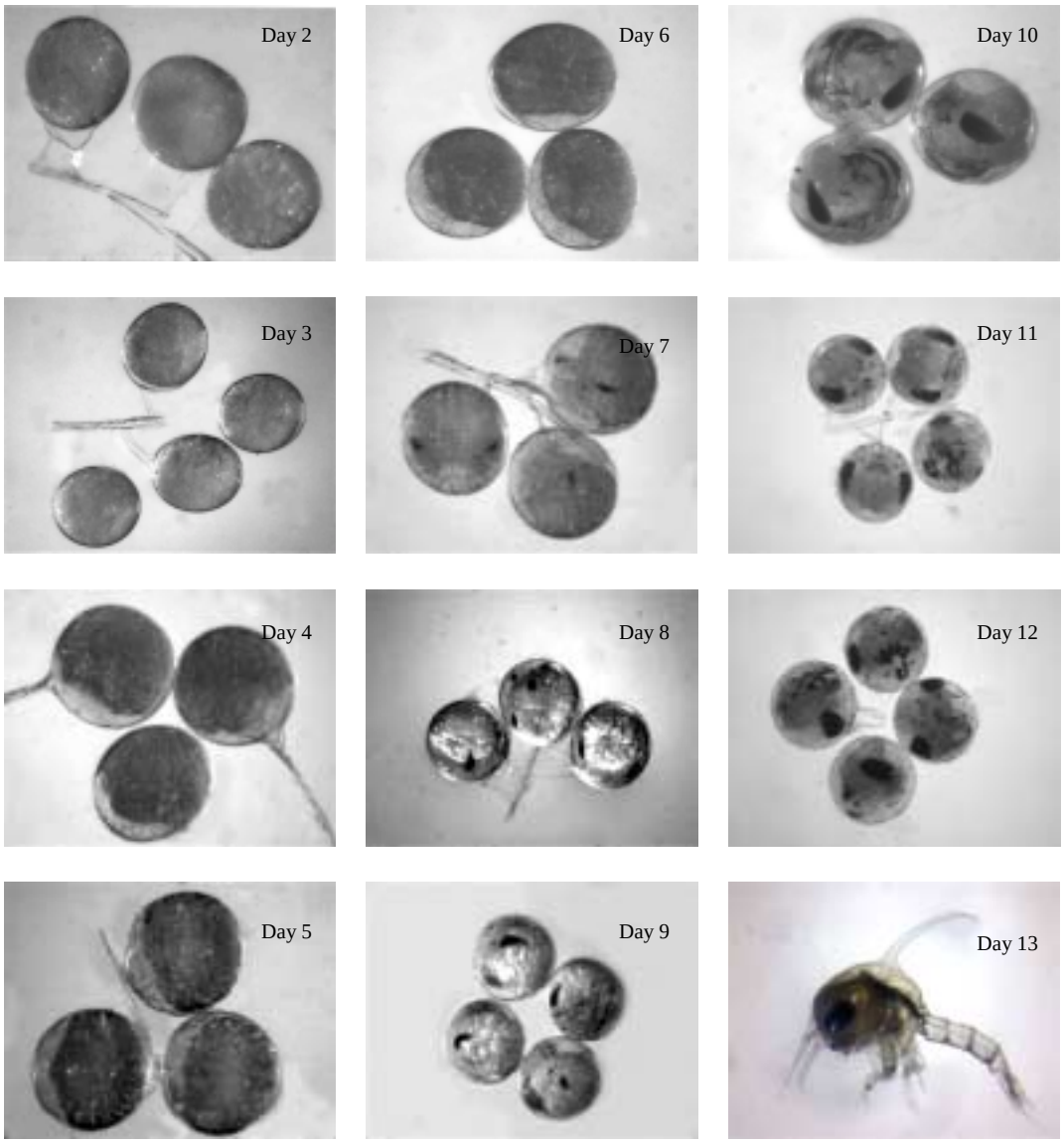

Fig. 2.2 Development of *Scylla serrata* eggs from fertilization until hatch at incubation temperature of 25-30ºC.

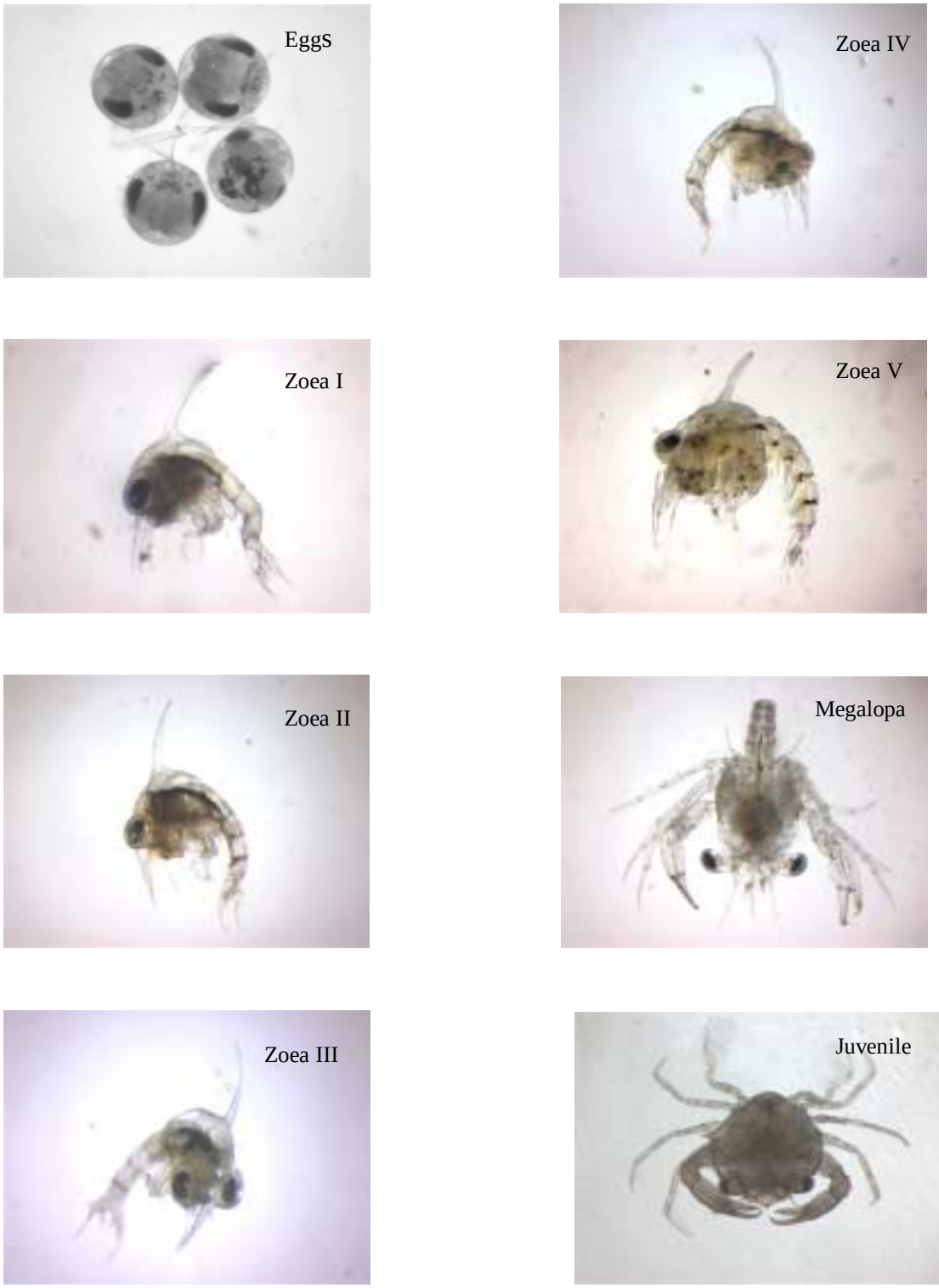

Fig. 2.3 Development of *Scylla serrata* larvae from zoea I to the first crab stage.

Table 2.1 Standard rearing conditions used for culturing mud crab larvae. Based on Genodepa et al. (2004a).

Larval stage	**Zoea I**	**Zoea II**	**Zoea III**	**Zoea IV**	**Zoea V**	**Megalopa**
Duration of culture	3-4	2-4	3-4	4-5	4-5	8-10
Brachionus sp. (individuals mL^{-1})	40-60	Gradually reduced to 10				
Artemia sp. (individuals mL^{-1})		Gradually increased form 0.5-5				
Nannochloropsis sp. (cells mL^{-1})		$5\text{-}10*10^4$				
Water exchange	20-25%		30-50%			
Salinity	20-22‰		22-24‰	24-25‰	25-28‰	
Temperature	25-29°C					
Antibiotics (mg L^{-1})*	10-15					

* Streptomycin was added only in association with initial stocking of zoea I.

Newly stocked larvae were fed rotifers (*Brachionus* sp.) at a density of 40-60 individuals mL^{-1} the first day of culture, and this ratio was maintained by daily addition of microalgae (*Nannochlorapsis* sp) (Harvey and Epifanio, 1997). The second day after the larvae molted to zoea II, newly hatched *Artemia* nauplii were introduced at a density of 0.5 individuals mL^{-1}. To match the size of the developing larvae, two different strains of brine shrimp were used; during zoea II a smaller strain containing a high level of HUFA (INVE Aquaculture NU, AF Specialty *Artemia*) (size ± 430 µm) was supplied, and as larvae molted to zoea III, a larger strain (Salt Creek Select Brine Shrimp) (size 490-510 µm) of Culture Selco® (INVE) enriched *Artemia* meta-nauplii was introduced to the larval cultures. As larvae grew the number of rotifers was reduced to a negligible level through discontinuous addition of microalgae, while the number of *Artemia* nauplii was gradually increased to 5 individuals mL^{-1}. Algae crumbs and dead larvae were siphoned from the bottoms of tanks every morning, and daily water exchange in the larval culture tanks increased from 10-15% at the two first zoeal stages, to 40-50% for zoea III onwards (Table 2.1).

2.4 Production of live food

2.4.1 Microalgae

Monospecific cultures of microalgae *(Nannochloropsis* sp.) were reared in semi-continuous cultures using 2000 L outdoor tanks under natural sunlight and temperature conditions. Prior to stocking, filtered seawater (approximately 20‰ salinity) was filled to 80% of the tank capacity, and the water was treated with 50 mg L^{-1} chlorine. After 24 h with vigorous aeration the water was fertilized with 30-40 g Aquasol plant fertilizer (Hortico Australia Pty. Ltd.) and the tanks were seeded with 400 L of algae. Within 4-7 days after inoculation the cell density increased to a concentration suitable for feeding to rotifer cultures, or to inoculate new culture tanks.

2.4.2 Rotifers

Rotifers, *Brachionus rotundiformis*, commonly referred to as S-type (Segers, 1995), were grown in continuous cultures (approximately 100-300 individuals mL^{-1}). The cultures were initiated by inoculating 100 L of microalgae with *B. rotudiformis*, using 300 L outdoor tanks kept in natural lighting conditions. The salinity and water temperature was kept at 35‰ and 25-30°C, respectively, and a daily water exchange of 30-40% was conducted to ensure good water quality. To prevent contamination and build up of bacteria and ciliates, a filter screen was suspended in the water to trap feces and dead algae. The filters were changed and washed on a daily basis, and every 10 days the culture tanks were completely drained and cleaned, and rotifers were either harvested or transferred to clean tanks to initiate new cultures. To ensure continuous growth in the culture and high nutritional value in each individual rotifer, consumption of microalgae was regularly monitored. When required, a portion of the culture water was removed from the rearing vessel and replaced with a similar volume of

Nannochlorapsis sp. culture. When harvesting rotifers for use in larval rearing, the required volume was sieved through a 60 µm mesh and rotifers were transferred to clean seawater before being introduced to the larval culture tanks.

2.4.3 Artemia

Two different strains of brine shrimp were used in the rearing of mud crab larvae as detailed in section 2.3. To ensure that the live prey was of maximum nutritional value when fed to the mud crab larvae, a new batch of *Artemia* nauplii was harvested every morning just prior to introduction to larval tanks. To decrease depletion of endogenous energy, brine shrimp not used immediately were kept under refrigeration until afternoon feeding.

Every morning a new batch of *Artemia* cysts were dehydrated for 2 h in fresh water treated with 1% chlorine. The solution was then poured through a sieve and cysts were washed thoroughly with fresh water before being transferred to a 20 L conically culture vessel. The hatching tank was filled with 1 µm filtered and UV treated seawater, and salinity and water temperature was kept at 27‰ and 25-30°C, respectively. The tank was supplied with strong aeration and illuminated with strong light for 24 h before harvesting, when newly hatched *Artemia* nauplii was separated from the cyst shells and bathed in fresh water for 5 minutes prior to being introduced to the mud crab larval culture tanks.

2.5 Preparation of microbound diets (MBD)

The diets used in experiments throughout this study where based primarily on the MBD successfully used by Sheen (2000) and Genodepa et al. (2004a) in previous experiments

with *S. serrata* larvae and juveniles. The amounts of some ingredients were altered in accordance with reported nutritional requirements of marine crustacean larvae: i.e. the amount of dietary lecithin was based on Teshima and Kanazawa (1983), Teshima et al. (1986), Kanazawa (1990) and Camara et al. (1997). The ingredients lists for each MBD are detailed in the appropriate chapters.

Before preparation of diets, all ingredients were grounded to a particle size of <100 µm. Lipid was extracted from squid meal using 2:1 chloroform-methanol (v/v) (Folch et al., 1957) in four successive treatments to remove lipid, and the levels of dietary corn starch and cellulose were manipulated to ensure that all experimental diets were iso-energetic. The MBD particles were prepared by combining and thoroughly mixing all dry and all moist ingredients in separate containers, before the binder (zein dissolved in 70% ethanol) was added, and all ingredients were combined into a homogenous mixture. The dough was spread thinly on an aluminum dish and oven dried at 50° for 24 h. Once the diet had the right consistency, it was ground using mortar and pestle and sieved to appropriate particle size (150-250 µm for zoea III and 400-600 µm for megalopa) (Genodepa et al., 2004b).

2.6 Experimental design: zoea feeding experiments

To ensure that all larvae used in the experiment had empty digestive tracts at the start of a feeding trial, hundreds of pre-molt zoea II larvae were removed individually from mass rearing tanks the night prior to the start of the experiment. These zoea II larvae were divided into four groups and were maintained under conditions identical to the subsequent feeding experiment. The following morning, larvae that had molted to the zoea III stage over night were randomly selected from the respective treatment group

and stocked into tall, conical-bottomed culture vessels filled with 1 L of 1 μm filtered and UV treated seawater. Twenty-five larvae were stocked into each culture vessel, and four replicate 1 L vessels were established for each treatment. Salinity and water temperature were maintained at 24-26‰ and 27-29°C, respectively, while photoperiod was maintained at 12 h L: 12 h D. Gentle aeration was supplied to the bottom of each culture vessel to prevent diet particles and larvae from settling.

As zoea larvae only ingest food particles suspended in the water column, a full ration was fed to each culture vessel four times daily at 09:00, 12:00, 15:00 and 18:00 h. A 100% water exchange was carried out every morning, when the number of molts and dead larvae for each culture vessel were recorded. A new ration of newly hatched *Artemia* nauplii was added to the vessel immediately after the water exchange. Any larvae that had successfully molted to zoea IV were removed during the daily water change, and the experiment was terminated when all larvae had either molted or died.

2.7 Experimental design: megalopa feeding experiments

Previous experiments with *S. serrata* have shown that larvae typically become cannibalistic when they reach the clawed megalopa stage, resulting in high mortality under communal rearing condition (Genodepa et al., 2004b). On this basis, megalopae were reared individually in separated containers during the experiment to avoid any potential confounding effects of cannibalism. To ensure that all megalopae used in experiments were at similar development stage, only megalopae that molted within the previous 24 h period were used. This was achieved by removal of newly molted megalopae from the mass rearing tanks in the evening. The following morning, megalopae were stocked individually into 250 mL, flat bottomed circular aquaria (6 cm

x 9.5 cm) filled with 1 μm filtered and UV treated seawater. Fifteen or twenty replicate megalopae were randomly assigned to the aquaria for each treatment, and because megalopae often suffer high mortality the day after metamorphosis (Genodepa, 2003), experiments were begun on the second day after molting to the megalopa stage. Two rations of 2 mg L^{-1} MBD daily (provided at 09:00 and 18:00 h) ensured that food was always available. Water temperature and salinity were maintained at 27°C and 25‰, respectively, and aquaria were subject to a 12 h L: 12 h D photoperiod. Each morning a 100% water exchange was conducted for each aquarium, and larval survival and molting were monitored and recorded during morning and evening feeding times. The experiment was terminated when all megalopae had either molted to the first crab stage or died.

2.8 Lipid analysis

Crab larvae and live foods were collected on nylon mesh and washed with distilled water to remove salt. After rinsing, excess water was removed by placing the mesh onto absorbent paper, and samples were snap-frozen in liquid nitrogen and stored at -80ºC until analyzed. Lipids were extracted from samples with chloroform/methanol by the method of Folch et al. (1957), and total lipid was determined gravimetrically from an aliquot of the extract by drying for 4 h at 80^{o}C in a pre-weighed glass vial.

The neutral lipid components of the extracts were removed by eluting with chloroform, and the phospholipids eluted with methanol and collected into a weighed glass vial (Christie, 1989). The fatty acids in the lipid extract were derivitised to their fatty acid methyl esters (FAME) using 14% boron trifluoride-methanol (Van Wijngaarden, 1967), and FAME were analyzed on an Agilent Technologies 6890 gas chromatograph using

split injection with helium carrier gas and a flame ionization detector. The column used was a DB23 fused silica capillary column, 30 m x 0.25 mm, with a 0.25 µm coating (Agilent Technologies, USA). Column oven temperature was held at 140°C for 5 minutes and then elevated at 3°C/minute to 210°C where it was held until all FAME of interest had been eluted. FAME were identified by comparing their retention times with those of authentic standards (Sigma-Aldrich Co, USA), and were quantified by comparison with the response of an internal standard, heneicosanoic acid.

Chapter 3

The use of microbound diets (MBD) as total or partial replacement of live feed for zoea III larvae, and specification of an optimal protein source in MBD for megalopae [1]

3.1 Introduction

Despite the clear potential of microbound diets (MBD) as a food source for *Scylla serrata* megalopae (Genodepa et al., 2004b), their potential as a food source for zoeal larvae of the same species has not previously been investigated. Research with penaeid and fish larvae has shown that early larval stages often have reduced digestive capabilities compared to older larvae, and that digestion is heavily reliant on enzymes obtained from live prey (Jones et al., 1997b; Kolkovski, 2001). On this basis, formulated diet particles are often fed to early larval stages in conjunction with live food organisms (e.g. Kanazawa et al., 1989), and this procedure is now broadly applied in the hatchery culture of many commercial species (e.g. Lauff and Hofer, 1984; Peron-Le Ruyet et al., 1993; Jones et al., 1993).

For *S. serrata* larvae, substantial advances in larval complexity and gut morphology has been identified as larvae develop to the zoea III stage. The gastric mill begins to form (Li et al., 2000), allowing the larvae to digest food more efficiently, and as a result the

[1] The data in this chapter is published in *Aquaculture* (2006) 257: 482-490. See Appendix 1

ingestion of MBD particles increases substantially (Genodepa et al., 2004a). On this basis it is reasonable to assume that partial replacement of live food with a formulated diet may be achievable from this larvae stage onwards. As opposed to the more benthic megalopa stage, however, zoea III larvae feed mainly on particles suspended in the water column (Genodepa, 2003). This is an important consideration that must be accounted for during development of appropriate feeding protocols and for the formulation of suitable practical diets. Prior research using radiotracers to determine digestion has shown that particles of semi-defined MBD containing dried rotifers were readily ingested by *S. serrata* zoea larvae (Genodepa et al., 2006). For megalopae this diet even supported similar rates of survival and development as those of megalopae fed live *Artemia* nauplii (Genodepa et al., 2004b). However, the potential for total or partial replacement of live feed in hatchery rearing of the zoea stages has not been investigated.

The clawed and sub-benthic megalopa stage is capable of feeding on larger food particles at the bottom, and the use of formulated diet particles has proven successful for this stage. The experimental MBD used in previous feeding experiments contained ^{14}C-labelled rotifers (39.7% of total dry weight) as a mean of estimating larval ingestion of MBD (Genodepa et al., 2004a, b), and although this diet supported good survival and development of mud crab megalopae (Genodepa et al., 2004b), live food production is both costly and time consuming. Development of a MBD without live food components would therefore be an important step towards development of a cheaper and more reliable diet for hatchery production of mud crab seeds.

This chapter reports on two feeding experiments assessing the potential of an experimental MBD developed in this laboratory for both zoea and megalopa larvae of *S.*

serrata. The first experiment evaluated the suitability of MBD for either total or partial replacement (i.e. co-feeding) of live foods for zoea III larvae. The second evaluated MBD containing various defined ingredients as a replacement for live food fed to *S. serrata* megalopae.

3.2 Materials and methods

3.2.1 Preparation of experimental MBD

The compositions of the experimental MBD used in this study were based on those developed by Genodepa et al. (2004b) which included dried rotifers as a major component (Table 3.1). In Experiment 1, the suitability of MBD as a complete or partial replacement for live food for to zoea III larvae was investigated using a MBD identical to that previously used by Genodepa (2004b) (Table 3.1). In Experiment 2 the potential for replacement of the rotifer component (39.7% of the dry weight) of the experimental MBD with more readily available protein sources was investigated, by comparing survival and development of megalopae fed MBD containing rotifer meal, *Artemia* meal, squid meal or fish meal.

Table 3.1 The composition (% dry weight) of the basal experimental diets used in feeding experiments with *Scylla serrata* zoea III and megalopae.

Ingredient	Source	Dry weight (%)
Squid meal	A	39.7
Dried rotifers*	B	39.7
Fish oil[a]	C	5.0
Corn oil[b]	C	1.0
Lecithin[c]	D	3.0
Cholesterol[d]	E	1.0
Dibasic calcium phosphate (DCP) [e]	C	0.6
Mineral mix[f]	F	4.0
Vitamin mix[g]	F	3.0
Zein[h]	C	3.0
Total		100

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Sources of ingredient: A) Skretting, Tasmania, B) Research Facilities, James Cook University, C) Sigma-Aldrich Pty. Ltd, D) Norganic Foods co, E) Labchem F) Rabar Pty Ltd [a]from menhaden, F8020. [b]C8267. [c]liquid soy lecithin. [d]1729. [e]C4131. [f]ZZ603 DO 067 DPI, each 1 kg contains: copper 1 g; cobalt 100 mg; magnesium 59.4 mg; manganese 5 g; iodine 800 mg; selenium 20 mg; iron 8 mg; zinc 20 g; aluminium 100 mg, and chromium 100 mg. [g]ZZ600 DPI, each 1 kg contains: Retinol (vit. A 2) mIU; Cholecaliferol (vit. D3) 0.8 mIU; Tocopherol (vit. E) 40 g; Phytomenadione (vit. K) 2.02 g; inositol (vit. Bh) 50 g; Niacin (vit. B3) 30.40 g, Pantothenic Acid (vit. B5) 9.18 g; Folic Acid (vit. B9) 2.56 g; Riboflavin (vit. B2) 4.48 g; Cyanocobalamin (vit. B12) 0.004 g; biotin (vit. H) 0.1g; Pyridoxine (vit. B6) 4 g; Thiamine (vit. B1) 3.4 g; Ascorbic Acid (vit. C) 44.4 g; para amino benzoic acid 20 g; tixosil 5 g and antioxidant 30 g. [h]from maize Z3625.

* Lipids were not extracted from the dried rotifers. In Experiment 1, dried rotifers were replaced by *Artemia* meal (Diet 2), squid meal (Diet 3) or fish meal (Diet 4).

3.2.2 Exp. 1. Replacement of live food with experimental MBD for zoea III

This experiment was conducted according to the methods detailed in section 2.6, and the following four dietary treatments were tested:

1) Unfed larvae (control)
2) Larvae fed a 100% ration of *Artemia* nauplii
3) Larvae fed a 100% ration of MBD
4) Larvae fed a diet of 50% MBD: 50% *Artemia* nauplii

For the purpose of this study, a 100% ration of *Artemia* nauplii was 5 individuals L^{-1} (Table 3.1). A 100% ration of MBD was 7.1 mg L^{-1}, which is the equivalent dry weight of the 100% *Artemia* nauplii ration. The feeding rates were based on results reported by Genodepa et al. (2004a).

