Studies on development of the formulated feed for the grow-out phase of mud crabs, *Scylla serrata* (Forskal 1775) and *S. paramamosain* (Estampador 1949)

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Thesis Declaration

I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy of the Charles Darwin University, is the result of my own investigations, and all references to ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not already been accepted in substance for any degree, and is not being currently submitted in candidature for any other degree.

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Signature Date

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Abstract

This thesis investigates some fundamental nutrient requirements for the development of a formulated feed for the grow-out phase of mud crabs, *Scylla serrata* (Forskal 1775) in Australia and *S. paramamosain* (Estampador 1949) in Vietnam.

Apparent digestibility (AD) for dry matter, protein, and energy were found for 7 ingredients in the diet of *S. serrata*, including defatted soybean meal, fish meal, blood meal, shrimp meal, cod liver oil, wheat flour, and alpha cellulose. AD for dry matter, protein, lipid, ash, and nitrogen free extract were determined for 13 ingredients in the diet of *S. paramamosain,* namely defatted soybean meal, full fat soybean meal, cassava meal, wheat flour, rice bran, fish meal, acetes meal, peanut oil cake meal, coconut oil cake meal, Korean fish oil, Korean squid oil, local pig fat oil, and vegetable oil.

Optimal requirements for digestible protein, total balanced essential amino acid, digestible energy, and two essential fatty acids, linoleic and linolenic acids, were determined. The optimal digestible protein requirement by juveniles of *S. serrata* was approximately 530 g kg^{-1} for maximum weight gain, and 460 g kg^{-1} for minimum feed conversion ratio. The optimal requirements of *S. serrata* for the total balanced essential amino acid and the digestible energy were 258 g kg^{-1} and 15.7 MJ kg^{-1}, respectively. The optimal requirement of juveniles of *S. paramamosain* for linolenic acid and linoleic acid were 8.1 g kg^{-1} and 11.5 g kg^{-1}, respectively.

These results were used to formulate a feed that was superior to trash fish (*Oreochromis mossambicus*) in terms of survival rate and weight gain, but inferior to the more expensive commercial vital prawn feed in terms of growth and feed conversion ratio.

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1.1 General introduction

The mud crabs, *Scylla* spp., are large edible portunid crabs of the genus *Scylla* de Haan which can be found throughout tropical to warm temperate zones in Asia and the Western Pacific. In recent years, hatchery seed production techniques and farming methods of mud crabs have been developed in many countries, particularly in South-East Asian countries and Australia (Keenan 1999a), although more intensive research on larval rearing techniques, nutrition, genetics and marketing need to be undertaken (Macintosh & Tan 1999). This thesis is devoted to further development of knowledge with regards to the nutritional aspects of mud crabs, *Scylla serrata* and *S. paramamosain* for the grow-out stage. This introduction describes the perspectives and challenges of contemporary mud crab aquaculture and states the aims and objectives of this study.

1.1.1 Perspectives and challenges of mud crab aquaculture

Strong demand for crabs in both domestic and international markets, and rapidly improving culture technology appear to bode well for the future growth of mud crab aquaculture. There has been strong demand for mud crabs from markets in the USA, Germany, Japan, Korea, Taiwan, Hong Kong, Guam, Malaysia, and Singapore (Sivasubramaniam & Angell 1991). In the domestic markets, the requirement for mud crabs is also very high. In Australia, the prices for live mud crab caught from the wild were reported to be A$8-12 per kg (Lee 2002b) and in Vietnam, prices have increased to A$ 5-10 per kg (Thach 2003).

In order to satisfy the increased demands, a variety of culture models have been used to farm crabs including pond culture (Dat 1999; Johnston & Keenan 1999), pen

culture (William & Abdullah 1999; Mwaluma 2002; Trino & Rodriguez 2002), and cage culture (Chandrasekaran & Perumal 1993). Furthermore, the mud crab has been proven as a viable alternative species to shrimp culture in areas where white spot syndrome virus (white spot disease) has occurred (Raghavulu *et al.* 1998). Additionally, the availability of mud crab juveniles is not a limiting factor for the development of the grow-out phase because they have been produced commercially in hatcheries. For example, in 2004, approximate 2 millions of crablets were produced from hatcheries in Vietnam (Lindner 2005).