3.2.3 Exp. 2. Use of defined MBD in rearing of megalopae

Six dietary treatments were assessed: (1) MBD prepared using dried rotifers; (2) MBD prepared using *Artemia* meal; (3) MBD prepared using squid meal; (4) MBD prepared using fish meal; (5) live *Artemia* nauplii; and (6) unfed.

3.2.4 Statistical analyses

In Experiment 1, the total number of molts and the intermolt period from zoea III to zoea IV from the different dietary treatments were compared using a Kruskal-Wallis non-parametric test due to the unequal sampling size. The Mann-Whitney test was used to show significant treatment effects. Daily percentage survival was compared using a one-way ANOVA, and specific differences among treatments were determined using Tukey's multiple comparison test at the 0.05 level of significance. In Experiment 2 the average development time to first crab stage (C1) for the six dietary treatments were compared using one-way ANOVA, and a specific differences among treatments were determined using Tukey's multiple comparison test at the 0.05 level of significance. Due to unequal sampling size, carapace width of newly metamorphosed C1 that emerged from different treatments was compared using a Kruskal-Wallis non-parametric test and significant treatment effects were determined using the Mann-Whitney test. The cannibalistic nature of the megalopae made individual culturing

necessary, and hence no replicated survival data were available. All statistical analysis were performed using SPSS for Windows, version 14.0.

3.3 Results

3.3.1 Exp. 1. Replacement of live food with experimental MBD for zoea III larvae

Highest survival (66.0%) of zoea III to the zoea IV stage was recorded for larvae fed the diet composed of 50% MBD: 50% *Artemia* nauplii (Fig. 3.1). Larvae fed 100% *Artemia* nauplii showed lower survival (51.0%) but the difference between the two treatments was not statistically significant (P >0.05) (Table 3.2). All unfed larvae died by day 7 and no molts were recorded in this treatment. However, among the larvae fed MBD exclusively, 3% of the larvae molted successfully to the zoea IV stage (Table 3.2) and the majority of larvae survived to day 10.

The shortest zoea III duration and greatest molting synchrony (5.5 ± 0.1 days) were again shown by larvae fed the diet composed of 50% MBD: 50% *Artemia* nauplii (Table 3.2). Although not significantly different, larvae fed 100% *Artemia* nauplii had a slightly longer intermolt period with unsynchronized molting, beginning on day 4 and lasting until day 13. All molts in the treatment receiving 100% MBD occurred at day 8 and, except for five larvae fed 100% *Artemia* nauplii, no larvae molted to the zoea IV stage after day 8.

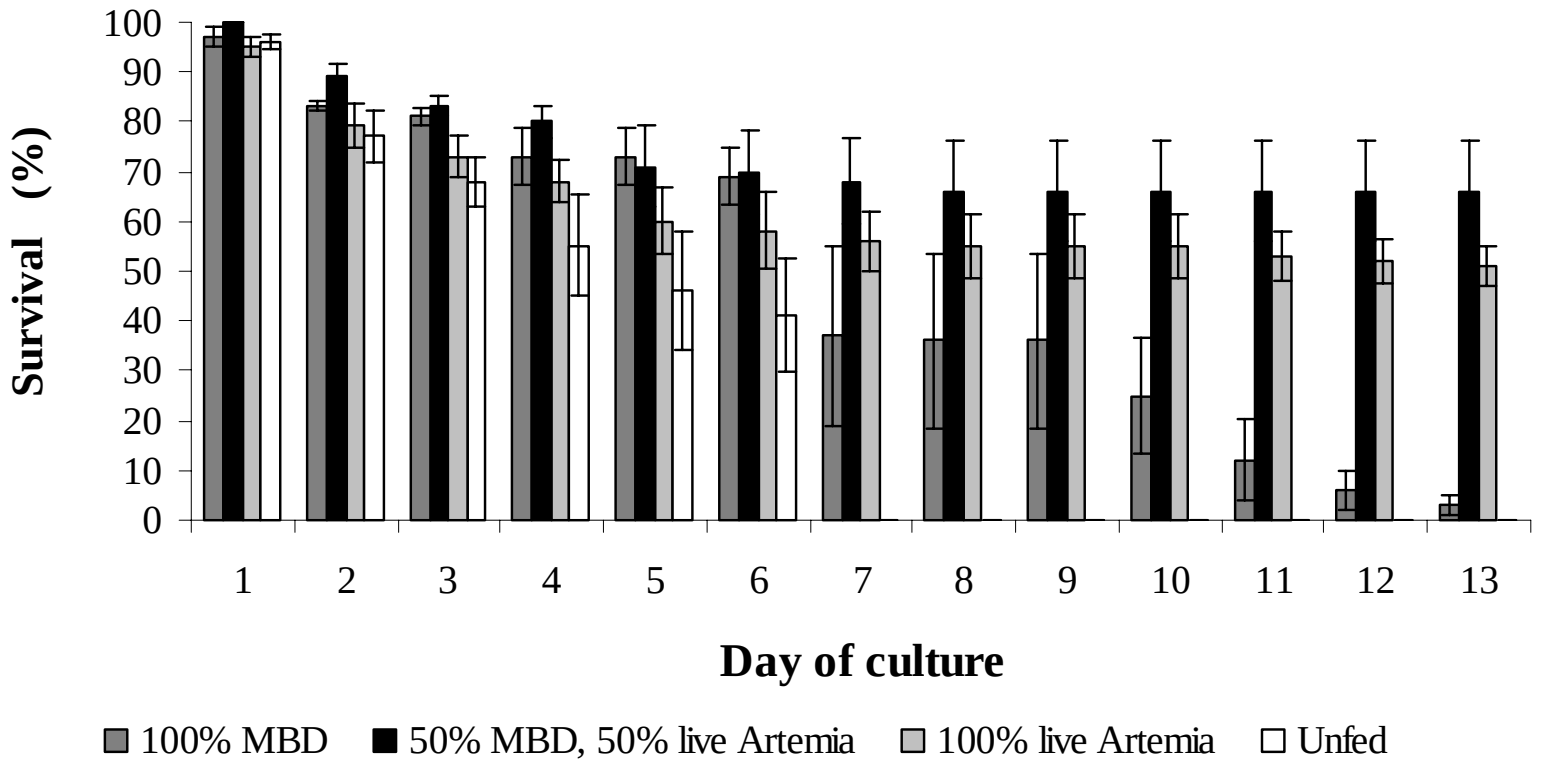

Fig. 3.1 Daily percentage (± SE) survival of *Scylla serrata* zoea III larvae fed diets containing four different levels of MBD and live *Artemia* nauplii. Survival at day 13 represents the total number of molts observed in each dietary treatment.

Table 3.2 Percentage survival to zoea IV stage and mean ± SE intermolt duration of zoea III larvae of *Scylla serrata* fed varying proportions of microbound diet (MBD) and live *Artemia* nauplii. Different superscripts in each column indicate significant differences between means (P <0.05).

Diet	Mean ± SE survival (%)	Mean ± SE development time (days)
100% MBD	3.0 ± 0.48 [a]	8.0 ± 0.0 [a,c]
50%:50% MBD/*Artemia*	66.0 ± 2.53 [b]	5.5 ± 0.1 [b]
100% *Artemia*	51.0 ± 0.95 [b]	5.6 ± 0.4 [b,c]
Unfed	0.0 [a]	-

3.3.2 Exp. 2. Use of defined MBD in rearing of Scylla serrata megalopae

Generally good survival (46.7-60.0%) was recorded for megalopae fed any of the four MBD. It is particularly worth noting that for megalopae fed MBD containing fish meal or squid meal, survival to C1 (60.0%) was higher than that of larvae fed MBD containing dry live feed, i.e. either *Artemia* meal or dried rotifers (Table 3.3). Larvae fed 100% live *Artemia* nauplii showed the highest survival (80.0%), while no larvae in the unfed treatment survived to C1 (Fig. 3.2). There were no significant differences in the average time required to reach C1 for megalopae fed any of the four MBD (Table 3.3). Shortest overall development time was recorded for megalopae fed live *Artemia* nauplii (6.2 ± 0.1 days). Among the megalopae fed MBD, shortest development time was recorded among those fed the squid meal based MBD (8.1 ± 0.1 days) (Table 3.3). Mean carapace widths of newly metamorphosed C1 from the different feeding treatments are shown in Fig. 3.3. Crabs that metamorphosed from megalopae fed live *Artemia* nauplii had significantly greater mean carapace width than those fed the MBD ($F_{4,39}$ = 31.629, P <001). Of the crabs that molted from MBD-fed megalopae, mean carapace width was significantly greater for those fed the MBD containing *Artemia*

meal. There was no significant difference in mean carapace width of crabs resulting from megalopae fed the other three MBD (Fig. 3.3).

Table 3.3 Percentage survival of *Scylla serrata* megalopae to the first crab stage and mean ± SE development time (days) when fed live *Artemia* nauplii and four experimental microbound diets. Different superscripts indicate significant differences between means (P <0.05).

Diet	**Survival**	**Mean ± SE development time**
Unfed	0	-
Rotifer meal MBD	46.7	8.4 ± 0.3^{a}
Artemia meal MBD	46.7	8.7 ± 0.2^{a}
Squid meal MBD	60.0	8.1 ± 0.1^{a}
Fish meal MBD	60.0	8.3 ± 0.4^{a}
Live *Artemia* nauplii	80.0	6.2 ± 0.1^{b}

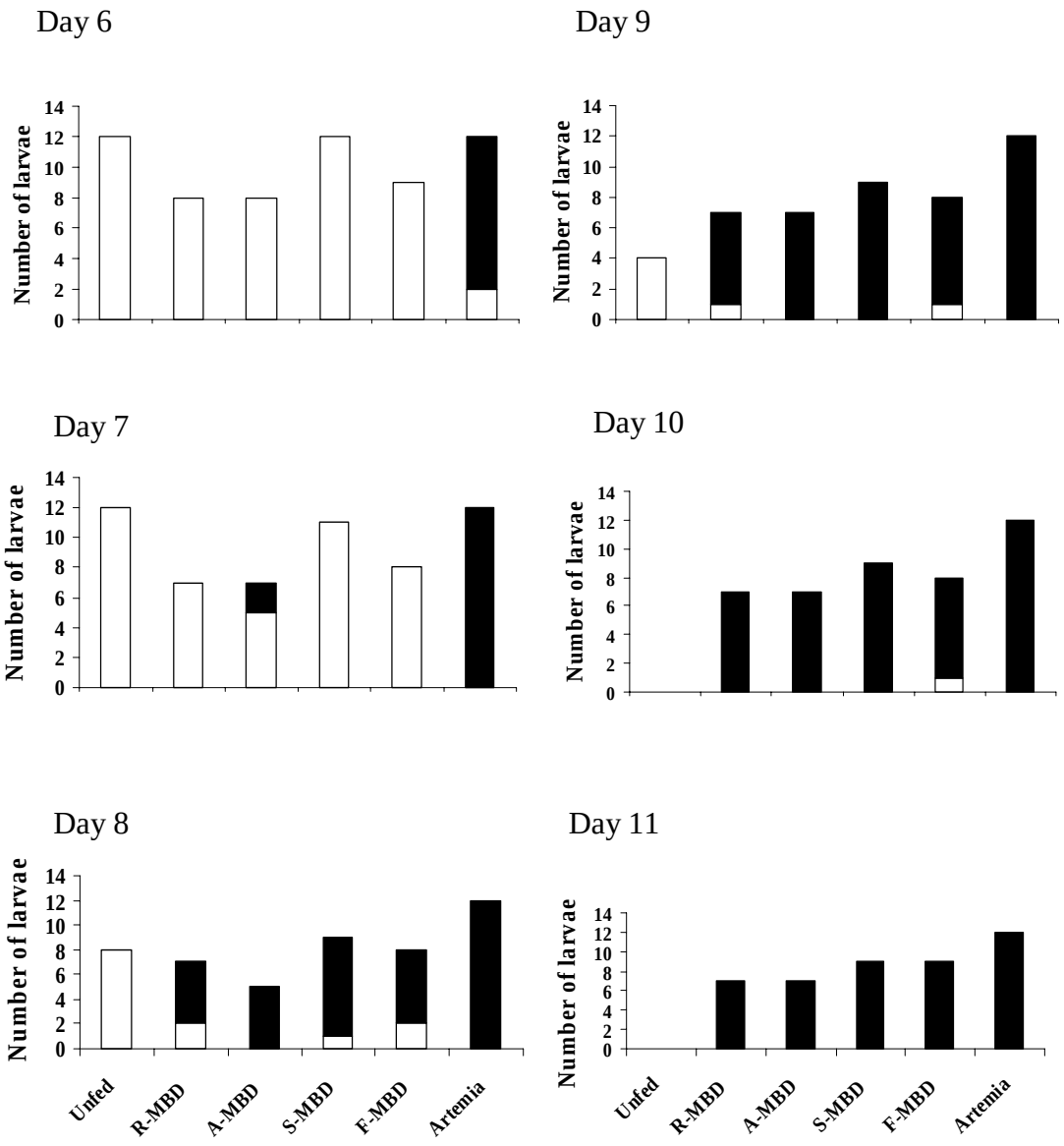

Fig. 3.2 The proportional number of *Scylla serrata* megalopae and juveniles present in each of the six dietary treatments from day 6 to day 11 of Experiment 1. □ = alive, ■ = molted. R-MBD = Rotifer meal MBD, A-MBD = *Artemia* meal MBD, S-MBD = Squid meal MBD, *Artemia* = live *Artemia* nauplii. The term 'alive' refers to megalopae that are alive, but not yet molted.

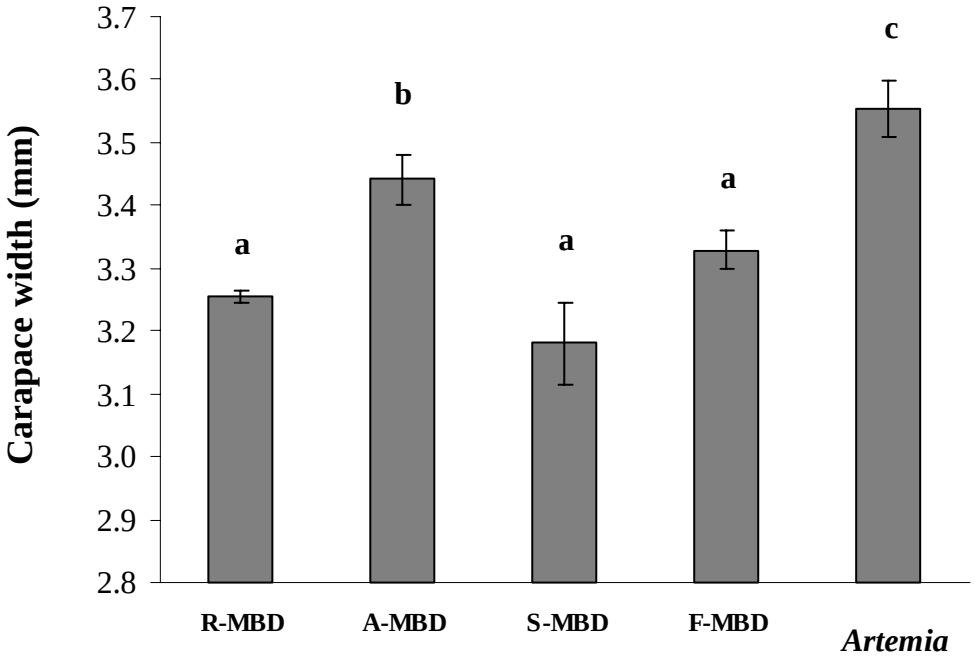

Fig. 3.3 Mean (± SE) carapace width of first stage crabs of *Scylla serrata* metamorphosed from megalopae fed MBD containing different nutrient sources. Means with different superscripts are significantly different (P <0.05). R-MBD = Rotifer meal MBD, A-MBD = *Artemia* meal MBD, S-MBD = Squid meal MBD, *Artemia* = live *Artemia* nauplii.

3.4 Discussion

The feeding experiment with zoea III larvae showed that survival and molting rates were higher when they were fed a diet composed of 50% MBD: 50% *Artemia* nauplii compared to those fed 100% *Artemia* nauplii. This indicates that the MBD contained nutrient(s) that are beneficial for larval development and survival, which are either lacking or present at limiting levels in *Artemia* nauplii. Total replacement of live prey with MBD supported development of 3% of the larvae to the zoea IV stage. These findings indicate that the larvae do ingest MBD particles and are able to assimilate

nutrients from formulated diet particles. At this stage, however, total replacement of live food with MBD for zoea III larvae of *S. serrata* appears unviable as it results in poor survival. More research is required to gain a better understanding of the degree to which zoea III larvae are able to utilize MBD particles. Similar results to those of this study have been reported for the larvae of other crustaceans, where total replacement of live foods with particulate food particles resulted in substantially reduced growth and survival and often resulted in higher incidence of deformities (Kanazawa et al., 1982; Jones et al., 1993). Quinitio et al. (1999) assessed the potential for replacement of live food with commercially formulated prawn feed for *S. serrata* larvae. While the fatty acid composition (particularly eicosapentaenoic acid 20:5*n*-3, EPA and docosahexaenoic acid 22:6*n*-3, DHA) of the formulated prawn diet was closer to that of crab zoea than live foods (rotifers and *Artemia* nauplii), larvae fed the formulated diet alone did not survive beyond the zoea II stage and only those fed live foods were able to molt to megalopa (Quinitio et al., 1999). The failure of the formulated feed was attributed to water fouling caused by the feed (Quinitio et al., 1999); however, it may also indicate that early zoeal larvae of mud crabs have a limited ability to digest formulated diet particles.

Previous studies have shown that movement and olfactory stimuli associated with live prey trigger an important prey-capture response in fish and crustacean larvae, which increases ingestion rates (Teshima et al., 2000). Kurmaly et al. (1990) reported that food capture by larvae of the clawed lobster, *Homarus gammarus*, resulted from chance encounter, but chemo-attractants appeared to have a role in determining edible from the inedible food particles. Crab zoeal are non-obligate visual feeders capable of feeding in the dark by either random encounter or chemosensory detection (Gardner and Maguire,

1998). Recent research has raised the question of whether mud crab zoea may also utilize visual cues for foraging during daytime (Rabbani and Zeng, 2005). However, very little is known about the specific foraging mechanisms utilized by crab zoea and particularly by *S. serrata* zoea. More research is needed to fully appreciate the potential roles of prey movement and feeding attractants in the further development and use of formulated food particles for this species.

Young fish and crustacean larvae are generally poorly developed and lack a fully functional digestive system. Most early larvae lack the enzymes required for efficient breakdown of food particles and therefore rely on live food organisms (such as rotifers and *Artemia* nauplii) to assist digestion and utilization of nutrients present in formulated food particles (Lauff and Hofer, 1984; Il'ina and Turesky, 1987; Bromage and Roberts, 1995). The benefits associated with co-feeding of live food with formulated food particles has been extensively studied and documented for the larvae of fish (e.g. Ehrlich et al., 1989; Jones et al., 1993) and several penaeid species (e.g. Jones et al., 1987; Tackaert et al., 1989; Biedenbach et al., 1990; Ottogali, 1991). More recently, the value of co-feeding has been shown for the larvae of prawn, *Penaeus setiferus* (Gallardo et al., 2002) and the American lobster, *Homarus americanus* (Fiore and Tlusty, 2005). The results of this study also suggested that co-feeding of MBD particles with live food can improve development and survival of zoea III larvae of *S. serrata*. However, for an MBD to be successful as a tool for nutritional requirement studies, complete replacement of live food is preferred. This allows for direct control of the dietary levels in the diets, and therefore a clear indication of optimum ratios of each nutrient. On this basis, megalopa was chosen as the most appropriated larvae stage for further experiments to specify the nutritional requirement of mud crab larvae.

Total replacement of live foods with formulated diets for crustacean larvae has been reported for penaeid prawn (Jones et al., 1979, 1987) and more recently for the freshwater prawn, *Macrobrachium rosenbergii*, from fifth larval stage onward (Kovalenko et al., 2002). The effects of different proportions of MBD and *Artemia* nauplii on survival and molting success of *S. serrata* megalopae was investigated by Genodepa et al. (2004b) who concluded that total replacement of live *Artemia* nauplii with MBD is possible for *S. serrata* megalopae when dried rotifers are incorporated into the diet.

The results from the present feeding experiment with *S. serrata* megalopae show that it is possible to replace the rotifer component of the experimental MBD used by Genodepa et al. (2004b) with commonly available and widely used nutrient sources, such as squid meal and fish meal, without affecting survival and development of megalopae. A higher proportion (60%) of megalopae successfully metamorphosed to C1 when they were fed MBD containing squid meal or fish meal compared to those fed MBD containing rotifer or *Artemia* meal (46.7%). Because the megalopa stage of *S. serrata* is substantially longer than the zoea stages (8-10 days versus 3-4 days), and is typically associated with higher feeding rates (Zeng, 1998), reduced reliance on live foods would be particularly advantageous during this stage of larval culture. Given the potential difficulties and high costs associated with rotifer and *Artemia* production (Southgate, 2003), the successful use of squid meal or fish meal in an experimental MBD is an important step towards development of a complete MBD that will enhance megalopal survival in mud crab hatcheries.

Highest overall survival of *S. serrata* megalopae was recorded for those fed live *Artemia* nauplii, a result that differs from those of similar prior experiments with *S. serrata* megalopae. Genodepa et al. (2004b) found that survival of larvae fed 100% MBD did not differ significantly from that of larvae fed 100% live *Artemia* nauplii. The exact reason for this variability between studies is unclear, but it may reflect physiological differences between different batches of larvae or differences in experimental conditions. High mortality of *S. serrata* is commonly associated with molting from zoea V to the megalopa stage (Genodepa et al., 2004b) and to overcome this, two-day old megalopae were selected for Experiment II. Mortality still occurred during the first few days of the feeding trial, especially for larvae fed MBD. Megalopae used in the experiment were fed *Artemia* nauplii from late zoea II stage to the start of the experiment. As such, larvae fed any of the MBD in Experiment II went through a weaning process during the first few days of the experiment which may have influenced overall survival. Previous research with *S. serrata* larvae has reported high mortality for the first few days after rapid change from live food to MBD, however, as larvae become accustomed to MBD, daily mortality was considerably reduced (Genodepa, 2003; Genodepa et al., 2004b). Similar observations have been made for the larvae of other crustaceans such as *M. rosenbergii* (Roustaian et al., 2001; Kamarudin and Roustaian, 2002), and it suggests that the stress of the weaning process may be reduced if an appropriate mixture of live and formulated foods is provided.

This experiment has shown that it is possible to rear *S. serrata* megalopae on a defined experimental MBD that contain only commercially available ingredients without affecting larval survival or development rates. This indicates considerable potential for MBD as a tool in subsequent studies to investigate the nutritional requirement of *S.*

serrata megalopae. Although both fish meal and squid meal based MBD supported good survival to C1, the squid meal diet resulted in shorter development time from megalopa to C1. On this basis squid meal was chosen as a suitable protein source for subsequent experiments in this study.

Chapter 4

An assessment of the dietary cholesterol requirement of *Scylla serrata* megalopae using semi-purified diets [2]

4.1 Introduction

Cholesterol is an important sterol, serving as a precursor for many physiological compounds such as sex and molting hormones, adrenal corticoids, bile acids and vitamin D (Sheen, 2000). Most animals can synthesize sterols from acetate, but crustaceans, like other arthropods, are incapable of *de novo* production of sterols (Teshima and Kanazawa, 1971; Sheen et al., 1994). Dietary cholesterol is therefore considered essential for good growth and high survival in crustaceans (Sheen, 2000), and the quantitative requirements for cholesterol in formulated diets for crustaceans have been studies since the early 1970's. Examples of the estimated cholesterol requirements of crustaceans range from 0.1% to 1.4% for juvenile *Penaeus japonicus* (Shudo et al., 1971), from 0% and 0.12%-0.5% for adult and juvenile marine lobster (*Homarus* sp.) (Castell and Covey, 1976), and between 0.23% and 0.42% for the white prawn *Penaeus vannamei* (Duerr and Walsh, 1996). Limited information is available on the cholesterol requirements of mud crabs, however, in experiments with *S. serrata* juveniles, significantly higher weight gain was observed for crabs fed diets containing

[2] The data in this chapter is published in *Aquaculture* (2006) 261: 1328-1334. See Appendix 2

0.5% and 0.79% dietary cholesterol (Sheen, 2000). No crabs fed the diet without dietary cholesterol survived, and the diet containing cholesterol levels higher than 1.12% had an adverse effect on juvenile mud crab growth.

The aim of this experiment was to determine the dietary cholesterol requirement for megalopae of *S. serrata* using semi-purified formulated diet particles. Five different levels of cholesterol were formulated into a basal microbound diet (MBD), and survival, dry weight, carapace width and development time to first crab stage (C1) in each treatment was used to evaluate the optimum dietary cholesterol level.

4.2 Materials and methods

4.2.1 Preparation of experimental microbound diets

The experiment was conducted following the methods detailed in section 2.7, and the compositions of the five experimental MBD are presented in Table 4.1. The formulations were based on those used in similar experiments with *S. serrata* juveniles by Sheen (2000) and *S. serrata* megalopae by Genodepa et al. (2004), and de-fatted squid meal (containing 76.15% crude protein) was used as protein source for the experimental MBD based on results from Chapter 3.

Table 4.1 The composition (% dry weight) of the experimental microbound diets used in feeding experiments with *Scylla serrata* megalopae. Formulation based on Sheen (2000), Genodepa et al. (2004) and the results from Chapter 3 of this thesis.

Ingredient	Source	Total cholesterol level in diet (% dry weight)				
		0.14%	0.20%	0.40%	0.80%	1.00%
Lipid-free squid meal	A	55.0	55.0	55.0	55.0	55.0
Fish oil [a]	B	4.0	4.0	4.0	4.0	4.0
Corn oil [b]	B	2.0	2.0	2.0	2.0	2.0
Zein [c]	B	3.0	3.0	3.0	3.0	3.0
Vitamin mix [d]	C	4.0	4.0	4.0	4.0	4.0
Mineral mix [e]	C	4.0	4.0	4.0	4.0	4.0
Lecithin [f]	B	2.6	2.6	2.6	2.6	2.6
Dibasic calcium phosphate (DCP) [g]	B	0.6	0.6	0.6	0.6	0.6
Choline chloride [h]	B	1.0	1.0	1.0	1.0	1.0
Cholesterol [i]	B	0.0	0.06	0.26	0.66	0.86
Starch [j]	B	18.9	18.78	18.33	17.44	17.0
Cellulose [k]	B	4.87	4.94	5.19	5.68	5.92

Sources of ingredient: A) Skretting Tasmania B) Sigma-Aldrich Pty. Ltd, C) Rabar Pty Ltd [a)] from menhaden F8020 [b)] C8267 [c)] from maize Z3625 [d)] ZZ600 DPI, each 1 kg contains: Retinol (vit. A 2) mIU; Cholecaliferol (vit. D3) 0.8 mIU; Tocopherol (vit. E) 40 g; Phytomenadione (vit. K) 2.02 g; inositol (vit. Bh) 50 g; Niacin (vit. B3) 30.40 g, Pantothenic Acid (vit. B5) 9.18 g; Folic Acid (vit. B9) 2.56 g; Riboflavin (vit. B2) 4.48 g; Cyanocobalamin (vit. B12) 0.004 g; biotin (vit. H) 0.1g; Pyridoxine (vit. B6) 4 g; Thiamine (vit. B1) 3.4 g; Ascorbic Acid (vit. C) 44.4 g; para amino benzoic acid 20 g; tixosil™ (anticoagulant) 5 g and antioxidant 30 g. [e)] ZZ603 DO 067 DPI, each 1 kg contained: copper 1 g, cobalt 100 mg, magnesium 59.4 mg, manganese 5 g iodine 800 mg, selenium 20 mg, iron 8 mg, zinc 20 g, aluminium 100 mg, chromium 100 mg [f)] L-A-phosphatidylcholine from soybean P7443 [g)] dibasic calcium phosphate C4131 [h)] 98% powder C7527, [i)] Sigma Grade 99% C8667 [j)] from corn S4126 [k)] alpha C8002.

Seven dietary treatments were used consisting of five iso-energetic, semi-purified MBD with different levels of cholesterol, one control group fed live *Artemia* nauplii, and one control group of unfed larvae. All dietary treatments had 15 replicates. Before preparation of the diets, lipids were extracted from the squid meal using 2:1 chloroform-methanol (v/v) (Folch et al., 1957), and to allow formulation of diets containing specific levels of cholesterol, the menhaden fish oil used in the diets was analyzed for endogenous cholesterol content prior to diet formulation. Supplemental cholesterol was added to each diet (with the exception of the basal diet) to give the required level of

dietary cholesterol (0.14%, 0.20%, 0.40%, 0.60%, 0.80% and 1.00%) (Table 4.1), while iso-caloricity was maintained between diets by manipulation of starch and cellulose contents (Table 4.1).

4.2.2 Statistical analysis

The mean development time from megalopa to first crab stage for the seven different dietary treatments were compared using a one-way ANOVA, and specific differences among the treatments were determined using Tukey's multiple comparison test at the 0.05 level of significance. Due to the unequal sample size, the carapace widths of megalopae fed different diets were compared using a Kruskal-Wallis non-parametric test and significant treatment effects were determined using the Mann-Whitney test. All statistical analysis were performed using SPSS for Windows, version 14.0.

4.3 Results

4.3.1 Survival from megalopa to the first crab stage

All the formulated diets supported development and survival of megalopae through to the first crab stage (C1) (Fig. 4.1). Best overall survival (73.3%) was recorded for megalopae fed the MBD containing 0.80% cholesterol, whereas no megalopae in the unfed control treatment metamorphosed into crabs.

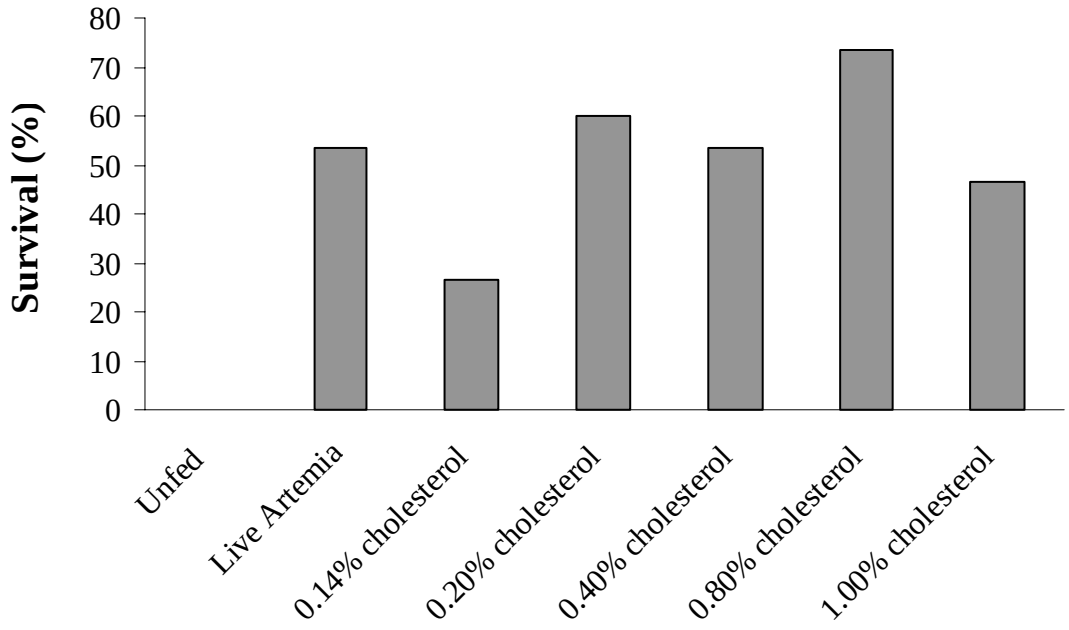

Fig. 4.1 Survival (%) of *Scylla serrata* from megalopae to the first crab stage when fed microbound diets containing different levels of cholesterol.

4.3.2 Carapace width of newly metamorphosed first stage crabs

Widest mean carapace width was recorded for C1 that molted from megalopae fed live *Artemia* nauplii (3.5 ± 0.08 mm) (Table 4.2). The second widest mean carapace width was recorded for crabs that molted from megalopae fed MBD containing 0.80% dietary cholesterol (3.3 ± 0.05 mm), which was significantly wider than the mean carapace width of crabs molting from megalopae fed MBD containing 1.00% total cholesterol. Neither the MBD containing 0.80% or 1.00% cholesterol gave significantly different mean carapace widths compared to the diets containing 0.14%, 0.20% or 0.40% cholesterol (Table 4.2).

Table 4.2 Percentage survival, mean ± SE final dry weight, carapace width and development time for juvenile *Scylla serrata* molted from megalopae fed semi-purified diets containing different levels of dietary cholesterol. Within columns, mean with different letters are significantly different (P <0.05).

Cholesterol level (%)	Survival (%)	Final dry weight (mg)	Carapace width (mm)	Development time (days)
0.14	26.7	12.0 ± 0.71 ^{bc}	3.2 ± 0.11 ^{bc}	11.0 ± 1.35 ^{bc}
0.20	60.0	13.8 ± 0.83 ^{c}	3.2 ± 0.07 ^{bc}	8.8 ± 0.91 ^{abc}
0.40	53.3	13.1 ± 1.55 ^{bc}	3.1 ± 0.05 ^{bc}	8.8 ± 0.94 ^{abc}
0.80	73.3	14.2 ± 1.18 ^{c}	3.3 ± 0.05 ^{b}	9.9 ± 1.27 ^{abc}
1.00	46.7	9.9 ± 0.59 ^{b}	3.2 ± 0.05 ^{c}	11.0 ± 1.24 ^{c}
Live *Artemia*	53.3	21.1 ± 0.22 ^{a}	3.5 ± 0.08 ^{a}	8.0 ± 1.36 ^{a}
Unfed	-	-	-	-

4.3.3 Dry weight of newly metamorphosed first stage crabs

Highest mean dry weight (21.1 ± 0.22 mg) was found among the newly molted C1 molting from megalopae fed live *Artemia* nauplii (Table 4.2), and the juveniles from this dietary treatment group were significantly heavier than megalopae from any other treatment. Second highest mean dry weight was recorded for crabs that molted from megalopae fed MBD containing 0.80% total cholesterol (14.2 ± 1.18 mg) (Table 4.2), a result that was significantly different from the mean dry weight of C1 molting from megalopae fed MBD containing 1.00% cholesterol (9.9 ± 0.59 mg). The mean dry weight of the juveniles that molted from megalopae fed the diet containing 1.00% cholesterol was, however, not significantly different from that of C1 resulting from megalopae fed the MBD containing 0.14% (12.0 ± 0.71 mg) or 0.40% (13.1 ± 1.55 mg) cholesterol (Table 4.2).

4.3.4 Development time from megalopa to first stage crabs

The development time from the start of the experiment until metamorphosis of megalopae from each treatment into the first crab stage is shown in Table 4.2. The first

molt was seen on day 5 of the feeding trial in the treatment fed live *Artemia* nauplii, and the last successful molt occurred on day 14 in the treatment receiving the MBD containing 0.80% total cholesterol. The mean development time from megalopae to C1 varied between the dietary treatments, and was shortest (between 8.0 and 9.9 days) for megalopae fed live *Artemia* nauplii or MBD containing 0.20%, 0.40% or 0.80% total cholesterol. Longest development time was recorded for the megalopae fed diets containing 0.14% or 1.00% cholesterol (both 11.0 days).

4.4 Discussion

A formulated MBD containing 0.80% total cholesterol supported better survival of *S. serrata* megalopae than live *Artemia* nauplii which is the standard live food for *S. serrata* megalopae (Dainteath and Quin, 1991). This encouraging result confirms the potential of MBD as a total replacement for live foods in hatchery culture of *S. serrata* megalopae, and although highest survival was recorded for megalopae fed the MBD containing 0.80% total cholesterol (74.3%), successful metamorphosis was also recorded for megalopae fed a diet without supplemented cholesterol (26.7%). These findings indicate that the endogenous cholesterol level (0.14% of total dry weight) of the basal MBD containing fish oil is sufficient to support development of some megalopae through to the first crab stage.

Similar results were reported in experiments with juvenile freshwater prawn, *Macrobrachium rosenbergii,* when fed semi-purified diets containing cod liver oil with 0.12% endogenous cholesterol (Briggs et al., 1988). In this study, little difference in survival of *M. rosenbergii* was seen when purified cholesterol was added to the diet (Briggs et al., 1988). This trend does not seem to be consistent for all crustacean

species, however, and work with juveniles of species such as marine prawns (*Penaeus* sp.), lobsters (*Homarus* sp.) and crayfish (*Pacifastacus leniusculus*) has shown that growth and survival was clearly improved when the diet was supplemented with cholesterol (Teshima and Kanazawa, 1983; D'Abramo et al., 1984; Sheen and D'Abramo, 1991; Sheen et al., 1994). These results emphasize the importance of determining the species-specific requirements for both cholesterol and other nutrients for commercially important species of crustacean, as the requirements established for one genus or species may not be directly transferable to the others.

On the basis of weight gain and broken line analysis, previous research on *S. serrata* concluded that the optimum dietary cholesterol level for juveniles is 0.51% (Sheen, (2000). In Sheen's study, however, highest overall survival (93%) was recorded for juveniles fed a diet containing 0.21% supplemental cholesterol, which was the lowest level tested. This suggests that even at the juvenile stage, *S. serrata* is able to survive on low levels of dietary cholesterol. The observed differences in preferred cholesterol levels between *S. serrata* megalopae and juveniles may reflect the expected variation between different development stages and between the experimental diets used (Teshima, 1997). Sheen (2000) found that *S. serrata* juveniles fed diets containing negligible levels of cholesterol (0.04%) did not survive indicating that dietary cholesterol is essential for *S. serrata* juveniles. The reduced survival and molting rate observed in crustacean larvae fed a cholesterol-free diet is believed to be linked to low biosynthesis of ecdysone. Cholesterol has been shown to be an important metabolic precursor for ecdysone biosynthesis (Watson and Spaziani, 1982), and studies have shown that Y-organ uptake of cholesterol is greatly improved at the time the molting sequence is initiated (Spazani and Kater, 1973). At the same time, too much cholesterol

in the diet has shown to adversely reduce growth and survival of crustacean (Sheen, 2000). Although not yet fully understood, this is believed to be a nutrient-response characteristic rather than the result of toxicity (Mercer, 1982). In this study, larvae fed the diet containing 1.00% cholesterol (the highest level tested) had generally lower final dry weight, and longer development time than any of the other diets supplemented with lower levels of cholesterol. However, the values for these parameters did not differ significantly from those for larvae fed diets containing lower levels of cholesterol, and further experiments are necessary to determine if a detrimental effect is associated with excess levels of dietary cholesterol for *S. serrata* megalopae.

All the MBD formulated for this experiment contained supplemental phospholipid at a level of 2.6% of total diet dry weight. Previous research with other crustaceans has shown that the digestion and assimilation of cholesterol is strongly influenced by total dietary lipid and phospholipid content (Teshima and Kanazawa, 1983; D'Abramo et al., 1985), and reduced absorption of cholesterol is commonly observed when marine larvae are fed lipid-free diets. This phenomenon has been reported for *Penaeus monodon* (Paibulkichakul et al., 1998), *P. japonicus* (Shudo et al., 1971), *P. vannamei* (Duerr and Walsh, 1996) and *S. serrata* (Sheen, 2000). Addition of dietary phospholipid, however, has been found to enhance digestibility of sterols (Conklin et al., 1980; Paibulkichakul et al., 1998; Thongrod and Boonyaratpulin, 1998). On this basis, it is possible that survival observed in the present study among *S. serrata* megalopae fed MBD containing low levels of dietary cholesterol may result from an interaction effect between dietary cholesterol and phospholipids.

In summary, this experiment indicate that mud crab *S. serrata* megalopae fed MBD containing 0.80% total dietary cholesterol show improved survival through to the C1 stage compared to megalopa fed the 'traditional' diet of live *Artemia* nauplii. Every MBD containing cholesterol at a level between 0.14% and 1.00% supported acceptable survival, suggesting that supplemented cholesterol is not strictly required for megalopae fed the experimental MBD containing phospholipids and fish oil. However, shorter development time, larger carapace width and higher dry weigh were achieved in C1 molting from megalopae fed MBD containing 0.80% total dietary cholesterol, compared to juvenile crabs molting from megalopae fed a diet without supplemented cholesterol. As these results are not statistically significant, further research is required to determine the optimum cholesterol level for *S. serrata* megalopae, and to gain further insight into the suspected interaction effect between different dietary lipids; this is addressed in Chapter 5.

Chapter 5

Assessment of dietary lecithin and cholesterol requirements of mud crab, *Scylla serrata*, megalopae using semi-purified microbound diets [3]

5.1 Introduction

Cholesterol and phospholipids are important nutrients that have been shown to enhance growth and survival of a range of marine crustaceans (Kanazawa et al., 1982; Teshima et al., 1982). Dietary requirements for cholesterol were investigated in Chapter 4, and the present chapter will focus on determining the requirement for dietary phospholipids, as well as assessing whether an interaction between these two nutrients exists. Phospholipids are compounds associated with the molecular organization of cells, particularly membranes (Hickmann et al., 1998). Although Kanazawa et al. (1985) reported that some penaeid species are capable of a slow rate of *de novo* synthesize of phospholipids, incorporation of supplemental phospholipids into crustacean diets (usually in the form of soybean lecithin) has been shown to improve survival and growth, and to enhance the success of larval metamorphosis in many crustacean species (i.e. Thompson et al., 2003). Supplemental dietary phospholipids have also shown to reduce the occurrence of molting death syndrome in crustacean larvae (Anderson and De Silva, 2003; Thompson et al., 2003).

[3] The data in this chapter is published in *Aquaculture Nutrition* (2007) 13: 413-423. See Appendix 3

A dietary source of phospholipids is important at all life stages for crustaceans, but larval stages are known to be particularly sensitive to phospholipids deficiency. Zoea I and II stages of *Penaeus japonicus*, for example, suffered 100% mortality before reaching the mysis stage when fed a phospholipid deficient diet (Kanazawa et al., 1985). At the same time, excessive levels of dietary phospholipids can be detrimental and experiments have shown that survival of *P. japonicus* larvae decreased when more than 3% soybean lecithin was added to the diet (Teshima et al., 1986b). Based on the above, it is clear that determination of appropriate levels of dietary phospholipids is important for development of an optimized formulated diet for *S. serrata* megalopae.

The purpose of this experiment was to use semi-purified microbound diets (MBD) to determine the effect of supplemental dietary cholesterol and lecithin on growth, development time and survival of *S. serrata* megalopae, and to determine a possible interaction between these two nutrients.

5.2 Materials and methods

5.2.1 Preparation of microbound diets

The formulation of the experimental MBD used in this study is shown in Table 5.1. Diet compositions were based on those used in similar experiments with *S. serrata* juveniles by Sheen (2000) and the results from Chapter 3 and 4 of this thesis. Lipids were extracted from the squid meal, using 2:1 chloroform-methanol (v/v) (Folch et al., 1957) before diet preparation, and the levels of dietary starch and cellulose were adjusted to render all diets iso-energetic.

5.2.2 Experimental design

Six semi-purified MBD were formulated to be iso-energetic and to contain three levels of supplemental lecithin (0.0, 2.0 and 4.0% of total diet dry weight) and two levels of supplemental cholesterol (0.0 and 0.7% of total diet dry weight). As the fish oil used in diet formulation contained endogenous cholesterol, the supplementation level of 0.7% cholesterol was used to formulate diets containing the optimum total dietary cholesterol level of 0.8% as defined in the previous chapter. Two control treatments were also included in the experiment; one treatment consisting of unfed megalopa, and one treatment consisting of megalopa fed the traditional diet of un-enriched, newly hatched live *Artemia* nauplii. Every dietary treatment had 15 replicates, and megalopae were randomly assigned to individual aquarium.

5.2.3 Statistical analysis

The interaction between cholesterol and lecithin on final dry weight and carapace width was analysed using two-way ANOVA. Significant differences between the treatments were determined using Tukey's multiple comparison test at the 0.05 level of significance. Survival from megalopae to the first crab stage (C1) was analyzed using Pearson Chi-square analysis (dead vs. alive) by diet (Diet 1-6). Development time from the start of the experiment to C1 for the different dietary treatments were compared using Kruskal-Wallis non-parametric test, and significant treatment effects were determined using the Mann-Whitney test. The Pearson Chi-square tests and the two-way ANOVA was performed using Systat 10 (SPSS), the one-way ANOVA and Kruskal-Wallis non-parametric test were performed using SPSS for Windows, version 14.

Table 5.1 Formulation (% dry weight) of experimental microbound diets used in feeding experiments with *Scylla serrata* megalopae and their analyzed total cholesterol, total phosphoplipid and total lipid contents. Formulation is based on diets used by Sheen (2000), Genodepa et al. (2004) and the results from Chapter 3 and 4 of this thesis.

Ingredient	**Source**	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**
De-fatted squid meal	A	55.0	55.0	55.0	55.0	55.0	55.0
Fish oil [a]	B	4.0	4.0	4.0	4.0	4.0	4.0
Corn oil [b]	B	2.0	2.0	2.0	2.0	2.0	2.0
Zein [c]	B	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin mix [d]	C	4.0	4.0	4.0	4.0	4.0	4.0
Mineral mix [e]	C	4.0	4.0	4.0	4.0	4.0	4.0
DCP [f]	B	0.6	0.6	0.6	0.6	0.6	0.6
Choline chloride [g]	B	1.0	1.0	1.0	1.0	1.0	1.0
Cholesterol [h]	B	0.0	0.7	0.0	0.7	0.0	0.7
Lecithin [i]	B	0.0	0.0	2.0	2.0	4.0	4.0
Starch [j]	B	17.0	15.5	12.6	11.1	8.1	6.7
Cellulose [k]	B	9.4	10.2	11.8	12.6	14.3	15.1
Dietary energy (MJ kg^{-1})[l]		26.91	26.91	26.91	26.91	26.91	26.91
Analyzed composition							
Cholesterol		0.0	0.8	0.0	1.1	0.1	0.9
Lecithin		0.6	0.5	2.3	2.8	4.0	4.4
Total lipid		6.5	6.0	8.4	8.5	10.5	10.8

A) Skretting Tasmania B) Sigma-Aldrich Pty. Ltd, C) Rabar Pty Ltd [a)] from menhaden F8020 [b)] C8267 [c)] from maize Z3625 [d)] ZZ600 DPI, each 1 kg contains: Retinol mIU; Cholecaliferol 0.8 mIU; Tocopherol 40 g; Phytomenadione 2.02 g; inositol 50 g; Niacin 30.40 g, Pantothenic Acid 9.18 g; Folic Acid 2.56 g; Riboflavin 4.48 g; Cyanocobalamin 0.004 g; biotin 0.1g; Pyridoxine 4 g; Thiamine 3.4 g; Ascorbic Acid 44.4 g; para amino benzoic acid 20 g; tixosil™ (anticoagulant) 5 g and antioxidant 30 g. [e)] ZZ603 DO 067 DPI, each 1 kg contains: copper 1 g, cobalt 100 mg, magnesium 59.4 mg, manganese 5 g iodine 800 mg, selenium 20 mg, iron 8 mg, zinc 20 g, aluminium 100 mg, chromium 100 mg [f)] dibasic calcium phosphate C4131 [g)] 98% powder C7527 [h)] Sigma Grade 99% C8667 [i)] L-A-phosphatidylcholine from soybean P7443 [j)] from corn S4126 [k)] alpha C8002 [l)] Calculated energy content, computed as 23.01, 38.07 and 17.15 MJ kg^{-1} of protein, lipid and carbohydrate, respectively (Anderson and De Silva, 2003).

5.3 Results

5.3.1 Diet compositions

The analyzed contents of total lipid, phospholipids and cholesterol of the MBD used in this experiment, and the level of these components for which the diets were formulated are shown in Table 5.1. Lipid analysis of the MBD showed that diets formulated with no supplemental phospholipid actually contained 0.6% and 0.5% (Diets 1 and 2). The

diets formulated with 2.0% supplemental phospholipid actually contained 2.3% and 2.8% phospholipid (Diets 3 and 4), and the diets formulated with 4.0% supplemental phospholipid actually contained 4.0 and 4.4% phospholipid (Diets 5 and 6). The diets formulated with no supplemental cholesterol actually contained 0.0, 0.0 and 0.1% (Diet 1, 3 and 5) and the diets formulated with 0.7% supplemental cholesterol contained 0.8, 1.1 and 0.9% cholesterol (Diet 2, 4 and 6).

5.3.2 Dry weight of newly metamorphosed first stage crabs

In terms of dry weight of newly molted C1, a highly significant (P = 0.005) interaction was detected between supplemental dietary cholesterol and supplemented dietary lecithin (Table 5.2). Highest mean dry weight (1.84 ± 0.07 mg) was recorded for the control treatment fed live *Artemia* nauplii, and these crabs were significantly heavier than those from all other treatments (Fig. 5.1). Among MBD treatments, the C1 that molted from megalopae fed MBD containing a high level of lecithin (Diet 5; lecithin 4.0%, cholesterol 0.1% and Diet 6; lecithin 4.4%, cholesterol 0.9%) showed significantly higher mean dry weights (1.39 ± 0.04 mg and 1.20 ± 0.02 mg for Diet 5 and 6, respectively) than crabs fed Diet 1 (lecithin 0.6%, cholesterol 0.0%) (0.70 ± 1.00 mg) or Diet 3 (lecithin 2.3%, cholesterol 0.0%) (0.80 ± 0.11 mg) (Fig. 5.1).