However, the grow-out phase of mud crab aquaculture may be the bottleneck for the progress of the industry due to the fact that most culture systems have been based on the use raw or unprocessed materials such as low value or "trash" fish (trash fish) as feeds. Trash fish may not always be available, particularly in the wet season, and there are extra costs associated with preventing spoilage such as electricity and a freezer (William & Abdullah 1999). Particularly, using trash fish could impact negatively on the sustainability of commercial fisheries due to the removal of juveniles of commercially valuable species (William & Fitzgerald 2002), and the environment as uneaten parts of trash fish could be a cause of water pollution and disease outbreaks (Thach 2003).

In Vietnam, the continued use of trash fish in mud crab aquaculture will impose much further pressure on fisheries resources, and it will reach a point where the need for diet development and formulated feeds will become necessary to sustain the industry. In Australia where using trash fish products is not an economical or environmentally acceptable option, the development of an artificial feed is critical to support the development of a mud crab aquaculture industry.

Recently, research towards the development of pelleted feeds for mud crabs has begun. For example, the dietary lipid and cholesterol levels required for growth of juvenile crabs have been determined by Sheen and Wu (1999) and Sheen (2000) respectively; the relationship between protein and lipid was investigated by Catacutan (2002), and a study on the digestibility of nutrients in feed components of mud crab diets as been completed by Catacutan *et al.* (2003).

The work presented in this thesis will add to the existing knowledge of nutrition of mud crabs. Its ultimate aim is to contribute towards the development of cost effective diets for mud crabs. However, the economic aspects of diet development will not be considered in this thesis.

In this thesis, a concept of "total balanced essential amino acids: TB-EAA" has been used. The TB-EAA is the sum of the total essential amino acids of the basal ingredients and the supplementary crystalline essential amino acids in a diet.

1.1.2 Thesis aims

1.1.2.1 General aims

This study aims to investigate the fundamental aspects of nutrition that will be required for the development of a formulated diet for mud crabs. This diet will be formulated using data on the digestibility of various ingredients, knowledge of the optimum levels of digestible protein, optimum total balanced essential amino acids, digestible energy, and the optimal levels of linoleic acid, and linolenic acid.

1.1.2.2 Specific objectives

The specific objectives of this study are:

- To determine the apparent digestibility (AD) of *S. serrata* for some feed ingredients in Australia and AD of *S. paramamosain* for common feed ingredients in Vietnam.
- To obtain data on the optimum specification for digestible protein in diets for juvenile mud crabs, *S. serrata*.
- To investigate optimum dietary specifications of juvenile mud crab *S. serrata* for total balanced essential amino acids (TB-EAA) and digestible energy.
- To determine quantitative requirements of juvenile mud crab, *S. paramamosain* for linoleic and linolenic acids.
- To develop pellet feed formulae for juvenile mud crabs (*S. paramamosain*) using a growth response trial.

1.1.3 Thesis outline

The thesis consists of eight Chapters. Chapter 1 provides a general introduction and a general literature review. Chapter 2 introduces in general terms, the materials and methods used in all of the experiments. Chapter 3 reports on two experiments conducted to determine apparent digestibility of 20 common nutrient ingredients. In Chapter 4 and 5, the optimal specifications of *S. serrata* for digestible protein, total essential amino acids, and digestible energy are reported. Chapter 6 presents optimal requirements of *S. paramamosain* for linoleic acid and linolenic acid. Chapter 7 shows the effects of three laboratory pellet feeds, trash fish, and prawn feeds fed for *S. paramamosain*. Finally, Chapter 8 summaries outcomes and recommends future works.

1.2 GENERAL LITERATURE REVIEW

The scope of this literature review will be limited to the information relevant to the development of formulated feeds for *Scylla* spp. This review is organised into seven major sections: (1) trends in global aquaculture feed production, (2) principles of feed formulation, (3) apparent digestibility of feed ingredients, (4) essential nutrient balance, (5) essential nutrient requirement, (6) an introduction to the biology and aquaculture of mud crabs, and (7) studies with formulated or artificial feeds.

1.2.1 Global aquaculture feed production and trend

Over the past few decades, aquaculture production has been growing more rapidly than all other animal food producing sectors, increasing at an annual rate of 9.2 % from 1970 to 2000, compared with just 1.4 % for terrestrial farmed meat production, and 2.8 % for capture fisheries (FAO 2002). However, the rapid growth of the world aquaculture is placing pressure on the global aquatic feed production in terms of both quantity and quality.

There are few official statistics on global manufactured aquatic feed (aquafeed) production. Tacon (2004) estimated that global compounded aquafeed production in 2002 was approximately 17.8 million metric tons (Mt) based mainly on global aquaculture production for each fed species and an estimated feed conversion ratio for that species. This production requires a huge amount of fish meal and fish oil, which are the main components of aquafeeds. With rapidly increasing commercial aquaculture production, the demand for fishmeal and fish oil is increasing and this led Pike (1998) to predict that the total use of fish meal and fish oil would be about 1.5 Mt and 1.1 Mt, respectively in 2010. This in turn puts significant pressure on fisheries production since fish meal and fish oil are made from raw materials

obtained from capture fisheries. Therefore, aquafeed composition and feed utilization are becoming increasingly important for sustainable aquaculture development. In particular, ingredients sourced from capture fisheries need to be replaced with alternatives, feed wastage needs to be reduced, and feeds need to be suitable for both specific species and for different farming systems (Tacon 1999).

1.2.1.1 Replacement of fisheries sources with alternative ingredients

It has also been predicted that with further expected expansion in aquaculture, the demand for fish meal and fish oil in aquaculture feeds might triple over the next 10 years (Allan *et al.* 1999b). Fish meal is an excellent source of essential amino acids such as lysine and methionine, which are deficient in plant sources. Fish oil is good source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both of which are found in some plant oils. Allan *et al.* (1999b) argued that aquaculture would only become a true contributor to food security in the long term if aquafeeds are based on more sustainable and renewable ingredients derived from terrestrial animals and plants.

Replacing fisheries ingredients with inexpensive and renewable ingredients derived mainly from plants and terrestrial animal by-products is now becoming a global trend in aquafeed development. Soybean meal, corn gluten meal, meat and bone meal have all been used as a basis for formulated aquafeeds (Smith *et al.* 2000). However, it is emphasised that practical diets without fish meal and fish oil should be balanced with essential amino acids as well as highly unsaturated fatty acids (Watanabe 2002).

1.2.1.2 Minimizing feed wastages in feed formulation

Uneaten feed causes pollution in aquatic environments by releasing solid wastes, nitrogen and phosphorus (Cho & Bureau 2001; Watanabe 2002). Uneaten feed waste

can be reduced in several ways. Firstly, sound management of the feeding regime to increase feed utilization and minimise food wastage (Cho & Bureau 2001). Secondly, the use of high quality feed, in terms of high digestibility and stability, can also reduce solid waste (Cho & Bureau 2001) and phosphorus waste (Watanabe 2002). Finally, a reduction in nitrogen waste outputs can be achieved by using diets low in the ratio of digestible protein to digestible energy (DP/DE) (Watanabe 2002).

1.2.1.3 Intended farming systems

It is clear that feed supplementation differs from species to species and from farm to farm. As a general principle, when farming carnivorous species, increasing density and yield requires more compounded feed (Avault 1998). Extensive and semi-intensive farms with lower stocking densities require less artificial feed (Figure 1.1).