Fig. 5.1 Dry weight of *Scylla serrata* first stage crabs molted from megalopae fed six different microbound diets formulated to contain three different levels of supplemental lecithin (0.0, 2.0 and 4.0%) and two different levels of supplemental cholesterol (0.0 and 0.7%). Means with different superscripts are significantly different (P <0.05).

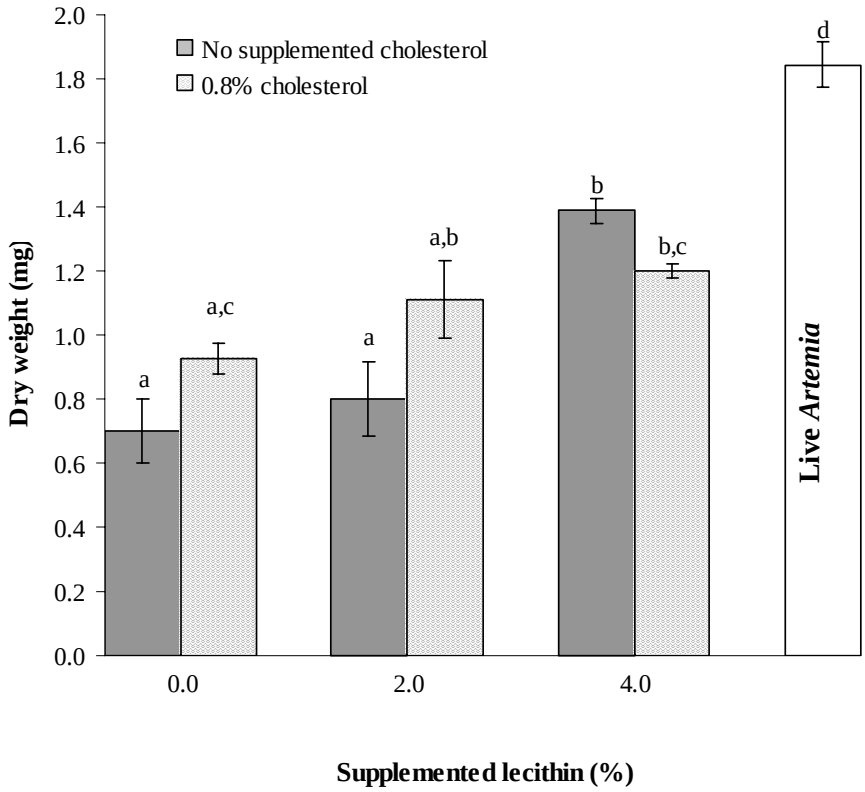

Table 5.2 Results from two-way ANOVA testing the interaction effect between lecithin and cholesterol on the dry weight (mg) and carapace width (mm) of newly molted *Scylla serrata* first stage crabs fed six different microbound diets formulated to contain three levels of supplemental lecithin (0.0, 2.0 and 4.0%) and two levels of supplemental cholesterol (0.0 and 0.7%).

Source	df	MS	*F*	*P*
Dry weight (mg)				
Lecithin	2	75.005	18.113	0.000
Cholesterol	1	10.750	2.596	0.117
Lecithin*Cholesterol	2	26.060	6.293	0.005
Error	32	4.141		
Carapace width (mm)				
Lecithin	2	0.250	9.503	0.001
Cholesterol	1	0.013	0.478	0.494
Lecithin*Cholesterol	2	0.052	1.972	0.156
Error	32	0.026		

5.3.3 Carapace width of newly metamorphosed first stage crabs

Based on two-way ANOVA, supplemental dietary cholesterol had no effect on the carapace width of newly molted crabs, and the carapace width was affected by supplemental dietary lecithin only (Table 5.3). The largest mean carapace width was recorded for C1 molting from megalopae fed live *Artemia* nauplii (3.33 ± 0.04 mm); however, this value was not significantly greater than the mean carapace width recorded for C1 resulting from megalopae fed the MBD containing 4.0% lecithin and 0.1% cholesterol (3.13 ± 0.05 mm) (Diet 5). Among MBD treatments, there was no significant difference between C1 that molted from megalopae fed low or medium levels of supplemental lecithin (Diet 1, 2, 3 or 4), but C1 fed MBD containing high levels of lecithin (4.0% and 4.4%) (Diet 5 and 6) had significantly greater carapace width (3.13 ± 0.05 mm and 3.05 ± 0.05 mm, respectively) than C1 resulting from megalopae fed Diet 2 (2.72 ± 0.06 mm) that containing the lowest level of lecithin (0.5%), but with added cholesterol (0.8%) (Table 5.3).

Table 5.3 Mean ± SE carapace width and development time of newly molted first stage crabs resulting from megalopae fed microbound diets containing various levels of supplemental dietary lecithin and cholesterol. Different subscripts within a column indicate significant differences between means (P <0.05).

Treatment	**n**	**Carapace Width (mm)**	**Development Time (days)**
Diet 1 (lecithin 0.6%, cholesterol 0.0%)	3	2.88 ± 0.07^{ab}	10.3 ± 0.9^{a}
Diet 2 (lecithin 0.5%, cholesterol 0.8%)	4	2.72 ± 0.06^{a}	12.5 ± 1.6^{a}
Diet 3 (lecithin 2.3%, cholesterol 0.0%)	5	2.85 ± 0.10^{ad}	10.8 ± 0.7^{a}
Diet 4 (lecithin 2.8%, cholesterol 1.1%)	8	2.97 ± 0.06^{ab}	10.4 ± 0.8^{a}
Diet 5 (lecithin 4.0%, cholesterol 0.1%)	9	3.13 ± 0.05^{bc}	9.8 ± 0.6^{a}
Diet 6 (lecithin 4.4%, cholesterol 0.9%)	9	3.05 ± 0.05^{bd}	9.9 ± 0.7^{a}
Live *Artemia* nauplii	9	3.33 ± 0.04^{c}	7.1 ± 0.4^{b}

5.3.4 Survival from megalopa to the first crab stage

All megalopae in the unfed control treatment died within 17 days of the start of the experiment without reaching the first crab stag. Although mortality in this treatment was observed from day 2, the majority of the unfed larvae died after day 10. Due to the cannibalistic nature of *S. serrata* megalopae, no replicate survival data were available; however, the outcome of Pearson Chi-square test suggested a trend in survival, given the marginal *P*-value of 0.083. Highest survival to the first crab stage (60%) was recorded for megalopae fed the two MBD containing the highest levels of lecithin (Diet 5: 4.0% lecithin, 0.1% cholesterol and Diet 6: 4.4% lecithin, 0.9% cholesterol) (Fig. 5.2). Survival of megalopae from these two treatments was the same as that of megalopae fed live *Artemia* nauplii. As the level of dietary lecithin was reduced to 2.3% (Diet 3; cholesterol 0.0%) and 2.8% (Diet 4; cholesterol 1.1%) survival decreased to 33% and 53%, respectively. Lowest overall survival (20%) was recorded for megalopae fed the MBD containing low total lecithin (0.6%) and low total cholesterol (0.0%) (Diet 1) (Fig. 5.2).

5.3.5 Development time from megalopa to first stage crabs

The first occurrence of C1 was found among megalopae fed live *Artemia* nauplii, where the mean development time to C1 from the beginning of the experiment was 7.1 ± 0.4 days (Table 5.3). This development time was significantly shorter than those for all MBD treatments. Among the MBD-fed megalopae, no significant difference in development time was detected (Table 5.3).

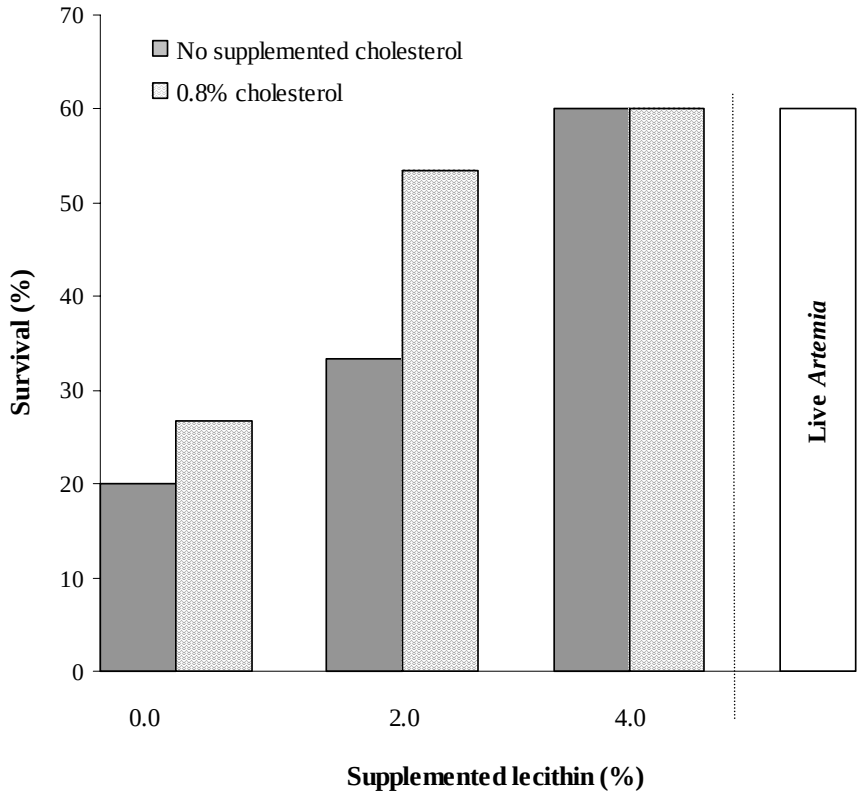

Fig. 5.2 Percentage successful metamorphosis to first stage crabs (C1) of *Scylla serrata* megalopae fed either live *Artemia* nauplii or one of six microbound diets supplemented with different levels of dietary lecithin and cholesterol. All larvae in the unfed treatment died before they molted to C1.

5.3.6 Total lipid and fatty acid compositions of microbound diet

As expected, total lipid level in the MBD increased with increasing level of supplemental lecithin. The lowest total lipid contents (6.5% and 6.0% diet dry weight) were found Diet 1 and Diet 2, respectively (Table 5.1). Dietary lipid level increased to 10.8% diet dry weight in Diet 6 which contained the highest phospholipid level (4.4%) (Table 5.1). The fatty acid compositions of the six MBD are shown in Table 5.4. The levels of linolenic acid (18:3*n*-3, LNA) and linoleic acid (18:2*n*-6, LN) increased with increasing levels of supplemental lecithin. The lowest level of LNA (1.3% of total fatty acid) was found in the diets containing 0.6% and 0.5% phospholipid (Diet 1 and 2) and this increased to 3.0% of total fatty acid in the diet containing 4.4% phospholipid (Diet

6). The lowest levels of LN (22.2% of total fatty acid) was found in the diets containing the lowest level of phospholipid (0.6% and 0.5% in Diet 1 and 2, respectively) and this increased to 37.6% of total fatty acid in the diet containing the highest level of phospholipid (4.4%) (Diet 6).

Table 5.4 Fatty acid composition (% of total fatty acid) of the six experimental microbound diets containing different level of supplemental lecithin and cholesterol. For diet abbreviation, see Table 5.3.

Fatty acid	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
14:0	4.2	4.3	3.8	3.2	3.0	2.8
15:0	0.4	0.4	0.4	0.3	0.3	0.3
16:0	16.4	16.8	16.3	16.0	17.0	16.3
17:0	0.5	0.5	0.5	0.4	0.4	0.4
18:0	3.4	3.4	3.6	3.5	3.8	3.7
20:0	0.3	0.3	0.3	0.3	0.3	0.2
22:0	0.2	0.2	0.2	0.1	0.2	0.1
24:0	0.2	0.1	0.1	0.1	0.1	0.1
16:1*n*-7	6.0	6.0	5.3	4.3	4.1	3.6
20:1*n*-11	0.2	0.2	0.1	0.1	0.1	0.1
18:1*n*-9	18.6	18.3	16.8	16.7	16.3	15.3
18:1*n*-7	2.5	2.4	2.4	2.2	2.2	2.1
20:1*n*-9	1.4	1.4	1.2	1.0	1.0	0.9
20:1*n*-7	0.2	0.2	0.2	0.1	0.1	0.1
22:1*n*-9	0.2	0.2	0.1	0.1	0.1	0.1
24:1*n*-9	0.4	0.4	0.3	0.2	0.2	0.2
18:2*n*-6, LN	22.2	22.2	27.5	33.4	33.6	37.6
20:2*n*-6	0.2	0.2	0.2	0.1	0.1	0.1
20:4*n*-6, AA	0.5	0.5	0.4	0.3	0.3	0.3
22:4*n*-6	0.1	0.1	0.1	0.1	0.1	0.1
22:5*n*-6	0.3	0.3	0.3	0.2	0.2	0.2
18:3*n*-3, LNA	1.3	1.3	2.0	2.4	2.5	3.0
18:4*n*-3	1.9	1.9	1.7	1.4	1.3	1.2
20:3*n*-3	0.2	0.2	0.1	0.1	0.1	0.1
20:4*n*-3	1.2	1.2	1.1	0.9	0.9	0.7
20:5*n*-3, EPA	6.6	6.6	5.8	4.7	4.5	3.9
22:5*n*-3	1.7	1.7	1.5	1.2	1.1	1.0
22:6*n*-3, DHA	8.9	8.8	7.8	6.2	6.0	5.2

5.4 Discussion

The results of this study suggest that an interaction exists between dietary lecithin and dietary cholesterol in developing *S. serrata* megalopae. Newly settled C1 fed MBD containing the highest level of total lecithin assessed (4.0-4.4%) showed significantly greater mean dry weight compared to C1 molting from megalopae fed MBD that lacked or contained very low levels of lecithin, or a diet containing a medium level of lecithin but lacking cholesterol. The results also suggest that high levels of dietary lecithin supported greater survival, and although not significant, shorter development time to C1 than megalopae fed MBD with lower levels of dietary lecithin, regardless of whether the diet was supplemented with cholesterol. Previous studies conducted with both larvae and juveniles of other crustaceans show that diets containing relatively high levels of dietary lecithin are generally superior. For example, Thongrod and Boonyaratpulin (1998) reported higher survival, better growth rates and higher feed efficiency in juveniles prawn, *Penaeus merguiensis,* when fed diets containing supplemental lecithin. These authors further reported that no apparent advantage was associated with addition of cholesterol to the basal diet containing 'high' levels of lecithin. Similarly, Conklin et al. (1980) showed that the absence of lecithin from the diet of juveniles lobster, *Homarus americanus,* reduced survival by 55%, while Kanazawa et al. (1985) reported that larvae of kuruma prawn, *P. japonicus,* failed to metamorphose when fed diets without supplemental phospholipids. More recently, Paibulkichakul et al. (1998) reported significantly greater growth and survival of larvae of the tiger prawn, *Penaeus monodon,* when fed diets containing the highest level of supplemental lecithin tested (1.5%). Similar dietary requirement for phospholipid was proven for *Penaeus vannamei* in experiments where the growth of juveniles was significantly enhanced when the level of dietary phospholipid was increased (Gong et al., 2000b). For juveniles of *Penaeus*

chinensis the optimal dietary lecithin level was found to be 2% (Kanazawa, 1993), and for *P. japonicus* best survival was achieved with diets containing 1.5% and 3.0% phospholipid (Teshima et al., 1986a; Camara et al., 1997). In contrast, no significant benefit to growth or survival resulted from supplemental dietary lecithin provided to juveniles of the freshwater prawn, *Macrobrachium rosenbergii* (Briggs et al., 1988; Kanazawa, 1993). As the best survival of *S. serrata* megalopae in the present study was achieved when fed MBD containing relatively high level of total lecithin (4.0% and 4.4%), it can be assumed that mud crab megalopae have a relatively high requirement for dietary phospholipid compared to other marine crustaceans.

Supplementation of cholesterol to the basal MBD used in this experiment did not result in any significant benefits to development or survival of *S. serrata* megalopae when a high level of dietary lecithin was present in the diet. For example, when comparing the performance of larvae fed Diet 5 and Diet 6 (4.0% and 4.4% phospholipid respectively), increasing the level of cholesterol from 0.1% (Diet 5) to 0.9% (Diet 6) did not improve mean dry weight or mean carapace width of newly molted crabs, mean development time from the start of the experiment to metamorphosis or survival. Similar observations was made in Chapter 4 of this thesis, where no significant differences in mean development time, mean carapace width or mean dry weight was recorded for *S. serrata* megalopae fed semi-purified MBD containing a constant level of lecithin (2.6%), but varying levels of cholesterol (0.14 to 1.00%). These findings suggest that *S. serrata* megalopae have a low requirement for dietary cholesterol when sufficient levels of lecithin are available in the diet. An interaction between dietary cholesterol and lecithin has previously been reported in the crab *Cancer borealis* (Lester et al., 1975), where the presence of lecithin in the diet was suggested to promote sterol solubilization, making

dietary cholesterol more available for absorption. D'Abramo et al. (1982) investigated this interaction further in experiments with *H. americanus* and reported that the presence of sufficient levels of phospholipid in the diet facilitated the movement of cholesterol from the hepatopancreas into the haemolymph. On this basis it is reasonable to assume that dietary cholesterol may become more available when supplemental dietary lecithin is supplied reducing the overall requirement for the dietary cholesterol.

Previous experiments with *S. serrata* juveniles have shown that this species is highly tolerant of elevated dietary lipid levels (Sheen and Wu, 1999). For example, when dietary lipid levels between 1.7% and 13.8% were assessed in formulated diets, best survival was achieved with diets containing between 5.3% and 13.8% dietary lipids (Sheen and Wu, 1999). This 'optimal' dietary lipid level is considerably higher than that for most other crustaceans studied, where the optimal range is approximately 2-10% (Deshiramu et al., 1979; Davis and Robinson, 1986; Sheen and D'Abramo, 1991; Sheen, 1997). In the present study, increases in the levels of dietary lecithin resulted in increases in the total lipid content of the MBD to a maximum of 10.5% and 10.8% of diet dry weight in the two diets containing the highest level of lecithin (4.0% and 4.4%). First stage crabs metamorphosing from megalopae fed these diets showed faster development time, greater mean carapace width and heavier mean final dry weight. As the diets were formulated to be iso-energetic (26.91 MJ/kg) it can be assumed that the growth enhancing effect was related to lipid content rather than energy content in the diets, which would mean that *S. serrata* megalopae, like juveniles of the same species, are able to utilize relatively high levels of dietary lipid which facilitate more rapid development and greater growth rates.

Soybean lecithin contains significant quantities of LNA and LN and diets supplemented with lecithin showed an expected increase in levels of these fatty acids. Previous studies have shown that both LNA and LN are important for growth in lobsters and prawns (Castell and Covey, 1976; Kanazawa et al., 1977), and these findings indicate that the C18 fatty acids have an important metabolic role in crustaceans. This may in turn be relate to the results of the present experiment, where a trend of increased final mean dry weight, faster development time of crabs molting from megalopae and higher overall survival was recorded for megalopae fed diets high in lecithin (Diets 5 and 6).

Similarly, the level of the *n*-3 fatty acids eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA) between diets, showing proportionally higher levels in Diet 1 and 2 than in the other diets. Due to the limited ability of marine crustaceans for *de novo* synthesis of highly unsaturated fatty acids (Sheen, 2000), appropriate levels of EPA and DHA are considered essential in the crustacean diet. Previous research has shown that *S. serrata* zoea III larvae fed un-enriched *Artemia* nauplii with a low level of DHA and EPA showed clear signs of essential fatty acid deficiency, such as prolonged intermolt period and low survival (Suprayudi et al., 2004). Similar results were found for the swimmer crab (*Portunus trituberculatus*) larvae fed rotifers containing a low level of *n*-3 HUFA (Hamasaki et al., 1998; Takeuchi et al., 1999; Suprayudi et al., 2002). Other studies have shown that elevated levels of EPA in live food resulted in abnormal development and mortality of the larvae at metamorphosis (Hamasaki et al., 2002), and in studies comparing different *Artemia* strains and *Artemia* enrichment products Mann et al. (2001) found no influence of the *n*-3 HUFA level on the ability of the larvae to complete development. The varied results may be linked to larval ability to build up tissue reserves during development, which

again influences growth and survival at life stages which require high levels of tissue synthesis. Regardless, these reports indicate that the DHA and EPA requirement of *Scylla* spp. are still not fully understood, and further research is required. In terms of the present findings, it should be noted that mean dry weight and overall survival of C1, as well as mean development time from megalopa to C1, may be influenced not only by the level of cholesterol and phospholipid in the diets, but also by the dietary level of the essential *n*-3 fatty acids.

In summary, the results of this experiment show a significant interaction between dietary lecithin and cholesterol on dry weight of newly settled C1 *S. serrata*, with supplemental cholesterol being beneficial only when an insufficient level of dietary lecithin is present.

Chapter 6

Survival, development and growth response of mud crab, *Scylla serrata* megalopae, fed semi-purified diets containing various fish oil:corn oil ratios [4]

6.1 Introduction

Lipids are important in aquaculture diets, not only for their sterol content, but also as a supply of essential and non-essential fatty acids (Sheen and Wu, 1999). Most animals require a wide range of different fatty acids for normal growth and functioning, and common practice in diet formulation for crustaceans has been to use a 2:1 ratio of marine and terrestrial oils (Millikin et al., 1980; Sheen, 1997; Kamarudin and Roustaian, 2002). Fish oil and cod liver oil are the most frequently used marine oils, as they contain a high level of polyunsaturated fatty acid of the *n*-3 series, while soybean oil and corn oils are the most commonly used terrestrial oils, containing a high level of *n*-6 fatty acids, particularly linoleic acid (18:2n-6, LN) (Castell and Covey, 1976).

Supplementation of dietary *n*-3 highly unsaturated fatty acid (HUFA) has been shown to improve growth and survival for several marine crustaceans, including the penaeids *P. japonicus* (Kanazawa et al., 1979a), *P. indicus* (Read, 1981) and *P. chinensis* (Xu et al., 1994). The requirement for *n*-6 fatty acids has been given less attention in the literature,

[4] The data in this chapter is published in *Aquaculture* (2007) 269: 4427-435. See Appendix 4

but recent research has shown that supplementation of dietary arachidonic acid (20:4*n*-6, AA) marginally improved the digestibility of neutral lipid in the diet of adult *Penaeus monodon* (Glencross and Smith, 2001). Inclusion of LN in the diet has also shown to be effective in promoting survival and growth in adult *P. japonicus* (Kanazawa et al., 1979c), and tissue analysis of the rock lobster, *Jasus edwardsii*, has shown that AA is conserved in the tissue of starved phyllosoma, indicating an important physiological role for this fatty acid (Smith et al., 2003). Furthermore, an interaction between dietary *n*-3 and *n*-6 fatty acids in crustacean diets has been identified by Glencross et al. (2002b), and an important role of the *n*-3 to *n*-6 fatty acids balance on crustacean growth has been suggested.

As marine oils are costly and often unavailable, vegetable oils and animal fats are now being investigated as a means of reducing reliance on marine oils in aquaculture diets (Borlongan and Parazo, 1991; Richard et al., 2006). Encouraging results has been reported, where work with species such as *Macrobrachium rosenbergii* has shown that 50% substitution of cod liver oil in diet formulation does not affect survival or growth of developing larvae (Kamarudin and Roustaian, 2002). However, the requirements for dietary *n*-3 and *n*-6 fatty acids varies between species, and species specific experimentation is required for mud crabs, a few studies have been conducted to specify fatty acid requirements (Sheen and Wu, 1999; Takeuchi, 2000; Hamasaki et al., 2002a; Suprayudi et al., 2004b; Suprayudi et al., 2004a; Nghia et al., 2007), but conflicting results have been reported (Nghia et al., 2007). Exact dietary requirements are therefore not fully understood and more research is required before a nutritionally optimized, cost-effective and sustainable diet can be developed for mud crab larvae.

The objective of this study was to determine the effects of various ratios of dietary fish oil and corn oil on survival, growth and development of mud crab megalopae, with the aims to identify the optimum *n*-3/*n*-6 fatty acid ratio in diets, and to evaluate the possibility of reduced use of fish oil in MBD for this species.