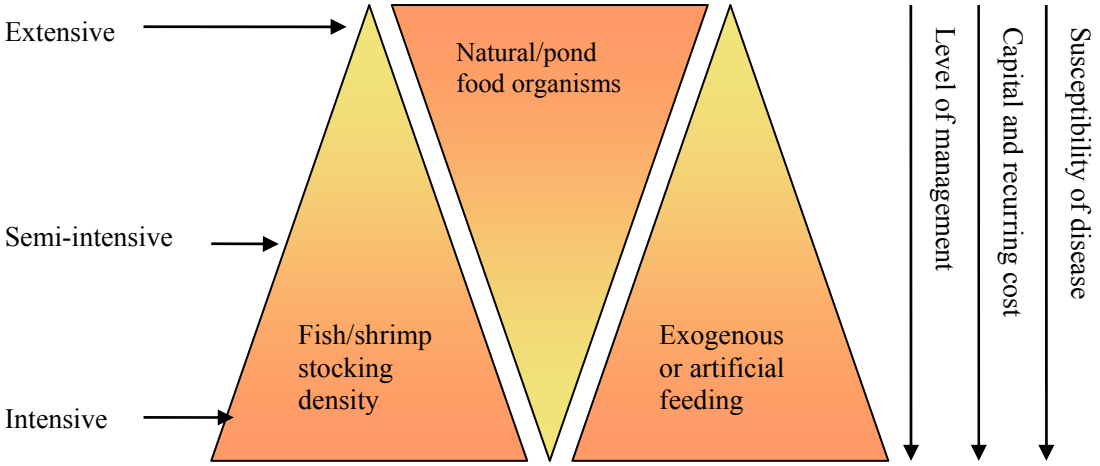

Figure 1.1 Representation of different aquaculture practices in relation to the inputs which was taken from De Silva (1993) who modified after Tacon (1990a)

Figure 1.1 shows that farming systems are basically divided into extensive, semi-intensive, and intensive models. The extensive farming system is stocked at low densities and is not provided with artificial feed, animals utilise natural food such as phytoplankton, zooplankton, and benthic organisms. In some cases, extensive ponds use a small amount of feed and fertilizer during certain seasons or stages to stimulate

the natural food chain. In semi-intensive farming system, animal densities are a little higher than those of extensive farming system and therefore some supplementary exogenous feeding is required. In intensive farming systems, on other hand, animal densities are high, and they depend almost entirely on artificial diets. Extensive and semi-intensive farming systems are employed in subsistence mode of farming, whereas intensive farms are usually commercial enterprises with high production. Intensive farming systems require high levels of management and technology, as well as high quality formulated feed (Figure 1.1). Formulated feeds (aquafeeds) often account for more than 50 % of the cost of running an intensive aquaculture business; therefore having the best aquafeed possible is paramount.

Currently, some aquafeeds are not always well-matched to the species or farming system where they are used. In principle, different feeds should be used for different species and for different farming systems. However, many extensive and semi-intensive pond farming systems with low stocking densities and natural food supplies are fed with nutritionally complete diets irrespective of the farm type and stocking density (Tacon & Barg 1998). This means that commercial aquafeeds used within these systems is generally over-formulated in terms of nutrition requirements, resulting in unnecessarily high feed costs and feed wastage.

1.2.2 Principles of feed formulation

Formulated feed should contain all the individual nutrients necessary for optimal growth and health of the aquaculture animals within the system in which they are cultured. This requires knowledge of the nutritional aspects of the species being cultured, which is still lacking for many species. As Houser and Akiyama (1997) wrote "*the formulation of feed for crustaceans is still more an art than a science. During the last 10 years, however, scientific research has yielded much information*

about the nutrient requirements of crustaceans and the suitability of various ingredients for feed. It is expected that during the next decade formulation of shrimp feed will evolve to being more of a science and less of an art".

Despite the significant amount of research on crustacean nutrition, feed development over the last decade has been based mainly on the experience of feed formulators (Houser & Akiyama 1997). Such commercial feeds normally contain protein, energy, vitamins, minerals, and feed additives (Akiyama *et al.* 1992; Houser & Akiyama 1997).