6.2 Materials and methods

6.2.1 Experimental design and preparation of experimental diets

The composition of the experimental MBD used in this study were based on previous research with *S. serrata* juveniles by Sheen (2000), and the results from Chapter 3, 4 and 5 of this thesis (Table 6.1). Six dietary treatments consisting of five iso-energetic, semi-purified diets with different levels of fish oil and corn oil, as well as a control group consisting of megalopae fed live *Artemia* nauplii, were used. Before preparation of the diet, lipids were extracted from the squid meal using 2:1 chloroform-methanol (v/v) (Folch et al., 1957) to minimize contribution of dietary lipid. Twenty replicate megalopae were used for each treatment, and as the results from Chapter 3 and 4 of this thesis showed that all unfed megalopae died before they reached the first crab stage (C1), unfed control treatments were not included in this experiment. The fatty acid composition of the fish oil and corn oil used in the formulation of experimental diets was determined analytically in accordance with the methods outlined in section 2.8 of this thesis.

6.2.2 Statistical analysis

Mean carapace width and log transformed mean dry weight of C1 molting from megalopae fed different diets were compared using a one-way ANOVA, where specific differences among treatments were determined using Tukey's multiple comparison test

at the 0.05 level of significance. Development time from megalopae to C1 was compared using Kruskal-Wallis non-parametric test. All statistical analyses were performed using SPSS for Windows, version 14.0.

6.3 Results

6.3.1 Survival and incidence of molting death syndrome

Highest survival (70%) was recorded for megalopae fed the MBD containing a fish oil:corn oil ratio of 1:1 (Diet 6) (Fig. 6.1). Slightly lower survival (65%) was recorded for megalopae fed diets containing fish oil:corn oil ratios of 3:1 (Diet 4) and 1:0 (Diet 5) (Fig. 6.1), while megalopae fed live *Artemia* nauplii and MBD containing a fish oil:corn oil ratio of 2:1 (Diet 3) had survival rates of 60%. Lower survival of 55% was recorded for megalopae fed the MBD with a lower fish oil:corn oil ratio of 1:2 (Diet 2), whereas the lowest overall survival (35%) was found for megalopae fed the MBD containing corn oil only (Diet 1) (Fig. 6.1).

Table 6.1 Formulation (% dry weight) of experimental microbound diets fed to mud crab, *Scylla serrata*, megalopae. Formulation based on results from Chapter 3, 4 and 5 of thesis, as well as Sheen (2000) and Genodepa et al. (2004).

Ingredient (%)	**Source**	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**
		Fish oil:corn oil ratio 0:1	*Fish oil corn oil ratio 1:2*	*Fish oil:corn oil ratio 2:1*	*Fish oil:corn oil ratio 3:1*	*Fish oil:corn oil ratio 1:0*	*Fish oil:corn oil ratio 1:1*
De-fatted squid meal	A	55.0	55.0	55.0	55.0	55.0	55.0
Fish oil [a]	B	0.0	2.0	4.0	4.5	6.0	3.0
Corn oil [b]	B	6.0	4.0	2.0	1.5	0.0	3.0
Zein [c]	B	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin mix [d]	C	4.0	4.0	4.0	4.0	4.0	4.0
Mineral mix [e]	C	4.0	4.0	4.0	4.0	4.0	4.0
DCP [f]	B	0.6	0.6	0.6	0.6	0.6	0.6
Choline chloride [g]	B	1.0	1.0	1.0	1.0	1.0	1.0
Cholesterol [h]	B	0.7	0.7	0.7	0.7	0.7	0.7
Lecithin [i]	B	2.0	2.0	2.0	2.0	2.0	2.0
Starch [j]	B	11.1	11.1	11.1	11.1	11.1	11.1
Cellulose [k]	B	12.6	12.6	12.6	12.6	12.6	12.6

A) Skretting Tasmania B) Sigma-Aldrich Pty. Ltd, C) Rabar Pty Ltd [a)] from menhaden F8020 [b)] C8267 [c)] from maize Z3625 [d)]ZZ603 DO 067 DPI, each 1kg contains: copper 1g, cobalt 100mg, magnesium 59.4mg, manganese 5g. iodine 800mg, selenium 20mg, iron 8mg, zinc 20g, aluminium 100mg, chromium 100mg [e)] ZZ600 DPI, each 1kg contains: vitamin A 2miu, vitamin D3 0.8miu, vitamin E 40g, vitamin K 2.02g, inositol 50g, vitamin B3 (niacin) 30.40g, vitamin B5 (pantothenic acid) 9.18g, vitamin B9 (folic acid,) 2.56g, vitamin B2 (riboflavin) 4.48g, vitamin B12 (cobalamin) 0.004g, biotin 0.1g, vitamin B6 (pyridoxine)4g, vitamin B1 (thiamine) 3.4g, vitamin C 44.4g, para amino benzoic acid 20g, tixosil 5g, antioxidant 30g [f)] dibasic calcium phosphate C4131 [g)] 98% powder C7527 [h)] Sigma Grade 99% C8667 [i)] L-A-phosphatidylcholine from soybean P7443 [j)] from corn S4126 [k)] alpha C8002

A substantially higher occurrence of molting death syndrome (MDS) resulted in high mortality of megalopae fed the diet containing corn oil only (Diet 1). In contrast, MDS was not recorded in other treatments. Megalopae inflicted by MDS had problems extracting claws, pereiopods or other parts of the body from the old exoskeleton, and when the new exoskeleton hardened the affected larvae were shackled to the old carapace, resulting in death shortly after molt. Although 35% of megalopae fed Diet 1 were able to metamorphose to the first crab stage (Fig. 6.1), newly settled crabs from this treatment had the lowest mean dry weight among treatments.

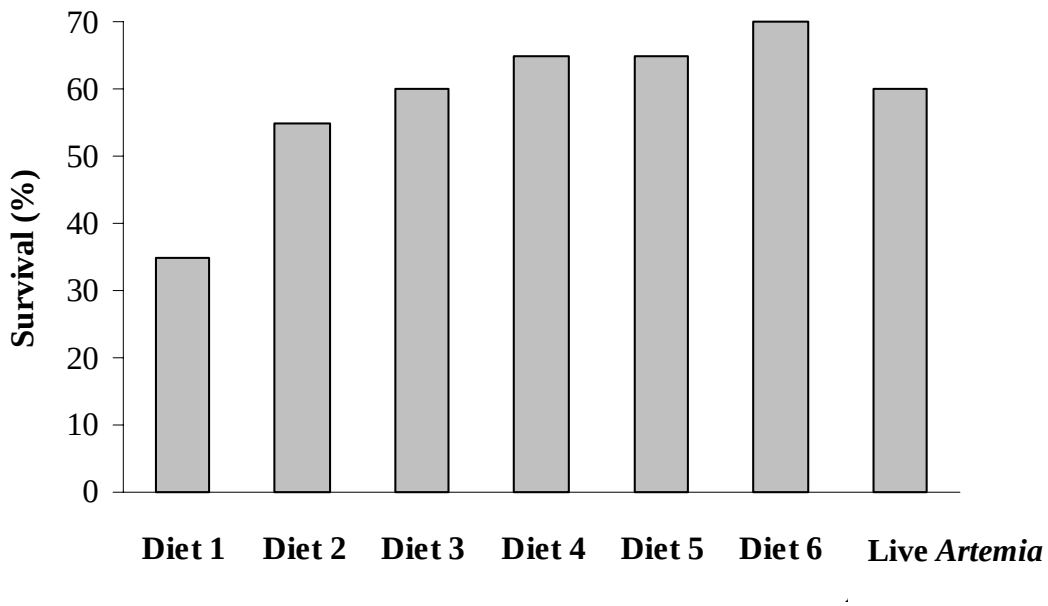

Fig. 6.1 Percentage successful metamorphosis of *Scylla serrata* from megalopae to the first crab stage, when fed either live *Artemia* nauplii or one of six microbound diets containing different ratios of fish and corn oil. For diet abbreviation refer to Table 6.1.

6.3.2 Mean carapace width and mean final dry weight

The greatest mean carapace width was recorded for C1 molting from megalopae fed live *Artemia* nauplii (3.51 ± 0.03 mm) (Table 6.2). This value was significantly greater than the mean carapace widths of crabs from all MBD-fed treatments. Crabs that molted from megalopa fed Diet 6 (fish oil:corn oil ratio 1:1) had the second largest mean carapace width (3.01 ± 0.04 mm), which was significantly greater than that of the crabs resulting from megalopae fed Diet 2 (fish oil:corn oil ratio 1:2) (2.84 ± 0.06 mm) and Diet 4 (fish oil:corn oil ratio 3:1) (2.85 ± 0.05 mm). The shortest mean carapace width was recorded for crabs that molted from larvae fed Diet 2, which had a fish oil:corn oil ratio of 1:2; however, this value did not differ significantly from that of crabs which received higher ratios of dietary fish oil:corn oil (Diets 4 and 5) (Table 6.2).

Significantly higher final mean dry weight was recorded for crabs that molted from megalopae fed live *Artemia* nauplii (2.14 ± 0.14 mg) when compared to those from MBD-fed treatments (Table 6.2). Although no statistically significant differences were detected for mean dry weight among crabs that molted from megalopae fed any of the six MBD, crabs that molted from megalopae fed Diet 5 and Diet 6 (fish oil:corn oil ratio of 1:0 and 1:1, respectively) showed the two highest mean dry weights among the MBD-fed megalopa (1.16 ± 0.06 mg and 1.14 ± 0.10 mg, respectively). The crabs molting from megalopa fed Diet 1 (fish oil:corn oil 0:1) showed the lowest overall mean dry weight (1.03 ± 0.14) (Table 6.2).

6.3.3 Development time from megalopa to first stage

Successful molts were observed over a period of 10 days, were the first molt occurred for larvae fed live *Artemia* nauplii on day 5, while the last successful molt took place on day 15 in the treatment receiving Diet 1 (fish oil:corn oil ratio 0:1). Shortest mean development time was recorded for megalopae fed live *Artemia* nauplii (6.8 ± 0.5 days) (Table 6.2). However, no significant difference in mean development time was observed among any of the treatments.

Table 6.2 Mean ± SE carapace width and mean ± SE dry weight of newly settled first stage crabs (C1), and mean development time from megalopa to C1 for *Scylla serrata*, fed semi-purified diets containing various fish oil:corn oil ratios. Within each column, means with different superscripts are statistically significant (P <0.05).

Treatment *(fish oil: corn oil ratio)*	**Carapace width** (mm)	**Dry weight** (mg)	**Development time** (days)
Diet 1 (*0:1)*	2.91 ± 0.03^{abc}	1.03 ± 0.14^{a}	7.1 ± 1.2^{a}
Diet 2 (*1:2)*	2.84 ± 0.06^{b}	1.06 ± 0.08^{a}	7.8 ± 0.4^{a}
Diet 3 (*2:1)*	3.00 ± 0.03^{ac}	1.07 ± 0.05^{a}	7.8 ± 0.5^{a}
Diet 4 (*3:1)*	2.85 ± 0.05^{ab}	1.06 ± 0.07^{a}	8.5 ± 0.5^{a}
Diet 5 (*1:0)*	2.99 ± 0.03^{abc}	1.16 ± 0.06^{a}	7.4 ± 0.3^{a}
Diet 6 (*1:1)*	3.01 ± 0.04^{c}	1.14 ± 0.10^{a}	8.2 ± 0.5^{a}
Live *Artemia* nauplii	3.51 ± 0.03^{d}	2.14 ± 0.14^{b}	6.8 ± 0.5^{a}

6.3.4 Fatty acid compositions of the fish oil and corn used in diet formulation

The fatty acid compositions of the fish oil and the corn oil used in this study are shown in Table 6.3. The corn oil contained predominantly C18 fatty acids, where oleic acid (18:1*n*-9) and linolenic acid (18:3*n*-3, LN) were predominant and made up 28.8% and 56.0% of total fatty acids, respectively. The fish oil had a more diverse fatty acid profile and contained C16, C18, C20 and C22 fatty acids. The fish oil further contained relatively large amounts of the essential fatty acids eicosapentaenoic acid (20:5*n*-3, EPA) (11.4% of total fatty acid) and docosahexaenoic acid (22:6*n*-3,

DHA) (15.2% of total fatty acid), as well as a low level of arachidonic acid (20:4*n*-6, AA) (0.8% of total fatty acid) (Table 6.3). The sums of *n*-3 and *n*-6 fatty acids were 36.5% and 0.9%, and 3.2% and 56.0% for fish oil and corn oil, respectively, resulting in a very high *n*-3/*n*-6 ratio in the fish oil (11.51), and a very low *n*-3/*n*-6 ratio in the corn oil (0.02) (Table 6.3).

Table 6.3 Fatty acids profile (% of total fatty acid) of the fish oil and corn oil used in formulation of microbound diets fed to mud crab *Scylla serrata* megalopae.

Fatty acid	**Fish oil**	**Corn oil**
14:0	6.7	-
15:0	0.6	-
16:0	20.2	11.0
17:0	0.7	-
18:0	4.0	2.0
20:0	0.2	0.4
24:0	-	0.2
16:1 *n*-7	9.5	-
18:1 *n*-7	3.5	0.6
18:1 *n*-9	11.3	28.8
20:1 *n*-7	0.3	-
20:1 *n*-9	2.2	0.2
22:1 *n*-9	0.2	-
22:1 *n*-11	0.2	-
24:1 *n*-9	0.5	-
18:3 *n*-3, LNA	1.5	0.9
18:4 *n*-3	3.3	-
20:3 *n*-3	0.2	-
20:4 *n*-3	2.1	-
20:5 *n*-3, EPA	11.4	-
22:5 *n*-3	2.8	-
22:6 *n*-3, DHA	15.2	-
18:2 *n*-6, LN	1.4	56.0
20:2 *n*-6	0.3	-
20:4 *n*-6, AA	0.8	-
22:4 *n*-6	0.2	-
22:5 *n*-6	0.5	-
Sum SFA	32.6	13.6
Sum MUFA	27.8	29.6
Sum HUFA	39.7	56.8
Sum *n*-3	36.5	0.9
Sum *n*-6	3.2	56.0
n-3/ *n*-6 ratio	11.51	0.02

6.4 Discussion

Among all dietary treatments in this experiment, including the *Artemia*-fed control, best survival of *S. serrata* megalopae to the first crab stage (C1) was achieved with a semi-purified formulated diet containing a fish oil:corn oil ratio of 1:1. This diet also resulted in the greatest mean carapace width and the second highest mean dry weight of newly settled C1 among all MBD-fed treatments, which indicates that a dietary fish oil:corn oil ratio of 1:1 is close to optimal for *S. serrata* megalopae. This differs from the ratio of 2:1 which has been used in a number of diet formulations for other crustaceans (Millikin et al., 1980; Kamarudin and Roustaian, 2002), including *S. serrata* (Sheen and Wu, 1999).

Several studies with marine crustaceans have reported better growth and survival when diets contain marine oils compared to oils derived from terrestrial sources (Kanazawa et al., 1977; Catacutan, 1991). As most marine crustaceans are incapable of *de novo* synthesizes of HUFA from C18 fatty acids (Kanazawa et al., 1979b; Bottino et al., 1980), the superior nutritional value of marine oils in crustacean diets has been linked to their higher content of the essential HUFA (Teshima et al., 1992; Merican and Shim, 1996). Particularly important among the HUFA are EPA which is reportedly effective in supporting larval survival (Levine and Sulkin, 1984), and DHA, which plays an important role in accelerating the intermolt duration and facilitates greater carapace width in *S. serrata* juveniles (Sheen and Wu, 1999). The varying functions of EPA and DHA are believed to be related to differences in their distribution within various tissues of the animal; DHA is found exclusively in neural tissue, such as brain and eyestalks, whereas EPA is distributed more evenly among a variety of tissues (Bell et al., 1995; Masuda et al., 1999).

Previous research reported that zoea III larvae of *S. serrata* fed un-enriched *Artemia* nauplii showed clear signs of essential fatty acid deficiency, including prolonged intermolt period, low survival and reduced swimming activity (Suprayudi et al., 2004a). Similar results were reported for larvae of the swimmer crab, *Portunus tributerculatus*, fed rotifers containing a low level of *n*-3 HUFA (Hamasaki et al., 1998). The importance of dietary *n*-3 fatty acids has also been confirmed by the growth enhancing response of supplemental dietary *n*-3 fatty acids in a wide range of other crustaceans, including *P. japonicus* (Kanazawa et al., 1979a), *Palaemon serratus* (Martin, 1980), *M. rosenbergii* (Kamarudin and Roustaian, 2002), *Jasus edwardsii* (Phleger et al., 2001), *Homarus americanus* (Castell and Covey, 1976), and the crabs, *Carcinus maenas* (Ponat and Adelung, 1980) and *Eurypanopeus depressus* (Levine, 1984). These findings are in agreement with the results from the present study where a high occurrence of molting death syndrome was observed among larvae fed MBD containing corn oil only. The megalopae fed the corn oil diet also showed lower survival compared to those fed diets containing fish oil, suggesting a nutritional requirement of *n*-3 HUFA for megalopae of *S. serrata*.

Given the importance of dietary *n*-3 HUFA for crustacean, including *S. serrata* (Sheen and Wu, 2003), it is perhaps surprising that megalopae fed MBD containing only corn oil (Diet 1) showed 35% survival to the first crab stage. As crustacean larvae have been shown to be able to accumulate lipids in their tissues as energy reserves (Phillips et al., 2006), it is possible that survival of megalopae fed Diet 1 was influenced by the relatively short duration of the megalopa stage in *S. serrata*, and the availability of essential fatty acids in tissue reserves accumulated during earlier larval stages. Previous research with crustacean larvae has shown that in periods of

starvation, larvae utilize stored lipids to maintain growth and development (Anger, 2001; Phillips et al., 2006), and it is reasonable to assume that similar mechanisms are utilized by larvae receiving nutrient deficient diets.

Despite reports on the growth-promoting effects of HUFA, it should be noted that excessive levels of HUFA in crustacean larval diets may negatively affect larval performance (Gonzalez-Felix et al., 2002). For example, Suprayudi et al. (2002) reported a high rate of abnormal molting when *S. serrata* larvae were fed rotifers containing a high level of EPA (2.4% dry basis). Hamasaki et al. (2002b) found similar results in experiments with the same species, where mass mortality of megalopae occurred when high concentrations of *Nannochloropsis* rich in EPA were added to the rearing tanks. Signs of excessive dietary HUFA were similarly observed in the present study, as survival was compromised in crabs that molted from megalopae fed Diets 3, 4 and 5 which contained high fish oil:corn oil ratios (2:1, 3:1 and 1:0, respectively) compared to Diet 6 which contained a 1:1 ratio of the two oils. These findings indicate that diets supplemented with high levels of fish oil may contain excessive HUFA levels, even though the total level of dietary lipids may be optimal for this species at 6% of total diet dry weight (Genodepa et al., 2004).

The importance of *n*-6 fatty acids in the crustacean diet is still subject to discussion, and contradicting results exist. For example, Liddy et al. (2004) showed that AA is conserved at a high level in the tissues of larval rock lobster, *Panulirus cygnus*, during starvation, indicating an important function during ontogeny of this species. However, experiments with adult *Penaeus monodon*, showed that dietary AA is not essential provided that dietary LN, the natural precursor for AA, is present in sufficient

amounts (Glencross et al., 2002a). These authors further suggested that the importance of dietary AA is related to the balance of dietary *n*-3 to *n*-6 fatty acids. In the present study, greatest survival was found among larvae fed Diet 6, which contained an equal amount of fish oil and corn oil, while megalopae fed the diet supplemented with corn oil only (Diet 1) performed poorly. These findings indicate an inability in *S. serrata* larvae to convert LN to AA, and it suggests that AA is an essential HUFA for larvae of this species. Further research is required to determine the absolute dietary AA requirement of *S. serrata* larvae, and to gain a better understanding of the interaction between dietary LN and AA.

In summary, this experiment showed that replacement of live food with an MBD containing a fish oil:corn oil ratio of 1:1 improved survival of *S. serrata* megalopae when compared to *Artemia* fed controls. At the same time, low survival and high occurrence of MDS was recorded for megalopae fed a diet supplemented solely with corn oil, indicating an importance of dietary *n*-3 for normal larval development. Compared to what has been determined as optimum for larvae of other crustacean species, a 1:1 ratio of fish oil to corn oil is relatively low. This may reflect a reduced requirement of *S. serrata* megalopa for dietary *n*-3 compared to most other crustaceans larvae studied. However, the results of Chapter 5 show increased survival and growth of *S. serrata* megalopa fed diets containing high levels of lecithin and, therefore, high levels of LNA and LN. On this basis it is likely that the optimum fish oil:corn oil ratio in the MBD fed to *S. serrata* megalopa is dependant on the level of supplemental lecithin in the diet.

Chapter 7

Ontogenetic changes in lipid and fatty acid composition in mud crab, *Scylla serrata*, larvae

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7.1 Introduction

Scylla serrata has a complex life cycle, consisting of five pelagic zoea stages, a transitional post-larval megalopa stage and benthic juvenile and adult stages (Raja Bai Naidu, 1955; Ong, 1964). As the endogenous nutrient supply is limited in newly hatched zoea I, immediate exogenous feeding is important to maximize larval survival and development (Genodepa et al., 2004). The nutritional requirements typically change as larvae develop, but due to the complexity of larval development and difficulties in conducting creditable research, little is known about the ontogenetic changes in metabolic or dietary requirements of *S. serrata* larvae.

In the wild, pelagic crustacean larvae are exposed to an enormous diversity of natural prey. The study of this natural diet would provide an useful insight into the nutritional requirements of *S. serrata* larvae, but as collection and examination of the gut content of small and fragile zooplankton is practically impossible (Nates and McKenny, 2000a), analysis of the biochemical changes occurring in the tissue of hatchery reared larvae offers an alternative method for estimation of dietary requirements (Ritar et al., 2003). These principles have previously been applied in experiments with the larvae of several other crustacean species with aquaculture potential, including spiny

lobsters, *Panulirus cygnus* (Liddy et al., 2004) and *Jasus edwardsii* (Smith et al., 2003), caridean prawn, *Palaemon serratus* (Narciso and Morais, 2001), the giant freshwater prawn, *Macrobrachium rosenbergii* (Sargent, 1995; Roustaian et al., 1999) and selected larval stages of *Scylla* spp. (Takeuchi, 2000; Hamasaki et al., 2002a).

Once prey has been ingested and enters the larval digestive tract, dietary lipids are hydrolyzed by digestive enzymes into mixtures of free fatty acids and monoglycerides which are either catabolised for energy, or used to maintain the functional integrity of biomembranes (Roustaian et al., 1999). These roles are particularly important during the complex morphological, physiological and biochemical changes that occur during larval development. As discussed in the Chapter 6, *S. serrata* juveniles, like many other marine crustaceans, lack the ability for *de novo* synthesis of long-chain highly unsaturated fatty acids (HUFA) (Sheen and Wu, 2003), and these fatty acids are therefore considered essential dietary components. A deficient supply of eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA) has been identified as a major cause of low survival and longer intermolt periods of many crustaceans, including *S. serrata* larvae (Suprayudi et al., 2004) and juveniles (Sheen and Wu, 1999). An inappropriate level of dietary HUFA has also been recognized as a possible cause of molting death syndrome, the major cause of mass mortality in mud crab hatcheries (Hamasaki et al., 2002b). Chapter 5 of this thesis highlighted the importance of dietary phospholipid in improving growth and survival of *S. serrata* megalopae. As hypothesised in that chapter, this may result from the high levels of linolenic acid (18:3*n*-3, LNA) and linoleic acid (18:2*n*-6, LN) in lecithin. Furthermore, the results of Chapter 6 indicated that *S. serrata* megalopa may have a lower relative requirement for *n*-3 HUFA when compared to other crustaceans. On

this basis it is clear that the nutritional requirements of mud crab larvae is still not fully understood, and a better understanding of lipid metabolism and the ontogenetic changes in *S. serrata* larvae is required for development of cost effective and nutritionally optimized hatchery diets for this species.

The objective of this study was to characterize the changes in lipid content, lipid class composition and fatty acid profile in developing *S. serrata* larvae reared under standard hatchery conditions, with the aim of providing a greater understanding of fatty acid metabolism in the early life stages.

7.2 Materials and methods

7.2.1 Sampling of larvae and live food

Larvae were reared following the protocol described in section 2.3, and whole body samples (approximately 500 mg wet weight) were collected for dry weight determination and lipid analysis at the zoea I, zoea III, zoea V and megalopa stages. Zoea I were collected immediately after hatch, while zoea III, zoea V and megalopa stages were collected when approximately 80% of the population had attained the required development stage. All larvae samples, as well as the three live foods used in their culture; rotifers (*Brachionus* sp.), AF Specialty *Artemia* nauplii and enriched *Artemia* meta-nauplii (Section 2.3 of this thesis), were collected for lipid analysis. Two separate batches of larvae from different females were cultured and sampled for lipid analysis, and the results in this chapter are presented as a mean of the two results.

7.2.2 Measurement of dry weight and ash free dry weight of larvae

All larval samples collected for dry weight (DW) and ash free dry weight (AFDW) determination were taken in triplicate. For each sample, twenty larvae were collected on nylon mesh, washed briefly with distilled water to remove salt, dried at 50ºC for 24 h, and weighed using a Cahn 33 microbalance. Samples were then heated for a further five hours at 500ºC and reweighed to determine ash weight (AW); AFDW was determined as the difference between DW and AW.

7.3 Results

7.3.1 Dry weight, ash-fee dry weight, weight increase and total tissue lipid

Mean dry weight of developing *S. serrata* larvae increased substantially from 12.11 ± 0.31 µg for newly hatched zoea I, to 1025.52 ± 87.11 µg for newly metamorphosed megalopa (Table 7.1). At the zoea I and megalopa stages, the organic dry weight measured approximately 60% of total dry weight, increasing to approximately 70% of total larvae dry weight at the intermediate larvae stages (zoea III and zoea V) (Table 7.1). These findings revealed a significant differences in percentage organic content among the larval stages (one-way ANOVA, $F_{4,20} = 3.233$, $P = 0.033$), and the results in this chapter relating to fatty acid composition of larvae are consequently presented on a per unit AFDW basis.

Table 7.1 Mean ± SE dry weight (DW), ash free dry weight (AFDW), dry weight increase (WI), organic content and total lipid content at various larvae stages of *Scylla serrata*.

Larvae stage	DW (µg)	AFDW (µg)	Organic content (%)	Total lipid (% of AFDW)
Zoea I	12.11 ± 0.31	7.05 ± 0.45	58.18 ± 2.37[a]	38.07 ± 3.51
Zoea III	47.53 ± 10.73	33.35 ± 7.66	70.16 ± 0.68 [b]	52.29 ± 2.73
Zoea V	339.21 ± 25.19	237.19(± 20.85	69.92 ± 2.16 [b]	57.87 ± 9.44
Megalopa	1025.52 ± 87.11	606.29 ± 79.56	59.12 ± 3.18 [a]	31.65 ± 14.36

7.3.2 Total lipid, total fatty acid and lipid class composition

The total lipid fraction of larval tissue varied as larvae developed (Table 7.1), starting at 38.07 ± 3.51% of AFDW in newly hatched zoea I, increasing to a high of 57.87 ± 9.44% of AFDW for newly molted zoea V. The total lipid level dropped to an overall low of 31.65 ± 14.36% as the larvae metamorphosed to megalopa. When presented as µg mg^{-1} AFDW, the total fatty acid content of larval tissues increased from 22.89 ± 3.72 µg mg^{-1} AFDW in newly hatched zoea I larvae, peaking at 31.38 ± 18.30 µg mg^{-1} AFDW in newly molted zoea V, before dropping down to 24.92 ± 13.56 µg mg^{-1} AFDW in newly metamorphosed megalopa (Table 7.2). The levels of fatty acids from the polar lipid fraction were relatively stable throughout larvae ontogeny (13.68 – 13.95 µg mg^{-1} AFDW), except from a clear peak at the zoea V stage (18.03 µg mg^{-1} AFDW) (Table 7.3). The levels of fatty acids from the neutral lipid fraction showed more variability, with a slight drop at the zoea II stage (7.81 µg mg^{-1} AFDW), followed by a substantial increase at the zoea V stage (13.35 µg mg^{-1} AFDW) (Table 7.4).

Table 7.2 Fatty acid composition (µg mg^{-1} ash free dry weight) of *Scylla serrata* larvae (mean ± SE; *n* = 2). Included in the table are the fatty acid composition of the larval food; rotifers, AF Specialty *Artemia* nauplii and enriched *Artemia* meta-nauplii presented as µg mg^{-1} dry weight (- indicates <1% of total fatty acid).

	Larval stage				**Live food**		
Fatty acid	Zoea I	Zoea III	Zoea V	Megalopa	Rotifer	AF *Artemia*	Enriched *Artemia*
Diet	*Rotifer*						
		AF Artemia					
			Enriched Artemia				
14:0	0.26 ± 0.07	0.43 ± 0.27	0.43 ± 0.34	0.29 ± 0.23	2.01	2.35	2.44
15:0	0.25 ± 0.08	0.41 ± 0.25	0.29 ± 0.23	0.17 ± 0.14	0.34	4.32	0.85
16:0	4.93 ± 0.85	4.36 ± 2.30	5.03 ± 3.20	3.60 ± 2.23	17.45	17.07	29.98
17:0	0.45 ± 0.14	0.64 ± 0.39	0.64 ± 0.45	0.34 ± 0.21	0.36	4.89	1.81
18:0	2.78 ± 0.31	1.92 ± 0.96	2.67 ± 1.42	2.27 ± 0.96	2.79	5.52	14.26
20:0	0.12 ± 0.02	0.01 ± 0.01	0.09 ± 0.06	0.05 ± 0.01	-	0.24	0.61
22:0	0.03 ± 0.03	0.08 ± 0.03	0.15 ± 0.10	0.10 ± 0.04	-	-	0.41
24:0	0.03 ± 0.00	0.07 ± 0.06	0.06 ± 0.04	0.13 ± 0.11	-	-	-
16:1*n*-7	1.04 ± 0.35	1.47 ± 0.74	1.28 ± 0.99	0.62 ± 0.41	9.02	16.80	5.48
17:1*n*-8	-	-	-	-	-	18.22	-
18:1*n*-7	1.03 ± 0.25	1.82 ± 0.98	2.53 ± 1.43	1.79 ± 0.73	2.23	15.57	9.21
18:1*n*-9	3.07 ± 0.84	3.30 ± 1.81	5.93 ± 2.80	5.31 ± 2.59	5.61	21.62	39.76
20:1*n*-7	0.14 ± 0.04	0.16 ± 0.11	0.16 ± 0.10	0.02 ± 0.02	0.23	1.26	-
20:1*n*-9	0.28 ± 0.01	0.32 ± 0.15	0.41 ± 0.21	0.34 ± 0.20	1.36	0.64	1.63
20:1*n*-11	0.15 ± 0.02	0.02 ± 0.02	0.01 ± 0.01	0.00 ± 0.00	0.41	0.58	-
22:1*n*-9	0.12 ± 0.07	0.53 ± 0.51	0.54 ± 0.53	0.49 ± 0.49	0.40	-	-
22:1*n*-11	-	-	-	-	-	-	0.31
24:1*n*-9	0.02 ± 0.01	0.02 ± 0.02	0.04 ± 0.04	0.00 ± 0.00	0.20	-	0.33
18:3*n*-3, LNA	0.04 ± 0.04	0.10± 0.06	2.64 ± 1.52	2.25 ± 1.10	-	1.55	37.69
18:4*n*-3	0.01 ± 0.01	0.00 ± 0.00	0.23 ± 0.16	0.14 ± 0.05	-	0.69	5.09
20:3*n*-3	0.04 ± 0.04	0.00 ± 0.00	0.24 ± 0.12	0.26 ± 1.14	-	-	1.53
20:5*n*-3, EPA	2.86 ± 0.49	3.02 ± 1.38	3.56 ± 1.96	2.54 ± 1.20	9.95	17.48	12.57
22:5*n*-3	0.44 ± 0.09	0.57 ± 0.28	0.54 ± 0.42	0.23 ± 0.18	3.97	-	1.37
22:6*n*-3, DHA	2.13 ± 0.10	0.25 ± 0.02	0.87 ± 0.37	1.40 ± 1.01	0.00	0.25	14.96
18:2*n*-6, LN	0.25 ± 0.02	0.78 ± 0.36	1.60 ± 1.02	1.52 ± 1.07	4.38	4.36	13.95
20:2*n*-6	0.30 ± 0.00	0.11 ± 0.02	0.20 ± 0.11	0.22 ± 0.14	0.74	-	-
20:4*n*-6, AA	1.62 ± 0.14	1.21 ± 0.43	1.20 ± 0.67	0.80 ± 0.32	3.98	5.79	2.62
22:2*n*-6	-	-	-	-	0.49	0.19	0.71
22:4*n*-6	0.28 ± 0.06	0.08 ± 0.01	0.04 ± 0.03	0.00 ± 0.00	-	-	-
22:5*n*-6	0.21 ± 0.07	0.01 ± 0.01	0.02 ± 0.02	0.05 ± 0.03	-	-	1.09
∑ SFA	8.85 ± 1.44	7.92 ± 2.25	9.35 ± 5.83	6.95 ± 3.92	22.96	34.39	50.36
∑ MUFA	5.86 ± 1.58	7.65 ± 4.26	10.89 ± 6.09	8.57 ± 3.92	19.46	74.67	56.72
∑ HUFA	7.88 ± 0.77	5.25 ± 2.13	6.67 ± 3.67	5.49 ± 3.02	19.14	23.71	34.84
∑ *n*-3	5.53 ± 0.46	3.95 ± 1.74	8.08 ± 4.55	6.82 ± 3.68	13.93	19.98	73.21
∑ *n*-6	2.66 ± 1.24	2.19 ± 0.81	3.06 ± 1.82	2.58 ± 1.56	9.59	10.34	18.36
n-6/*n*-3 ratio	2.08 ± 0.02	1.75 ± 0.15	2.71 ± 0.12	2.81 ± 0.27	1.45	1.93	3.99
Total	22.89 ± 3.72	21.70 ±11.06	31.38 ± 18.30	24.92 ± 13.56	65.45	139.18	196.86

Table 7.3 Ontogenetic changes in fatty acid composition of the polar lipid fraction (µg mg^{-1} ash free dry weight) of *Scylla serrata* larvae (mean ± SE; *n* = 2) (- indicates <1% of total fatty acid).

Fatty acid	Zoea I	Zoea III	Zoea V	Megalopa
14:0	0.07 ± 0.01	0.18 ± 0.10	0.16 ± 0.11	0.11 ± 0.08
15:0	0.10 ± 0.03	0.20 ± 0.11	0.13 ± 0.09	0.09 ± 0.07
16:0	2.28 ± 0.18	2.44 ± 1.12	2.73 ± 1.46	2.03 ± 1.06
17:0	0.27 ± 0.07	0.38 ± 0.21	0.37 ± 0.22	0.21 ± 0.11
18:0	1.88 ± 0.13	1.44 ± 0.64	2.00 ± 0.94	1.62 ± 0.61
20:0	0.04 ± 0.00	0.01 ± 0.01	0.04 ± 0.03	0.01 ± 0.01
22:0	0.01 ± 0.01	0.01 ± 0.01	0.04 ± 0.03	0.01 ± 0.01
24:0	0.02 ± 0.01	-	-	0.34 ± 0.03
16:1*n*-7	0.35 ± 0.07	0.69 ± 0.17	0.40 ± 0.24	0.15 ± 0.05
18:1*n*-7	0.50 ± 0.06	1.06 ± 0.43	1.35 ± 0.57	0.93 ± 0.23
18:1*n*-9	1.54 ± 0.22	1.88 ± 0.77	2.83 ± 0.95	2.23 ± 0.61
20:1*n*-7	0.04 ± 0.00	0.09 ± 0.06	0.10 ± 0.05	0.01 ± 0.01
20:1*n*-9	0.15 ± 0.00	0.19 ± 0.05	0.22 ± 0.08	0.16 ± 0.06
20:1*n*-11	0.04 ± 0.00	0.02 ± 0.02	0.01 ± 0.01	-
22:1*n*-9	0.07 ± 0.07	0.49 ± 0.48	0.50 ± 0.49	0.49 ± 0.49
24:1*n*-9	-	-	-	-
18:3*n*-3, LNA	0.02 ± 0.02	0.05 ± 0.02	1.19 ± 0.47	0.87 ± 0.14
18:4*n*-3	-	-	0.08 ± 0.04	0.02 ± 0.02
20:3*n*-3	0.01 ± 0.01	-	0.18 ± 0.08	0.18 ± 0.09
20:5*n*-3, EPA	2.54 ± 0.40	2.43 ± 0.96	2.79 ± 1.33	1.95 ± 0.81
22:5*n*-3	0.28 ± 0.06	0.49 ± 0.22	0.43 ± 0.32	0.15 ± 0.10
22:6*n*-3, DHA	1.68 ± 0.08	0.24 ± 0.03	0.64 ± 0.20	1.02 ± 0.66
18:2*n*-6, LN	0.14 ± 0.03	0.48 ± 0.14	0.78 ± 0.04	0.62 ± 0.32
20:2*n*-6	0.19 ± 0.01	0.11 ± 0.02	0.15 ± 0.07	0.16 ± 0.10
20:4*n*-6, AA	1.42 ± 0.11	0.91 ± 0.23	0.86 ± 0.40	0.58 ± 0.20
22:4*n*-6	0.14 ± 0.02	0.07 ± 0.01	0.04 ± 0.03	-
22:5*n*-6	0.14 ± 0.04	0.01 ± 0.01	0.01 ± 0.01	0.05 ± 0.03
∑ SFA	4.67 ± 0.42	4.66 ± 2.16	5.48 ± 2.88	4.11 ± 1.94
∑ MUFA	2.70 ± 0.42	4.42 ± 1.95	5.40 ± 2.39	3.98 ± 1.42
∑ HUFA	6.42 ± 0.56	4.27 ± 1.46	5.10 ± 2.42	4.08 ± 1.99
∑ *n*-3	4.55 ± 0.36	3.22 ± 1.23	5.30 ± 2.44	4.19 ± 1.78
∑ *n*-6	2.04 ± 0.14	1.59 ± 0.40	1.85 ± 0.88	1.41 ± 0.64
Total	13.95 ± 1.35	13.89 ± 5.73	18.03 ± 8.59	13.68 ± 5.79

Table 7.4 Ontogenetic changes in fatty acid composition of the neutral lipid fraction (µg mg^{-1} ash free dry weight) of *Scylla serrata* larvae (mean ± SE; *n* = 2) (- indicates <1% of total fatty acid).

Fatty acid	Zoea I	Zoea III	Zoea V	Megalopa
14:0	0.19 ± 0.06	0.25 ± 0.17	0.27 ± 0.23	0.18 ± 0.15
15:0	0.15 ± 0.06	0.21 ± 0.14	0.16 ± 0.14	0.08 ± 0.07
16:0	2.65 ± 0.67	1.92 ± 1.18	2.30 ± 1.73	1.57 ± 1.17
17:0	0.18 ± 0.08	0.26 ± 0.18	0.27 ± 0.23	0.13 ± 0.09
18:0	0.90 ± 0.18	0.48 ± 0.32	0.67 ± 0.47	0.65 ± 0.35
20:0	0.08 ± 0.02	0.01 ± 0.01	0.04 ± 0.03	0.04 ± 0.02
22:0	0.02 ± 0.02	0.07 ± 0.04	0.11 ± 0.07	0.09 ± 0.05
24:0	0.01 ± 0.01	0.06 ± 0.06	0.05 ± 0.04	0.09 ± 0.08
16:1*n*-7	0.69 ± 0.28	0.77 ± 0.56	0.88 ± 0.75	0.47 ± 0.36
18:1*n*-7	0.53 ± 0.19	0.76 ± 0.56	1.17 ± 0.86	0.86 ± 0.50
18:1*n*-9	1.54 ± 0.62	1.42 ± 1.04	3.10 ± 1.85	3.09 ± 1.98
20:1*n*-7	0.11 ± 0.04	0.07 ± 0.05	0.06 ± 0.05	-
20:1*n*-9	0.13 ± 0.01	0.14 ± 0.09	0.19 ± 0.13	0.18 ± 0.14
20:1*n*-11	0.11 ± 0.02	0.01 ± 0.01	-	-
22:1*n*-9	0.05 ± 0.00	0.04 ± 0.03	0.04 ± 0.04	-
24:1*n*-9	0.02 ± 0.00	0.01 ± 0.01	0.04 ± 0.04	-
18:3*n*-3, LNA	0.01 ± 0.01	0.04 ± 0.04	1.45 ± 1.05	1.38 ± 0.96
18:4*n*-3	0.01 ± 0.01	-	0.15 ± 0.12	0.12 ± 0.07
20:3*n*-3	0.02 ± 0.02	-	0.06 ± 0.05	0.07 ± 0.05
20:5*n*-3, EPA	0.32 ± 0.09	0.60 ± 0.42	0.77 ± 0.63	0.59 ± 0.39
22:5*n*-3	0.16 ± 0.03	0.08 ± 0.06	0.11 ± 0.10	0.08 ± 0.08
22:6*n*-3, DHA	0.44 ± 0.02	-	0.24 ± 0.17	0.38 ± 0.34
18:2*n*-6, LN	0.10 ± 0.01	0.30 ± 0.22	0.82 ± 0.63	0.89 ± 0.75
20:2*n*-6	0.10 ± 0.00	-	0.05 ± 0.04	0.06 ± 0.05
20:4*n*-6, AA	0.20 ± 0.03	0.29 ± 0.20	0.34 ± 0.27	0.22 ± 0.12
22:4*n*-6	0.14 ± 0.04	0.01 ± 0.01	-	-
22:5*n*-6	0.07 ± 0.02	-	-	-
∑ SFA	4.19 ± 1.02	3.26 ± 2.09	3.87 ± 2.95	2.83 ± 1.98
∑ MUFA	3.16 ± 1.16	3.78 ± 2.48	4.48 ± 3.47	3.12 ± 2.19
∑ HUFA	1.47 ± 0.21	4.33 ± 2.90	5.49 ± 4.19	3.89 ± 2.63
∑ *n*-3	0.98 ± 0.09	3.84 ± 2.76	6.29 ± 4.31	5.41 ± 3.43
∑ *n*-6	0.61 ± 0.10	3.64 ± 2.63	6.08 ± 4.13	5.28 ± 3.34
Total	8.94 ± 2.37	7.81 ± 5.33	13.35 ± 9.70	11.23 ± 7.77

7.3.3 Major fatty acids

The total fatty acids composition of *S. serrata* larvae are shown in Table 7.2. At the zoea I stage, saturated fatty acids (SFA) and highly unsaturated fatty acids (HUFA) dominated the fatty acid profile, while monounsaturated fatty acids (MUFA) were less abundant. As the larvae developed, this profile changed, showing a decrease in HUFA at zoea III, and an increase in MUFA at the zoea V and megalopa stages (Table 7.2).

The most abundant fatty acids in newly hatched zoea I, in sequence of abundance, were palmitic acid (16:0), oleic acid (18:1*n*-9), eicosapentaenoic acid (20:5*n*-3, EPA), stearic acid (18:0), docosahexaenoic acid (22:6*n*-3, DHA) and arachidonic acid (20:4*n*-6, AA). These fatty acids were dominant throughout larval development, although the levels of AA, EPA and DHA were substantially reduced by the time the larvae reached the megalopa stage (Table 7.2). At the same time, the level of linolenic acid (18:3*n*-3, LNA) and linoleic acid (18:2*n*-6, LN) increased considerably, making LN and LNA major fatty acids at the zoea V and megalopa stages (Fig. 7.1). The total level of *n*-3 and *n*-6 fatty acids fluctuated during larval development, mainly in accordance with the changing *n*-3/*n*-6 fatty acid ratio in the three types of live prey provided as food (Table 7.2). The *n*-3/*n*-6 ratio was relatively high in newly hatched zoea I (2.08) when maternally derived fatty acids were present, but dropped to 1.75 at the zoea III stage. The *n*-3/*n*-6 ratio later increased again, reaching a high of 2.81 at the megalopa stage (Table 7.2).

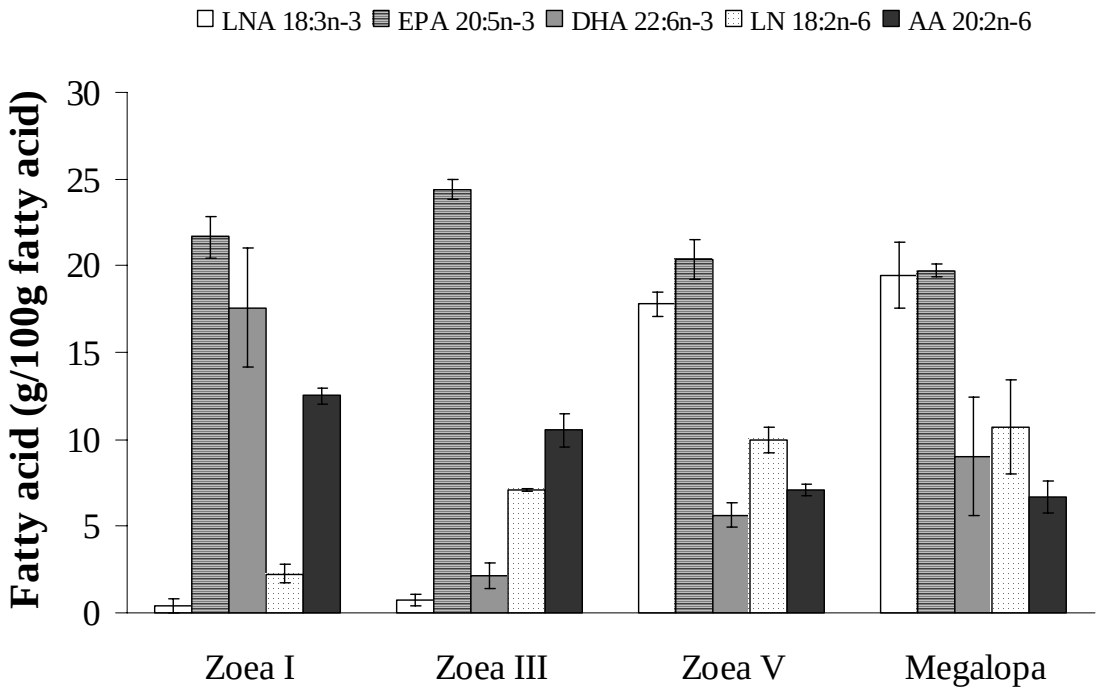

Fig. 7.1 Changes in levels (g 100g $^{-1}$ fatty acid) of linolenic acid (LNA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid (LN) and arachidonic acid (AA). in developing *Scylla serrata* larvae fed rotifers, *Artemia* nauplii and enriched *Artemia* meta-nauplii.

The fatty acid profiles (µg mg^{-1} DW) of rotifers, AF Specialty *Artemia* nauplii and enriched *Artemia* meta-nauplii are shown in Table 7.2. Rotifers contained the lowest total fatty acid content among the three live feeds (65.45 µg mg^{-1} DW), with the lowest *n*-3/*n*-6 ratio (1.45). The fatty acid profile of rotifers was dominated by 16:0, EPA, 16:1*n*-7, and 18:1*n*-9. AF Specialty *Artemia* nauplii contained twice the fatty acid content of rotifers (139.18 µg mg^{-1} DW), and the fatty acid profile was dominated by MUFA. The *n*-3/*n*-6 fatty acid ratio of AF Specialty *Artemia* nauplii was 1.93. The highest level of total fatty acids (196.86 µg mg^{-1} DW) was found in enriched *Artemia* meta-nauplii, where 34.84 µg mg^{-1} DW was HUFA, and the majority of the HUFA were *n*-3 fatty acid (Table 7.2). Enriched *Artemia* meta-nauplii also contained the highest *n*-3/*n*-6 ratio among the three live foods (3.99).

7.4 Discussion

A number of studies have reported on changes in lipid content, lipid class composition or fatty acid compositions in larval crustaceans. The majority have reported their results on the basis of changes in lipid component per unit dry weight of larvae (Roustaian et al., 1999; Narciso and Morais, 2001; Smith et al., 2003; Liddy et al., 2004; Calado et al., 2005; Phillips et al., 2006; Nghia et al., 2007). However, considerable proportion of the dry weight of crustacean larvae is composed of the exoskeleton which has a significant inorganic component. This was clearly shown in this study where the organic content of *S. serrata* larvae ranged between approximately 58% and 70% of dry weight, and varied significantly between larval stages. Under these circumstances, the level of each lipid component is more appropriately expressed per unit of ash fee dry weigh (AFDW), and this method was adopted for the current experiment.

Growth in decapod crustacean larvae can be described as a stepwise pattern, with rapid size increases at each instar due to absorption of large quantities of water and incorporation of new tissue (Nates and McKenny, 2000a; Southgate and Lucas, 2003). Each of these molts is energetically expensive and successful growth and development is dependent not only on the essential nutrients needed for tissue formation, but also a sufficient supply of energy (Roustaian et al., 1999). *S. serrata*, like other crustaceans, undergoes nutrient transfer from broodstock to oocytes, and the maternally-derived yolk typically contains a high level of lipid to sustain embryonic development (Olsen, 1998; Genodepa, 2004). In contrast to lecithotropic larvae, however, mud crab larvae undergo a prolonged egg incubation period (Zeng, 2007),

resulting in limited yolk reserves for post-hatch development and strong reliance on immediate post-hatch feeding (Davis et al., 2005).