1.2.2.1 Protein sources

The nutritive value of a dietary protein is governed by the extent to which its amino acid content reflects the need of the growing animal. Tacon and Akiyama (1997) suggested that protein ingredients in feed should have an overall chemical composition similarly to that of the whole body of a crustacean and, in particular they should have the same essential amino acid profile as the animal. Panaflorida (1989) used an essential amino acid index (EAAI) to formulate feed for shrimp. The author based on the ratio of essential amino acid (EAA) in individual feed ingredients and the profile of those EAAs in tissue of the shrimp *P. monodon*:

$$EAAI = \sqrt[n]{\frac{aa_1}{AA_1} x \frac{aa_2}{AA_2} x \frac{aa_3}{AA_3} x \ldots\ldots\ldots x \frac{aa_n}{AA_n}}$$

where EAAI is the essential amino acid index of ingredient (the n^{th} root of the product of the ratios of each essential amino acid in the feed to that of a reference protein), aa_n is the A/E ratio of a particular amino acid in the feed (A/E is a ratio of an essential amino acid by weight divide total essential amino acids plus tyrosine and

cystine by weight and multiplying with 1000) and AA_n is the A/E ratio in the whole shrimp tissue.

Fortunately, EAA profiles for most common ingredients in aquafeeds have been summarised by Tacon (1990b). In addition to the EAAIs, the apparent digestibility of amino acids (or biological availability), particle size, density, water solubility, and pellet ability of ingredients are also important considerations in selecting protein sources (Tacon & Akiyama 1997).

1.2.2.2 Energy sources

All organic compounds contain energy but in variable amounts. The energy is not a nutrient, but is released during the breakdown (metabolic oxidation) of carbohydrates, proteins and fats. Energy is required by the body in anabolic metabolism whereby amino acids and other simple compounds are made into muscle, enzymes, hormones, etc., which are necessary for life.

Protein is a good source of energy since only 20% of dietary protein is retained as body protein in most crustaceans, and that growth generally is faster with high protein diets than with low protein diets. It implies that crustaceans are preferentially using protein as an energy source. Hence, protein is not only important as a source of amino acids but it is also an important energy source. However, ingredients with high protein content are usually more expensive than sources of energy rich in carbohydrate and lipid. Sound knowledge of energy utilization by growing animals is essential for formulating cost-effective diets, to ensure that protein is used almost exclusively for tissue synthesis, rather than as a source of energy in itself (Cuzon & Guillaume 1997).

Carbohydrates and lipids are the other main sources of energy in feeds. Wheat and wheat by-products are the most common source of carbohydrate used in crustacean feed because they contain high levels of carbohydrate and the carbohydrate improves physical quality of the pellets (Houser & Akiyama 1997). Additionally, lipids are a source of energy, a source of fatty acids (saturated fatty acids, monounsaturated fatty acids, and essential polyunsaturated fatty acids) and a carrier of several fat-soluble vitamins, notably Vitamins A, E, D, and K (Parker 2000). Fish, squid, linseed, and sunflower oils are all good sources of both energy and essential fatty acids (Glencross & Smith 1999; Glencross & Smith 2001; Glencross *et al.* 2002b).

1.2.2.3 Vitamins

Vitamins are required for normal growth, reproduction, and the general health of the animals. Vitamins can be divided into two groups, water soluble and fat soluble. Water soluble vitamins include thiamine, riboflavin, pyridoxine, pantothenic, niacin, biotin, folate, Vitamin B12, choline, myoinositol, and Vitamin C. Most water-soluble vitamins serve as coenzymes of biochemical reactions in the body. Most enzymes are specific to a particular biochemical reaction. Fat-soluble vitamins include Vitamin A, Vitamin D, Vitamin E, and Vitamin K. The functions of fat-soluble vitamins are quite specific. For example, Vitamin A is necessary for sight, proper growth, and reproduction, while Vitamin D helps the body mobilize, absorb and use calcium and phosphorus. Although vitamins represent only a small part of feed by weight, they are critical for maintaining normal metabolic and physiological functions (Parker 2000).

There is little information about the specific vitamin requirement of individual crustacean species. It is difficult to determine their vitamin requirement because of leaching from the pellets into water and their slow feeding rate. Existing information

on the vitamin requirements of crustaceans have been summarised by Conklin (1997). In extensive and semi-intensive farms, where animals can access natural food, it may not be necessary to supply vitamins, but a vitamin supplement is essential in feed for intensive systems (Pillay & Kutty 2005). For example, in intensive culture, coated Vitamin C (ascorbic acid), a stable form of Vitamin C as has been used at a level of 1000 mg kg^{-1} (Akiyama *et al.* 1992).