The neutral lipids are generally the most common storage lipid class in animals, and in accordance with this, the neutral lipid levels in larval in this experiment fluctuated with changing feeding conditions (Liddy et al., 2004). Polar lipids, on the other hand, are structurally bound in membranes where they fulfill crucial physiological and structural functions (Chapelle, 1986) and are heavily involved in lipid transport in the hemolymph (Teshima and Kanazawa, 1980). As a result, polar lipids are preferentially conserved in the tissue and remain at relatively stable levels under variable nutritional situations (Koven et al., 1989). Such findings have been reported for lobster, *Jasus edwardsii*, (Phleger et al., 2001; Nelson et al., 2003) and *Panulirus cygnus*, phyllosoma (Liddy et al., 2004), larvae of the ghost shrimp, *Lepidophthalmus louisianensis* (Nates and McKenny, 2000b) and larvae of the stone crab, *Menippe adina* (Nates and McKenny, 2000a). A similar trend of polar lipid retention was also noted in the present experiment, indicting that *S. serrata* has a similar lipid metabolism to other marine crustaceans.

However, lipid analysis of mud crab larvae indicated accumulation of fatty acids in both the neutral and the polar lipid fraction at the zoea V stage. Although not common, similar finings has been reported for *J. edwardsii* phyllosoma (Smith et al., 2003) and American lobster, *Homarus americanus*, larvae (Sasaki, 1984), and these authors suggested that lipid accumulation in larvae tissue is linked to subsequent energetically challenging molts. Considering the substantial morphological and physiological changes associated with the metamorphosis from zoea V to megalopa

stage in *S. serrata*, it is possible that the peak in total lipids at the zoea V stage resembles active uptake or accumulation of fatty acids in preparation for this metamorphosis. At this stage in development the larvae experience massive weight gain, and develop from the free swimming planktonic zoea state to a clawed and sub-benthic megalopa stage (Ong, 1964).

Although lipid constituted a relatively large proportion of *S. serrata* larval tissue compared to larvae of many other crustacean species (Phleger et al., 2001; Roustaian and Kamarudin, 2001; Ritar et al., 2003), a decline in tissue lipid was observed as the larvae molted to the megalopa stage. This depletion resulted in a substantially reduced lipid level in tissue of megalopae compared to earlier zoeal stages, indicating that lipid most likely functioned as the major energy reserve to meet the demands of the growing and continuously swimming pelagic zoeal larvae. Based on a similar decrease in the caloric content measured during larval development of the freshwater prawn, *Macrobrachium rosenbergii*, Stephenson and Knight (1980) also suggested that stores of caloric-rich lipids were used to support the rapid morphological changes as well as the perpetual swimming behavior associated with food gathering. This mode of lipid metabolism has also been documented for marine crustaceans larvae (Barnes, 1965; Teshima and Kanazawa, 1982; Roustaian and Kamarudin, 2001; Ritar et al., 2003), and for species with lifecycles similar to that of *S. serrata* it has been suggested that decline in tissue lipids during ontogeny may be linked to larval buoyancy. Fat reserves reduce the amount of energy needed to maintain position in the water column, and consequently planktonic larvae generally have higher lipid reserves than animals adapted to a more benthic habitat (Roustaian and Kamarudin, 2001). As the substantial decrease in lipid reserves in the present experiment was

associated with the molt from the planktonic zoea V to the sub-benthic megalopa stage, it is possible that this is an active mechanism adapted to cope with the altered buoyancy requirements when larvae become more benthic.

The major fatty acids in tissue of *S. serrata* larvae include 16:0, 18:0, 18:1*n*-9, AA, DHA and EPA. This fatty acid composition is comparable to that of the live foods, and is similar to those reported for larvae of other crustaceans, i.e. pureulus of *J. edwardsii* (Phleger et al., 2001), *Jasus verreauxi* peruli (Jeffs et al., 2002), *P. cygnus* phyllosoma (Liddy et al., 2004) and juvenile Chinese mitten crab, *Eriocheir sinensis* (Wen et al., 2006). However, in *S. serrata* larvae a substantial increase in linolenic acid (18:3*n*-3, LNA), and to a lesser degree, linoleic acid (18:2*n*-6, LN), was recorded during development. Previous radiotracers studies with larvae of *Penaeus japonicus* showed that *de novo* synthesis of LNA does not occur in larvae of this species (Jones et al., 1979). If the same is true for *S. serrata* larvae, the substantial increase in LNA content in *S. serrata* larvae at this stage may be directly linked to the high LNA content in the diet. Compared to earlier larvae stages, the zoea V larvae have a well developed gut and more functional swimming appendages. These adaptations enable zoea V larva to efficiently catch and digest the faster swimming *Artemia* meta-nauplii which contained a high level of LNA. An important biochemical role of dietary LNA has previously been reported for juveniles of *M. rosenbergii* (Reigh and Stickney, 1989; D'Abramo and Sheen, 1993) and several species of penaeid (Merican and Shim, 1996), but despite a clear growth enhancing effect of dietary LNA, the specific role of this fatty acid in crustaceans is still not fully understood. Further research using radiotracers is therefore required to identify the specific biological and physiological functions of LNA. As there is a possibility that the increased LNA uptake may be

related to active accumulation of this fatty acid as an energy reserve for subsequent molts, research to evaluate the effects of dietary LNA, and the LNA content of larval tissues on survival and growth of the resulting C1 should also be undertaken.

The essential HUFA have an important role in maintaining the structural and functional integrity of cellular membranes (Fox et al., 1994; Sargent, 1995). Most important are the *n*-3 HUFA, where EPA is known to maintain larval survival, and DHA plays an important role in accelerating the intermolt period and producing wider carapace widths in *S. serrata* juveniles (Sheen and Wu, 2003). Although largely neglected in the literature, recent research has suggested that *n*-6 HUFA, such as AA, also play important roles as essential nutrients for many crustacean species, including mud crabs (Suprayudi et al., 2004; Nghia et al., 2007). The specific biochemical role of this fatty acid is not fully established, but as AA acts as a precursor to the 2-series prostaglandis, which appear to be linked to molting in adult penaeid prawns (Koskela et al., 1992), it is possible that AA in the diet may help accelerate larval development (Ritar et al., 2003). In this experiment, the level of AA was highest at the zoea I stage (1.62 ± 0.14 µg m^{-1} AFDW) dropping to a low of 0.80 ± 0.32 µg m^{-1} AFDW at the megalopa stage. This decrease may result from the low level of AA in the enriched *Artemia* meta-nauplii used to feed zoea III and later stages. As addition of appropriate amounts of AA to the diet has been reported to have a positive effect on growth and survival in the developing larvae of other species, further research into the exact AA requirement of *S. serrata* larvae should be conducted.

The level of total HUFA in *S. serrata* tissue was high in newly hatched zoea I, when maternally-derived lipids were still present. However, as larvae developed the level of

HUFA fluctuated, and for DHA in particularly, a significant drop at zoea III stage was followed by a subsequent increase during the zoea V and megalopa stages. In relation to the total fatty acid content of larval tissue, the relative abundance of DHA, EPA and AA was clearly decreased, while the level of LN and LNA showed a substantial increase. This relative depletion of HUFA in larvae is not consistent with the common belief that HUFA have a primary structural role and therefore are conserved in the tissue (Koven et al., 1989; Liddy et al., 2004). Considering the relatively high level of HUFA present in enriched *Artemia* meta-nauplii, this may indicate that HUFA are stored in live prey in a form that is not readily available to the developing larvae. This phenomenon has previously been reported from studies on *P. cygnus* larvae, where the DHA level in tissue of larvae fed enriched *Artemia* was low compared to the level in *Artemia* itself (Liddy et al., 2004). This led to the suggestion that *Artemia* retain HUFA in a form that can not easily be absorb by poorly developed crustacean larvae (Dhert et al., 1993; Danielsen et al., 1995; Triantaphyllidis et al., 1995). Alternatively, that low DHA level in larvae fed enriched *Artemia* could also be related to *Artemia*'s documented ability to convert DHA into EPA shortly after enrichment (Han et al., 2001). However, a recent study by Nghia et al. (2007) evaluated the influence of HUFA content in live food on larviculture of *Scylla paramamosain* and showed that elevated HUFA levels in enriched rotifers and *Artemia* resulted in significant increases in the levels of these fatty acids in the crab larvae. With this taken into account, the results from the present study, where HUFA levels decreased during larvae development, may indicate that the crab larvae utilize HUFA for energy. Although not common, breakdown of HUFA (DHA) as an energy source has been reported for *J. edwardsii* larvae (Smith et al., 2003), and together with the current

results this indicates that the requirement for HUFA may decreases during the later part of larval development in some crustacean species.

The ratio of *n*-3 to *n*-6 fatty acids has long been used as a indicator of growth in fish (Watanabe, 1982) and is gaining support as an important factor in fatty acid metabolism and lipid nutrition in crustaceans (Xu et al., 1994; Glencross et al., 2002). In this study the *n*-3/*n*-6 fatty acid ratio in mud crab larvae was clearly influenced by the *n*-3/*n*-6 fatty acid ratio of the diet. An initially high ratio of 2.08 in zoea I suggested accumulation of significant levels of *n*-3 fatty acids in oocyte yolk, but due to low *n*-3 levels in the rotifers fed to the early zoea larvae, the *n*-3/*n*-6 fatty acid ratio in zoea III was reduced to 1.75. However, when *Artemia* nauplii (*n*-3/*n*-6 fatty acid ratio of 1.93), and enriched *Artemia* meta-nauplii (*n*-3/*n*-6 fatty acid ratio of 3.99) were introduced as a larval food source, the *n*-3/*n*-6 fatty acid ratio of larvae increased accordingly, reaching 2.81 at the megalopa stage. These results indicate that diet, rather than nutritional superiority determines the balance between *n*-3 and *n*-6 fatty acid ratio in *S. serrata* larvae.

In summary, this experiment showed that ontogenetic changes in lipid composition of *S. serrata* larvae is best expressed on an ash free dry weight basis, to correct for the variability in the organic content of different larvae stages. Depletion of lipids during development signified use of lipid reserves to sustain the rapid morphological changes associated with larval growth, and the declining levels of EPA, DHA and AA suggested that HUFA requirements were reduced during the later larval stages. A major increase in the LNA and LN content at the zoea V stage was recorded, and although the exact nutritional and physiological implication of such increases are still

not fully understood, it seems likely that this is linked to the high level of LNA and LN in enriched *Artemia* meta-nauplii used as food at this stage. A similar link was observed between the *n*-3/*n*-6 fatty acid ratio in larval tissue and live food. Increase in both total lipid and total fatty acid levels was observed prior to metamorphoses at the zoea V stage, an accumulation that may be related to the energetically high cost of this challenging molt. Overall, the results from this experiment provide new insight into lipid and fatty acid metabolism of *S. serrata* during larval development, which again is important for identification of key fatty acids and dietary lipid requirements of larvae.

Chapter 8

Effects of starvation on lipid class and fatty acid profile of zoea V and megalopae larvae of mud crab, *Scylla serrata*

8.1 Introduction

When subject to periods of starvation, crustacean larvae initially metabolize energy rich lipid reserves to sustain metabolic functioning and survival (Anger, 2001). This is reflected by decreasing lipid levels in the tissue, which is typical during short term food deprivation. However, significant proportions of the lipid pool are bound in critical cell structures and membranes, and are therefore unavailable for energy metabolism (Anger, 2001). As a result, larvae subject to prolonged starvation would rely predominantly on protein catabolism for energy, and it is only at the final phase, just prior to death, that structural lipids may be degraded (Ritar et al., 2003). This characterizes a critical condition known as 'the point of no return', where larvae will not recover even after re-feeding (Mikami et al., 1995; Anger, 2001). On this basis, the role of lipids in crustacean nutrition can be considered dual; to provide energy for growth and development, and to supply fatty acids to maintain the integrity and structure of cellular membranes.

The fatty acid and lipid analysis of developing *S. serrata* larvae reported in Chapter 7 indicated significant shifts in the fatty acid profile and lipid metabolism at the time when zoea V larvae metamorphosed to the megalopa stage. This was reflected by

accumulation of fatty acids in both the polar and the neutral lipid fractions of newly molted zoea V larvae, mainly caused by increased levels of docosahexaenoic acid (22:6*n*-3, DHA), linolenic acid (18:3*n*-3, LNA) and linoleic acid (18:2*n*-6, LN). At the same time, an interesting decrease in the levels of the highly unsaturated fatty acids (HUFA); eicosapentaenoic acid (20:5*n*-3, EPA) and arachidonic acid (20:4-*n*-6, AA) was observed. These findings gave new insights into fatty acid utilization in mud crab larvae, however, they did not establish whether this pattern of fatty acid metabolism was a result of a change in larval nutritional requirements, or rather an effect of change in dietary intake. On this basis it is worth investigating whether the observed shift in lipid metabolism reflected a shift in dietary fatty acid requirements at the time of this important metamorphosis, which again could be linked to the high occurrence of molting death syndrome commonly observed during the metamorphosing process.

The object of this experiment was to investigate the effect of starvation on the lipid composition of mud crab zoea V and megalopa larvae. This was done by comparing patterns of conservation and utilization of stored fatty acids in starved and fed larvae, with the aim to further clarify the trends of fatty acid utilization observed in Chapter 7, and to provide information assisting formulation of suitable diets for later stage *S. serrata* larvae.

8.2 Materials and methods

8.2.1 Experimental dietary treatments

Larvae were reared in mass culture tanks following the methods detailed in section 2.3. When 80% of the population reached the zoea V stage, a sample (approximately 500 mg wet weight) was collected and snap-frozen using liquid nitrogen for later lipid

analysis. A large number of the reminding zoea V were manually removed from the communal rearing tanks and divided into fed and unfed treatments. Fed larvae were transferred to tanks containing seawater and enriched *Artemia* meta-nauplii, while the unfed larvae were moved to tanks containing clear seawater only. The larvae were kept under these conditions for 4 days, before being sampled and frozen in the same manner as the newly molted zoea V.

A similar experimental design was used for larvae at the megalopa stage, where larvae were kept in mass-culture tanks until 80% of the population had molted into the megalopa stage. A sample of newly molted megalopa was collected, while a large number of the remaining megalopa were manually removed from the mass-culture tanks and subject to 4 days of feeding or starvation. However, due to their cannibalistic behavior, starved megalopa were reared in clear seawater in individual cubicles, and as the megalopa stage lasts longer than the zoea stages (8-10 days versus 4-5 for zoea V), an additional 6 day starvation treatment was included at the megalopa stage in order to identify the effect of prolonged starvation. All larvae were sampled and analyzed following the procedures detailed in section 2.8 of this thesis, although technical difficulties prevented repeated analysis of tissue sample.

8.3 Results

8.3.1 Major fatty acids

Thirty individual fatty acids were identified in the tissue of fed and starved *S. serrata* zoea V and megalopae where the most abundant fatty acids were, in sequence of abundance; oleic acid (18:1*n*-9), palmitic acid (16:0), eicosapentaenoic acid (20:5*n*-3, EPA), linolenic acid (18:3*n*-3, LNA), stearic acid (18:0), vaccenic acid (18:1*n*-7) and

linolenic acid (18:2*n*-6, LN) (Table1). Saturated fatty acids (SFA), mono unsaturated (MUFA), C18 fatty acids and HUFA (including EPA, DHA and AA) were all depleted during starvation, although the largest decrease was observed among the MUFA (Table 8.1).

Table 8.1 Fatty acid composition (µg mg^{-1} ash free dry weight) of fed and starved *Scylla serrata* zoea V and megalopae. Samples were collected immediately after molt and following 4 days of feeding or starvation. An additional 6 day starvation treatment was included for the megalopa stage (- indicates <1% of total fatty acid).

	Zoea V			**Megalopa**			
Fatty acid	Newly molted	Starved (4 days)	Fed (4 days)	Newly molted	Starved (4 days)	Starved (6 days)	Fed (4 days)
14:0	0.77	0.18	0.42	0.53	0.21	0.24	0.21
15:0	0.52	0.15	0.26	0.31	0.15	0.16	0.11
16:0	8.23	2.36	5.72	5.83	1.93	2.04	4.04
17:0	1.09	0.30	0.62	0.55	0.14	0.14	0.38
18:0	4.08	1.92	3.57	3.23	1.48	1.42	3.15
20:0	0.14	-	0.06	0.06	-	-	0.08
22:0	0.25	-	0.14	0.13	-	-	0.14
24:0	0.10	0.03	0.16	0.24	0.15	0.20	0.07
16:1*n*-7	2.27	0.11	0.90	1.03	-	-	0.53
18:1*n*-7	3.95	0.74	2.83	2.52	0.36	0.32	2.61
18:1*n*-9	8.73	1.48	6.38	7.90	1.10	1.06	6.60
20:1*n*-7	0.26	0.06	0.09	-	-	-	0.08
20:1*n*-9	0.62	0.14	0.43	0.54	0.15	0.09	0.44
20:1*n*-11	-	-	-	-	-	-	0.06
22:1*n*-9	1.07	0.55	0.77	0.98	0.69	0.93	0.27
22:1*n*-11	-	-	-	-	-	-	-
24:1*n*-9	0.07	-	-	-	-	-	-
18:3*n*-3, LNA	4.16	0.34	3.42	3.35	0.30	0.30	4.53
18:4*n*-3	0.40	-	0.24	0.19	-	-	0.28
20:3*n*-3	0.36	0.13	0.46	0.40	0.13	0.11	0.44
20:5*n*-3, EPA	5.52	2.78	4.40	3.74	1.68	1.55	2.86
22:5*n*-3	0.96	0.39	0.53	0.41	0.09	0.08	0.23
22:6*n*-3, DHA	1.25	0.79	1.47	2.40	0.87	0.77	2.23
18:2*n*-6, LN	2.62	0.44	1.95	2.58	0.26	0.23	2.15
20:2*n*-6, AA	0.32	0.16	0.31	0.36	0.14	0.12	0.33
20:4*n*-6	1.87	0.90	1.41	1.11	0.58	0.56	0.88
22:4*n*-6	0.07	-	0.04	-	-	-	-
22:5*n*-6	-	-	0.06	0.08	-	-	0.14
∑ SFA	15.18	4.94	10.95	10.87	4.07	4.20	8.19
∑ MUFA	16.98	3.08	11.40	12.97	2.30	2.40	10.58
∑ HUFA	12.65	5.43	10.32	10.73	3.61	3.30	8.93
∑ *n*-3	12.64	4.43	10.52	10.50	3.07	2.81	10.58
∑ *n*-6	4.88	1.50	3.77	4.13	0.97	0.91	3.49
n-3/*n*-6	2.59	2.95	2.79	2.54	3.16	3.07	3.03
Total	49.67	13.94	36.64	38.47	10.40	10.32	32.84

8.3.2 Total fatty acid and lipid class composition in fed and starved larvae

The highest total fatty acid content (µg mg^{-1} AFDW) was recorded for newly molted zoea V (49.67 µg mg^{-1} AFDW) and newly molted megalopae (38.47 µg mg^{-1} AFDW) (Table 8.1). After 4 days of starvation, these levels were reduced by 71.93% (to 13.94 µg mg^{-1} AFDW) in zoea V larvae, and by 72.96% (to 10.40 µg mg^{-1} AFDW) in megalopae. When subjected to a further 2 days of starvation, however, the fatty acid content of megalopae was practically unchanged (Table 8.1). Fed larvae also showed a slight decrease in fatty acid content, and the final fatty acid contents of fed zoea V and megalopae were 36.64 µg mg^{-1} AFDW and 32.84 µg mg^{-1} AFDW, respectively (Table 8.1).

Fatty acids from the polar lipid fraction dominated the tissue of both fed and unfed larvae at both larvae stages (Tables 8.2). During starvation the fatty acids from the neutral lipid fraction functioned as the main energy store, resulting in a reduction from 23.05 to 1.23 µg mg^{-1} AFDW in zoea V larvae, and from 19.00 to 1.27 µg mg^{-1} AFDW in megalopae (Table 8.3). Fatty acids from the polar lipid fraction were reduced at a slower rate, from 26.62 to 12.71 µg mg^{-1} AFDW in starved zoea V and from 19.47 to 9.05 µg mg^{-1} AFDW in starved megalopa. A full list of the polar and neutral fraction of the lipid pool in larval tissue is shown in Table 8.2 and 8.3.

Table 8.2 Changes in fatty acid composition (µg mg^{-1} ash free dry weight) of the polar lipid fraction in fed and starved *Scylla serrata* zoea V and megalopae. Samples were collected immediately after molt and following 4 days of feeding or starvation. An additional 6 day starvation treatment was included for the megalopa stage (- indicates <1% of total fatty acid).

	Zoea V			**Megalopa**			
Fatty acid	Newly molted	Starved (4 days)	Fed (4 days)	Newly molted	Starved (4 days)	Starved (6 days)	Fed (4 days)
14:0	0.26	0.11	0.21	0.20	0.12	0.14	0.08
15:0	0.23	0.10	0.16	0.16	0.09	0.11	0.05
16:0	4.19	1.99	3.86	3.08	1.54	1.63	2.39
17:0	0.59	0.27	0.44	0.33	0.14	0.14	0.26
18:0	2.94	1.74	2.88	2.23	1.25	1.25	2.32
20:0	0.07	-	0.06	-	-	-	0.04
22:0	0.07	-	0.06	-	-	-	0.04
24:0	-	-	0.05	0.07	0.07	0.09	0.02
16:1*n*-7	0.64	0.11	0.42	0.20	-	-	0.16
18:1*n*-7	1.93	0.68	1.84	1.16	0.36	0.32	1.44
18:1*n*-9	3.78	1.31	3.69	2.84	0.99	0.95	2.93
20:1*n*-7	0.15	0.06	0.09	-	-	-	0.04
20:1*n*-9	0.30	0.14	0.26	0.22	0.11	0.09	0.22
20:1*n*-11	-	-	-	-	-	-	0.02
22:1*n*-9	0.99	0.55	0.77	0.98	0.69	0.93	0.24
22:1*n*-11	-	-	-	-	-	-	-
24:1*n*-9	-	-	-	-	-	-	-
18:3*n*-3, LNA	1.66	0.34	1.93	1.01	0.20	0.17	2.02
18:4*n*-3	0.12	-	0.10	-	-	-	0.09
20:3*n*-3	0.25	0.13	0.37	0.27	0.13	0.11	0.30
20:5*n*-3, EPA	4.12	2.64	3.74	2.76	1.63	1.48	2.20
22:5*n*-3	0.75	0.39	0.45	0.25	0.09	0.08	0.15
22:6*n*-3, DHA	0.84	0.75	1.16	1.68	0.87	0.77	1.73
18:2*n*-6, LN	1.18	0.39	1.19	0.94	0.26	0.23	1.06
20:2*n*-6	0.22	0.16	0.25	0.25	0.14	0.12	0.23
20:4*n*-6, AA	1.26	0.85	1.13	0.78	0.48	0.43	0.62
22:4*n*-6	0.07	-	0.04	-	-	-	-
22:5*n*-6	-	-	0.06	0.08	-	-	0.09
∑ SFA	8.36	4.21	7.71	6.06	3.22	3.37	5.19
∑ MUFA	7.79	2.85	7.07	5.40	2.14	2.29	5.06
∑ HUFA	7.51	4.93	7.20	6.07	3.34	2.99	5.30
∑ *n*-3	7.74	4.25	7.76	5.97	2.92	2.62	6.48
∑ *n*-6	2.73	1.40	2.67	2.05	0.87	0.78	1.99
Total	26.62	12.71	25.21	19.47	9.15	9.05	18.72

Table 8.3 Changes in fatty acid composition (µg mg^{-1} ash free dry weight) of the neutral lipid fraction in fed and starved *Scylla serrata* zoea V and megalopae. Samples were collected immediately after molt and following 4 days of feeding or starvation. An additional 6 day starvation treatment was included for the megalopa stage (- indicates <1% of total fatty acid).

	Zoea V			**Megalopa**			
Fatty acid	Newly molted	Starved (4 days)	Fed (4 days)	Newly molted	Starved (4 days)	Starved (6 days)	Fed (4 days)
14:0	0.50	0.07	0.21	0.33	0.09	0.10	0.13
15:0	0.30	0.04	0.10	0.16	0.06	0.06	0.05
16:0	4.03	0.37	1.86	2.75	0.39	0.41	1.65
17:0	0.50	0.03	0.17	0.22	-	-	0.13
18:0	1.14	0.18	0.69	1.00	0.23	0.17	0.83
20:0	0.08	-	-	0.06	-	-	0.04
22:0	0.18	-	0.09	0.13	-	-	0.11
24:0	0.10	0.03	0.11	0.17	0.08	0.11	0.05
16:1*n*-7	1.63	-	0.48	0.83	-	-	0.37
18:1*n*-7	2.02	0.06	0.98	1.36	-	-	1.17
18:1*n*-9	4.95	0.17	2.69	5.07	0.11	0.11	3.67
20:1*n*-7	0.11	-	-	-	-	-	0.03
20:1*n*-9	0.33	-	0.17	0.32	0.04	-	0.23
20:1*n*-11	-	-	-	-	-	-	0.03
22:1*n*-9	0.08	-	-	-	-	-	0.02
22:1*n*-11	-	-	-	-	-	-	-
24:1*n*-9	0.07	-	-	-	-	-	-
18:3*n*-3, LNA	2.50	-	1.49	2.34	0.09	0.12	2.51
18:4*n*-3	0.27	-	0.13	0.19	-	-	0.19
20:3*n*-3	0.11	-	0.09	0.13	-	-	0.15
20:5*n*-3, EPA	1.40	0.14	0.66	0.98	0.05	0.07	0.66
22:5*n*-3	0.21	-	0.08	0.16	-	-	0.08
22:6*n*-3, DHA	0.41	0.04	0.31	0.73	-	-	0.51
18:2*n*-6, LN	1.45	0.05	0.75	1.64	-	-	1.09
20:2*n*-6	0.09	-	0.06	0.11	-	-	0.10
20:4*n*-6. AA	0.61	0.05	0.28	0.34	0.10	0.13	0.26
22:4*n*-6	-	-	-	-	-	-	-
22:5*n*-6	-	-	-	-	-	-	0.05
∑ SFA	6.82	0.73	3.24	4.81	0.85	0.84	3.00
∑ MUFA	9.19	0.23	4.33	7.57	0.15	0.11	5.52
∑ HUFA	2.82	0.22	1.49	2.44	0.15	0.20	1.81
∑ *n*-3	4.90	0.17	2.77	4.53	0.15	0.19	4.10
∑ *n*-6	2.15	0.09	1.10	2.09	0.10	0.13	1.49
Total	23.05	1.23	11.43	19.00	1.25	1.27	14.11

8.4 Discussion

In nature, pelagic crab larvae often rely on tides and currents for cross-shelf transportation, an adaptation that allows larvae to cover large areas at a minimal

metabolic cost (Epifano, 1988). Although this mode of transport is energetically-effective for poorly developed larvae with limited swimming abilities, it exposes them to patches of highly variable prey-densities (Wen et al., 2006). Most crustacean larvae have adapted to this highly variable habitat, and are able to efficiently catabolize stored lipid to satisfy energy requirements during periods of food deprivation (Narciso and Morais, 2001; Smith et al., 2003; Wen et al., 2006). The impact of this type of periodical starvation is species-specific and depends on the nutritional status of the larvae prior to food deprivation, as well as the duration of the starvation period.

The results from this experiment shows that lipid reserves were drastically reduced in crab zoea V and megalopa during a 4 day starvation period, indicating that unfed *S. serrata*, like other crustacean larvae, efficiently utilized stored lipid reserves for energy. When subjected to longer starvation periods, however, the fatty acid concentration in larval tissue remained practically unchanged. This might suggest that larvae had depleted available lipid reserves and, as a result began to utilize other nutrients for energy, sparing the remaining lipids for critical metamorphosis at later larval stages. Although a common mechanism among some decapod larvae (Anger et al., 1985; Volgt et al., 1985; Anger, 2001), this mode of lipid sparing has been mainly reported for species with long, non-feeding larval stages. As *S. serrata* larvae feed continuously throughout ontogeny, it was possible that the observed decrease in lipid utilization during prolonged starvation may rather be a result of reduced lipase activity. This phenomenon was investigated by Johnston et al. (2004), who reported that starved *J. edwardsii* phyllosoma showed significant reduction in lipase activity compared to fed larvae. The authors suggested a link between starvation and damage to the digestive gland epithelia, which again compromised larval ability to produce

lipases. Without these enzymes the digestive captivity is reduced, and larvae were unable to actively catabolize lipid for energy. However, as very few studies have been conducted on enzyme activity in *S. serrata* larvae, further research is needed to determine the effects of starvation on enzyme activity of this species.

In accordance with the findings reported in Chapter 7, the results in the present experiment showed that fatty acids from the polar lipid fraction dominated the lipid content of mud crab larvae. Although these fatty acids seem to function as structural lipids in *S. serrata* larvae, a slight decrease in polar lipids was also observed during starvation. This suggests that fatty acids from the polar lipid fraction can be mobilized for energy production, at least to a degree, when exogenous feeding subsides. However, the largest and most prevailing reduction in fatty acids in the present experiment was recorded in the neutral lipid fraction, where approximately 94% of the fatty acid level was utilized during the 4 day period of starvation. This use of neutral lipids as a short-term energy reserve is consistent with most other marine crustaceans (Sasaki, 1984; Fraser, 1989; Anger, 1998; Jeffs et al., 2001), whereas the use of polar lipids for energy is usually only seen in species with long, nektonic larval stages, including the spiny lobsters *J. edwardsii* (Phleger et al., 2001) and *P. cygnus* (Liddy et al., 2004).

The role of dietary HUFA in survival and development of marine larvae was demonstrated by Lèger et al. (1985), who concluded that the main factor affecting the nutritional value of *Artemia* for marine prawn larvae was EPA content. Subsequent research has attempted to specify the optimal HUFA requirements for a range of crustacean larvae (Sorgeloos et al., 1998), and for *S. serrata*, deficiency in dietary

supply of HUFA has been identified as a major cause of low survival, longer intermolt period and narrower carapace width in both larvae (Suprayudi et al., 2004) and juveniles (Sheen and Wu, 1999). Despite these findings, problems with larvae culture of *S. serrata* have hampered nutritional research considerably, and contradictory results have been reported. Hamasaki et al. (2002) found that the elevated levels of EPA in live food resulted in abnormal development and mortality of the larvae at metamorphosis, while in a study comparing different *Artemia* strains and the effects of *Artemia* enrichments, Mann et al. (2001) found no influence of the *n*-3 HUFA level on the ability of the larvae to complete development. Furthermore, a recent study conducted with *Scylla paramamosain* larvae reported that high level of HUFA (particularly EPA) in the diet was neither needed nor beneficial for larval performance (Nghia et al., 2007). The results of the present experiment show that both saturated and unsaturated fatty acids were catabolized for energy production during food deprivation. These findings are consistent with the findings in Chapter 7, where depletion of both SFA, MUFA and HUFA was associated with ontogenetic development. Traditionally, the depletion of a particular fatty acids during food deprivation has been viewed as a sign of limited nutritional value, whereas retention of specific fatty acid is interpreted as a requirement for the fatty acid in question (Olsen, 1998). If this is true for *S. serrata* larvae, the results from this experiment indicate that HUFA is less important in larval tissue than previously suggested, or that the requirement for EPA, DHA and AA is reduced during the last two stages of larval development.

Another group of fatty acids that has been given limited attention in the literature are the C18 fatty acids; LNA and LN. It has been suggested that both play an important

role as essential fatty acids (Kanazawa et al., 1977), although the effect of LNA on growth is believed to be superior to that of LN (Kanazawa et al., 1979). Previous studies have shown that some larvae crustaceans are unable to synthesize LNA *de novo* (Jones et al., 1979), while other studies have shown that many crustacean larvae rely on dietary LNA for normal growth and development (Reigh and Stickney, 1989; D'Abramo and Sheen, 1993; Merican and Shim, 1996). In Chapter 7 a substantial increase in LNA level was recorded in newly molted zoea V compared to earlier larval stages, and based on this it would be reasonable to assume that LNA plays an important role in larval mud crab nutrition. However, the present study showed a rapid and clear depletion of LNA during starvation, and as the fatty acid content in this study has been expressed on the basis of organic content, this indicates that LNA was actively catabolized as an energy source when feeding subsided. On this basis it is probable that the high LNA levels in fed larvae reflects the high level of this fatty acid in enriched *Artemia* meta-nauplii, rather than an increased nutritional requirement. Alternatively, the increased LNA content may result from active accumulation of this fatty acid to function as an energy reserve during subsequent molts. However, as the role of dietary LNA in larval nutrition is still not fully understood, further investigation using radiotracers is required before the fate of dietary LNA in *S. serrata* can be described in detail.

In summary, this experiment demonstrated that fatty acids play an important role in energy storage in *S. serrata* larvae during short-term starvation. This mode of lipid utilization did not persist when starvation was prolonged to 6 days for megalopae, indicating that other nutrient sources were catabolized for energy to sustain larvae during extended food deprivation. Fatty acids from the polar lipid fraction dominated

larval tissue, and compared to the fatty acids from the neutral lipid fraction, these fatty acids were preferentially conserved to maintain metabolic functioning during starvation. Reduction of SFA, MUFA and HUFA was observed among larvae in the unfed treatment, and in contrast to common belief, depletion of DHA, EPA and AA during ontogeny indicated possible decreasing requirement for these fatty acids during the final part of larval development. Fed larvae, on the other hand, maintained more stable fatty acid concentrations, and they were able to sequester a range of fatty acids from live rotifer and *Artemia.* This might explain the initially high level of LNA and LN in newly molted zoea V and megalopa cultured with enriched *Artemia* meta-nauplii. However, as the level of these C18 was depleted in larvae during starvation, LNA and LN may play important roles as energy reserves during periods of low food availability, and when extra energy is required to support energetically challenging molts.

Chapter 9

General Discussion

9.1 General discussion

Development of a nutritionally optimized and inexpensive, 'off-the-shelf' dry diet that can be used in *Scylla serrata* hatcheries would be an important milestone in mud crab aquaculture. Significant savings can be realized with reduced need for labor, specialized facilities and electricity, while hatchery production becomes more predictable and reliable. Microbound diets (MBD) can be formulated to meet dietary requirements which will sustain high larval survival, rapid growth and reduced occurrences of molting death syndrome (Hamasaki et al., 2002a). They also provide an ideal vehicle for the introduction of prophylactic probionts and immunostimulants to replace antibiotics in larval culture (Cox and Johnston, 2003). This will result in a steady supply of hatchery reared seed to mud crab farms, more reliable production of commercial sized crabs for local and export markets, and a sustainable mud crab industry throughout the Indo-Pacific.

Before an optimized dry diet can be formulated, knowledge of the nutritional requirements of the mud crab larvae must be established. However, due to difficulties in mud crab larval rearing, their nutritional requirements are not well documented. In recent years, some work has been conducted using different enrichment media to culture live prey such as rotifers and *Artemia*, and based on larval survival and

development time, nutritional requirement for certain nutrients has been suggested (Hamasaki et al., 2002b; Suprayudi et al., 2004). However, conflicting results have been reported, and clearly defined nutritional requirements have yet to be established (Nghia et al., 2007). On this basis, this thesis aimed to use MBD as a tool to investigate nutritional requirements of mud crab larvae with the aim to fill gaps in our current knowledge relating to lipid requirements of *S. serrata* larvae. The major outcomes are summarized and discussed in a broader context in this Chapter, and the importance and applications of the results are summarized in Table. 9.1.

Table 9.1 Schematic overview of the results and implications of this study.

Experiment	**Main result/recommendation**
Use of MBD as partial replacement for zoea III larvae (Chapter3).	50% replacement of live food results in improved growth and survival from zoea III to zoea IV larvae, while total replacement results in poor larvae survival.
Evaluation of 4 protein sources to replace live food component in original experimental MBD for megalopa (Chapter 3).	The use of fish meal and squid meal as replacement of dry rotifer component in experimental MBD resulted in better survival and improved growth to C1 compared to the existing experimental diet.
The effect of supplemental dietary cholesterol on growth, development and survival of megalopa fed semi-purified MBD (Chapter 4).	Megalopa molted successfully to C1 in all dietary treatments, while highest survival was recorded for the megalopa fed a diet containing 0.80% total dietary cholesterol.
Assessment of dietary lecithin and cholesterol requirements of megalopa using semi-purified MBD (Chapter 5).	Interaction between dietary lecithin and cholesterol was documented, and a combination of 0.8% supplemented cholesterol and 2.0% supplemented lecithin was recommended.
Survival, development and growth response megalopae, fed semi-purified diets containing various fish oil:corn oil ratios (Chapter 6).	Megalopa were able to utilize both *n*-3 and *n*-6 fatty acids, but highest survival were achieved with a fish oil: corn oil ratio of 1:1. This allows for formulation of cheaper diets and reduced reliance on marine oils in diet formulation.
Ontogenetic changes in lipid and fatty acid composition in tissue of mud crab larvae (Chapter 7).	Lipids are used to sustain larval development, while decline in HUFA levels indicate reduced requirement for EPA, DHA and AA during later larvae stages. LNA becomes a significant fatty acid in zoea V, and accumulation of fatty acids prior to molt to megalopa was observed.
Effects of starvation and feeding on the fatty acid profile of zoea V and megalopa (Chapter 8).	Starved larvae deplete lipid reserves for energy during initial starvation only. Depletion of both DHA, EPA, AA and LNA show active use of both HUFA and C18 fatty acid during food deprivation, suggesting limited nutritional value, which again indicate reduced reliance of expensive *n*-3 HUFA at this stage of larvae development.

Previous research has shown that MBD has a clear potential as a complete replacement of live food for *S. serrata* megalopa (Genodepa et al., 2004). The potential of MBD as a food source for the zoea larvae of this species had, however, never been evaluated. The results from Chapter 3 were therefore the first to demonstrate that 50% replacement of live food with MBD is possible for zoea III larvae. This dietary treatment resulted in highest survival and fastest development to the zoea IV stage, while only a few successful molts were found among larvae fed MBD exclusively. Although those findings imply that zoea III larvae rely on external digestive enzymes from live prey to help assimilate the consumed food particles, these encouraging results also show that the experimental MBD in the past contained certain beneficial nutrients lacking in *Artemia* and that co-feeding the MBD with *Artemia* may enhance larval survival and development. Still, it is apparent that total replacement of live food with the experimental MBD will result in poor survival of zoea III larvae, and formulated diet particles should be fed to early zoea stages in conjunction with live food organisms. The reliance on external enzymes from live prey can, however, be reduced by incorporation of digestive enzymes into the formulated diets. Research with fish larvae has shown that inclusion of digestive enzymes into diet particles improve nutrient assimilation and growth by up to 30% (Kolkovski et al., 1993). Based on this it is possible that addition of digestive enzymes and chemoattractants in formulated diets represents an avenue for reduced use of live food in mud crab zoeal culture, a topic that warrants further research.

As zoea larvae still rely on the presence of live prey, the megalopa stage was chosen for further nutritional requirement studies. Total replacement of live food with MBD is possible at this stage (Genodepa et al., 2004), and this allows for close monitoring

of the quantity and quality of ingested nutrients. Additionally, the megalopa stage also represents an important stage for diet development and larval nutritional studies as it has a substantially longer duration and higher feeding rates than the zoeal stages, and is associated with the highest occurrence of molting death syndrome. Although previous research has shown that mud crab megalopae readily ingest and assimilate formulated diet particles, the experimental MBD used contained a high proportion of dried rotifers (Genodepa et al., 2004), and as a result megalopa could not be reared without the use of cultured food. Identification of a commercially available protein source to replace the dry rotifers in this MBD was therefore necessary, and comparison of MBD formulated with four different marine meals confirmed that megalopa reared on MBD containing squid meal and fish meal showed higher survival to the first crab stage (C1) than those fed a diet containing dried rotifers and *Artemia* (Chapter 3). Based on these findings, the use of fish meal and squid meal offers great possibilities for production of commercially viable hatchery diets for mud crab larvae, and in the meantime it provided a suitable baseline diet for further nutritional requirements studies.

Cholesterol is considered an essential nutrient for marine crustaceans, as it serves as a precursor for many physiological compounds such as sex and molting hormones, adrenal corticoids, bile acids and vitamin D (Sheen, 2000). Previous research on other crustaceans have shown that cholesterol requirements vary greatly among species, highlighting the importance of species-specific identification of such requirements (Paibulkichakul et al., 1998). Although Sheen and Wu (1999) specified the dietary cholesterol requirements for mud crab juveniles, the present study was the first to show that *S. serrata* megalopa were able to metamorphose into the first crab stage on

minimal levels of dietary cholesterol (0.14% of diet dry weight). Nevertheless, clear benefits of supplemented cholesterol were demonstrated, and highest survival was achieved with an MBD containing 0.80% total cholesterol (Chapter 4).

As the dietary requirement of cholesterol is greatly affected by the presence of dietary phospholipid (Gong et al., 2000), multi-factorial experimentation was needed to pinpoint a possible interaction between these two nutrients for *S. serrata* megalopa. Phospholipid itself is an essential nutrient in crustacean diets, associated with molecular organization of cells and membranes (Hickmann et al., 1998), and research has shown that supplementation of phospholipid in diets accelerates the level of cholesterol in the hepatopancreas and haemolymph of some crustacean species (Teshima et al., 1986). This interaction had not previously been investigated for *S. serrata* larvae, and so the findings in Chapter 5 were the first to demonstrate that high dietary phospholipid levels greatly reduced the need for supplementary dietary cholesterol for larvae of this species. A significant interaction between the two nutrients was established as highest survival and mean dry weight of newly settled crabs was recorded for megalopa fed diets containing the highest levels of dietary lecithin (3.97% and 4.41% of total diet dry weight) regardless of whether diets were supplemented with cholesterol. The results indicate that supplemental dietary cholesterol may not be essential for *S. serrata* megalopa when fed a diet containing fish oil and sufficient levels of dietary phospholipids.

Oils are extensively used in diet formulation for aquatic species, and in addition to being an important source of sterols, they also function as the main supply of non-essential and essential fatty acids (Castell and Covey, 1976). The most common

practice in diet formulation for crustaceans has been to use a 2:1 ratio of marine to terrestrial oils, as this provides a wide range of various *n*-3 and *n*-6 fatty acids (Millikin et al., 1980; Sheen, 1997; Kamarudin and Roustaian, 2002). High quality marine oils are, however, costly and the sustainability of their long term use is aquaculture diets has been questioned (FAO, 2004). As a result, vegetable oils are now being investigated as a means of reducing reliance on marine oils (Borlongan and Parazo, 1991; Richard et al., 2006). The present work suggests that mud crab megalopa require both *n*-3 and *n*-6 fatty acids to sustain survival and growth, as megalopa from all dietary treatments (fish oil:corn oil ratios of 0:1, 1:2, 2:1, 3:1, 1:0, 1:1) successfully molted into C1. However, best survival to C1 (70%) was achieved when fed an MBD containing a fish oil:corn oil ratio of 1:1, indicating that corn oil can be used to partially substitute costly fish oil in MBD formulated for *S. serrata* larvae. The study further showed that complete replacement of fish oil with corn oil in the formulated diet resulted in a high occurrence of molting death syndrome (MDS)-related mortality, indicating that a certain level of dietary HUFA and C18 is essential for *S. serrata* megalopa and that a link exists between HUFA availability and the occurrence of MDS.

Analysis of the biochemical changes occurring in the tissue as larvae progress through the various stages of ontogeny has previously been used to identify nutritional requirements of crustacean species like *Panulirus cygnus* (Liddy et al., 2004), *Jasus edwardsii* (Ritar et al., 2003) and *Macrobrachium rosenbergii* (Roustaian et al., 1999). This research has provided useful information for determining suitable diets for larval culture of several crustacean species with aquaculture potential, however, a comprehensive record of the changes in lipid content, lipid class composition and

fatty acid profile in of developing *S. serrata* larvae reared under hatchery conditions had not previously been reported. Depletion of lipids during development signified utilization of lipid reserves to sustain the rapid morphological changes associated with larval development and growth. Declining tissue levels of eicosapentaenoic acid (20:5*n*-3, EPA), docosahexaenoic acid (22:6*n*-3, DHA) and arachidonic acid (20:4*n*-6, AA) at the later larval stages, suggests that *S. serrata* is unable to synthesize HUFA *de novo,* and indicate that the requirement for these fatty acids may be reduced as the larvae becomes more developed. A major increase in linolenic acids (18:3*n*-3, LNA) content at the zoea V stage was recorded, and has been linked to high level of LNA in enriched *Artemia* meta-nauplii used as feed for later larval stages. A similar link was observed between the *n*-3/*n*-6 fatty acid ratio in larval tissue and live food. Increase in both total lipid and total fatty acid levels were observed at zoea V, prior to metamorphosis to the megalopa stage, an accumulation that may be related to the energetically high cost of this challenging molt.

To get a better understating of whether the observed patterns of fatty acid catabolism and metabolism were a result of a change in nutritional requirements, or rather an effect of the change in dietary intake, the biochemical changes occurring in larvae during starvation was compared at the zoea V and megalopa stages. During periods of food deprivation, crustacean larvae usually rely on stored nutrient reserves to sustain metabolic functioning (Anger, 2001), and the most common strategy among marine crustaceans is to utilize energy-rich lipid reserves. Starvation experiments are therefore an effective method for determining nutritional requirements of fish and crustacean larvae, with the retention of specific fatty acids being interpreted as a requirement for that specific fatty acid (Olsen, 1998). The results in Chapter 8 showed

that *S. serrata* zoea V and megalopa actively utilized stored fatty acids for energy during short-term starvation; however, other nutrient sources were catabolized for energy when starvation was prolonged. Fatty acids from the polar lipid fraction dominated larval tissue, and these fatty acids seemed to be preferentially conserved during starvation. Reduction of SFA, MUFA and HUFA was observed among larvae in the unfed treatment, an observation that is in accordance with the results from Chapter 8, where depletion of DHA, EPA and AA indicated a decreasing requirement of these fatty acids at the last part of larval development. This reduced reliance of HUFA can similarly be related to the findings of Chapter 5, where highest survival to C1 was obtained when megalopa were fed a diet containing a diet with a moderate level of HUFA (1:1 fish oil:corn oil ratio). Together these results show great potential for development of cost-effective and environmentally-sustainable diets with reduced reliance on *n*-3 HUFA and marine oils for mud crab larvae.

9.2 Future directions

An appropriate diet must meet the nutritional requirements of the animal in question, at the same time as it is sustainable and economically viable. Formulated diets have been identified as the best way to achieve these goals, and the problems associated with live food have highlighted the importance and urgency of development of a nutritionally suited diet for the commercial success of mud crab hatchery culture.

A fundamental understanding of the nutritional requirements of developing larvae is needed before such diets can be developed. While this thesis has provided insight into the lipid, sterol and fatty acid metabolism of mud crab larvae, some aspects of lipid utilization and fatty acid requirement still needs further research. For example, the

substantial increase of LNA during zoea development may be a direct result of increased LNA in the diet, or it may be a sign of active accumulation of this fatty acid to sustain development to subsequent stages. As this can have significant influence on diet formulation, radiotracers studies should be conducted to document the fate of dietary LNA in developing *S. serrata* larvae. Evaluation of the effect LNA content has on survival and growth of C1 would also be of interest. Similarly, the observed decrease in HUFA requirements during larvae development will have significant implications for diet development, and further work using MBD containing purified DHA, EPA and AA to determine specific dietary HUFA requirements would clearly be beneficial.

Research should also be conducted to specify an optimum lipid level in MBD particles. Although good survival was achieved with MBD containing 6% lipid in this study, work on juvenile *S. serrata* has shown that mud crabs have tolerance to relatively high lipid levels (Sheen and Wu, 1999), where no significant difference in survival or weight gain was observed among juveniles fed between 5.3% and 13.8% total lipid. If the same is true for *S. serrata* larvae, development of high lipid diets may lead to an elevated level of protein sparing, which again allows for production of cheaper diets. At the same time, dietary requirements for other nutrients such as protein, amino acids, carbohydrates and micronutrients are still largely unknown for mud crab larvae, and laboratory trials examining enzyme activity in developing larvae may be used as a basis for dietary formulation to be tested in culture. In addition to formulating a nutritionally optimized diet, the physical characteristics of diet particles needs refinement, and key characteristics such as texture, color, size, shape taste and consistency must be explored (Cox and Johnston, 2003).

Once a suitable diet has been developed, enormous potential exists for future development of the mud crab industry. This thesis addressed important aspects of lipid nutrition of *S. serrata* larvae, resulting in an improved understanding of dietary lipid requirements of mud crab larvae that will function as a guide for further development of suitable diets. The current study is an important step towards more reliable juvenile production, and through continuous research project like this, the goals of a sustainable, reliable and profitable mud crab industry can be realistically achieved.

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