1.2.2.4 Minerals

Minerals may be divided into two groups based on the level of their requirement, namely macro-minerals (required in larger amounts) and micro-minerals (required only in small amounts). Macro-minerals include calcium, chloride, magnesium, phosphorus, potassium, and sodium, while copper, iodine, iron, manganese, selenium and zinc can be regarded as micro-minerals (Parker 2000).

Unlike terrestrial animals, aquatic animals may utilise some dissolved minerals, hence determination of their mineral requirements is therefore difficult (Lall 1989). As with vitamins, little specific information is available for the mineral requirements of crustacean species (Pillay & Kutty 2005). The few studies on mineral requirements of crustaceans have been summarised by Davis and Lawrence (1997).

1.2.2.5 Carotenoids

In general, animals need only small amounts of carotenoids, although they play important roles as antioxidants, in provitamin A activities, in enhancing immune responses, in reproduction, and in early larval development (Torrissen 1990).

Many crustacean species have a significant requirement for astaxanthin (Meyers & Latscha 1997). Two different strategies have been recommended for astaxanthin supplements in diets for black tiger shrimp, *P. monodon*: supplementation with 50-

100 mg kg^{-1} astaxanthin in the last 4-8 weeks before harvesting; or adding 30-70 mg kg^{-1} astaxanthin for entire culture period (Meyers & Latscha 1997).

1.2.2.6 Attractants

Formulated feeds often contain attractants such as squid meal, mussel flesh, shrimp meal and waste, short-neck clam flesh, marine polychaete worms, fish protein and hydrolysates to encourage the animals to eat. For crustaceans, purified substances such as mixture L-amino acids glycine, alanine, proline, histidine, or betaine may be more effective as attractants (Tacon 1990b). Recently, Smith *et al.* (2005a) revealed that crustacean and krill meals were useful not only as attractants but also as nutrient sources in the black tiger shrimp.

1.2.2.7 Binders

Crustacean species eat slowly, so feed should be stable in water to minimise nutrient losses through fragmentation and leaching. The process of extrusion used in the manufacture of many formulated feeds is effective in reducing leaching, but it is costly. Alternatively, binding agents such as starch, alginate, plant gum, agar, high-gluten wheat flour, and urea-formaldehyde are used in crustacean feed (Houser & Akiyama 1997).

1.2.3 Apparent digestibility for feed ingredients

1.2.3.1 Digestive enzymes

Proteins, lipids, and carbohydrates from diets are digested by digestive enzymes in the gut. They are broken down into smaller sub-units (amino acids, fatty acids, glucose, and glycerol) (De Silva & Anderson 1995). The sub-units are involved in the many chemical reactions in animal body.

In general, the digestive enzymes in crustaceans are similar to those in the stomach of fish, but unlike fish, pepsin is absent and trypsin or serine protease is always present at high to very high activity (Guillaume 1997). Moreover, endogenous cellulase activities have been reported in the guts of crayfish, *Cherax quadricarinatus*, (Xue *et al.* 1999; Figueiredo *et al.* 2001), in white shrimp, *L. vannamei,* (Moss *et al.* 2001), in fresh water prawn, *Macrobrachium rosenbergii,* (Gonzalez-Pena *et al.* 2002). Furthermore, Johnston and Freeman (2005) recently found two enzymes, cellulase and chitinase, in the velvet crab, *Nectocarcinus tuberculosus*, and green crab, *Carcinus maenas*. A number of enzymes, including protease, amylase, cellulase, and xylanase, have also been found in the mid-gut gland of mud crab, *S. serrata,* (Pavasovic *et al.* 2004).

1.2.3.2 Importance of apparent digestibility for formulated feed

The digestibility of a feed is theoretically the sum of the digestibility of its component ingredients multiplied by their proportional contribution to the feed. The digestibility of feed and each of its ingredients is important because it is one of the main factors that influence how much of the feed is converted into body mass and how much is lost as faecal waste. The quality of ingredients can be evaluated though determining their apparent digestibility (Akiyama *et al.* 1992). High digestibility is fundamental to the formulation of feeds with low cost and minimum wastage (De Silva & Anderson 1995). The digestibility of different ingredients for crustacean feeds is therefore attracting increasing attention of nutritionists and aquaculturists (Lee & Lawrence 1997).

It is pertinent to include definitions of true digestibility and apparent digestibility here for the sake of clarity. True digestibility (TD) describes the portion of feed that is absorbed, minus the materials that are lost by the gut in the process of ingestion

and digestion. These losses are known collectively as metabolic faecal losses. In contrast, apparent digestibility describes the portion of the feed that is absorbed and it is based on the difference between the amount of feed ingested and the amount of faeces. In this case, faeces contain other components such as gut mucosa cells, bacteria and digestive enzymes. Apparent digestibility (AD) is the most commonly determined index using empirical methods because it is much harder to measure true digestibility.

1.2.3.3 Methods used to determine the apparent digestibility

A number of techniques have been used to determine AD. These include in vivo methods: gravimetric (total collection) and inert marker, in vitro digestibility, nutrient composition correlations, and radioactive tracers (Lee & Lawrence 1997). In general, each method has its own advantages and disadvantages. However, the gravimetric methods are generally considered the best for estimating AD, but this method requires the collection of all faeces and the exact amount of feed intake must be known. This requires a significant amount of labour, hence, is costly. Thus, two other techniques, using an inert marker (*in vivo*) and *in vitro* digestion, have also been widely used.

1.2.3.3.1 In vivo method

The *in vivo* method uses a series of inert markers such as chromic oxide (Kots & Luckey 1972), cholestane (Ishikawa *et al.* 1996), or yttrium oxide (Carter *et al.* 2003). A good inert marker should not move through the gut differentially from that of the feed. Also, the good marker should not be toxic to the animal. As a result, chromic oxide (Cr_2O_3) is the most commonly used marker for the *in vivo* method (De Silva & Anderson 1995), and it has been used widely to measure the apparent

digestibility of fish (Sullivan & Reigh 1995; McGoogan & Reigh 1996; Allan *et al.* 2000). Chromic oxide has also been used as an inert marker to estimate the digestibility of shrimps (Akiyama *et al.* 1989; Reigh *et al.* 1990; Brunson *et al.* 1997), crayfish (Jones & De Silva 1997; Campana-Torres *et al.* 2006), the Chinese hairy crab, *Eriocheir sinensis* (Mu *et al.* 2000), and the mud crab, *S. serrata*, (Catacutan *et al.* 2003).

The main concerns with using chromic oxide as an inert marker are the homogeneity of its distribution in the faeces, and the cost associated with the use of perchloric acid in the digestion of samples. However, if samples are collected and handled properly, this method is quantitative and reproducible (Lee & Lawrence 1997). When chromic oxide is used as an inert marker in crustaceans, Lee and Lawrence (1997) recommended the following protocol:

- Sample collection: most faeces should be collected, and then pooled daily from several individuals.
- Sample handling: the sample must not be contaminated with feed and micro-organisms. The sample should be dried at a low temperature (lower than 40^{o}C) to avoid the loss of volatile components.
- Feed formulation: feed should contain 0.5 to 1.0% chromic oxide, which should be distributed homogenously throughout the feed, it should be well bound.
- Animal behaviour: animals should be acclimated to trial conditions for at least five days then fed with the experimental diets
- Analytical procedure: all parameters should be recorded on a dry weight basis. The dry matter, protein, and energy digestibility coefficients should be

determined and, where possible, amino acid and mineral digestibility coefficients should also be determined.

1.2.3.3.2 In vitro method

Unlike the *in vivo* method, the *in vitro* method uses enzyme activity to test the digestibility of ingredients. The *in vitro* method has been used to determine apparent digestibility discus fish, *Symphysodon aequifasciata* (Chong *et al.* 2002), rainbow trout, *Oncorhynchus mykiss,* (Cheng & Hardy 2002), and in the South African abalone, *Haliotis midae* (Shipton & Britz 2002). It has also been used as an alternative method for digestibility determination for the southern blue fin tuna, *Thunnus maccoyii,* (Carter *et al.* 1999). The *in vitro* method has also been used to estimate digestibility in the Indian white shrimp, *Penaeus indicus*, (Omondi & Stark 1996), and *P. vannamei* (Cousin *et al.* 1996; Ezquerra *et al.* 1998).

The main advantage of this method is that it is quick and relatively inexpensive for analytical consumables. On the other hand, the *in vitro* method requires a high level of analytical skill. There is also some question about its reliability. For example, Chong *et al.* (2002) reported that the *in vitro* method gave lower values for apparent digestibility than the *in vivo* method. Values of apparent digestibility obtained by the *in vitro* method appear to be also influenced by biological and environmental factors. Divakaran *et al.* (2004) indicated that when shrimp, *L. vannamei*, were fed different feed as a cause to obtain different protein digestibility values for the same ingredient. Further comparative studies of the *in vitro*, *in vivo* and gravimetric methods for estimating apparent digestibility are needed to assess the reliability of the *in vitro* method.

1.2.4 Essential nutrient balance

1.2.4.1 Protein and amino acid balance

Proteins are long chains of amino acids linked by peptide bonds. Proteins provide aquatic animal with source of energy as well as amino acids (Parker 2000). Twenty standard amino acids are found in animal proteins (De Silva & Anderson 1995). These amino acids are divided into two groups, essential amino acids and non-essential amino acids. Essential amino acids are those that cannot be synthesized by an animal whereas non-essential amino acids can be synthesized from other components by the animal. The ten essential amino acids are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. These must all be supplied by the diet. Two non-essential amino acids can be used in some metabolic progress instead of phenylalanine and methionine, tyrosine and cystine, respectively and hence reduce the requirement for these amino acids (Guillaume 1997).

The essential amino acid composition of fish is strongly correlated with the essential amino acid profile in their diets (Cho *et al.* 1985; Wilson & Poe 1985). This is also likely to be the case for crustaceans (Tacon 1990a; Akiyama *et al.* 1992; Tacon & Akiyama 1997). Balancing essential amino acids is therefore one of the most important steps in feed formulating.

The EAA balance can be improved by adding crystalline amino acids to otherwise deficient protein sources. This has been demonstrated in casein based diets for juvenile Atlantic salmon, *Salmon salar* L, (Rollin *et al.* 1994), in soybean-based diets for rainbow trout, *Oncorhynchus mykiss,* (Davies & Morris 1997), and for juvenile American lobster, *Homarus americanus,* (Floreto *et al.* 2000).

Where possible, it would be better to combine different protein sources to obtain the right essential amino acid balance instead of using crystalline amino acids. Different protein sources have different EAA profiles, so it may be possible to match the EAA balance of the target species by a suitable mix of protein sources (Houser & Akiyama 1997). However, this requires a range of protein ingredients (with proper EAA profiles) and computer software support.

1.2.4.2 Essential amino acid and energy balance

Energy is required by the anabolic metabolism through which the animals synthesize complex compounds (such as muscles, enzymes, hormones) using amino acids and other components.

The level of energy in a diet has significant influence on the EAA requirement and hence growth rate. If the diet does not contain enough energy, surplus EAAs cannot be utilised for protein synthesis, but are broken down and used as an energy source (Batterham 1992). On the other hand, if EAAs are deficient then surplus energy is converted to fat (Batterham 1992).

1.2.4.3 Essential fatty acid balance

The fatty acids with a chain length 18 or more carbon atoms, and with more than one double bond are called polyunsaturated fatty acids (PUFAs). Further, the PUFAs that have more than three double bonds and contain 20 or more carbon atoms are referred to as highly unsaturated fatty acids (HUFAs).

Although the lipid of marine crustaceans usually contains higher levels of HUFAs and PUFAs than that of fresh water crustaceans (Castell 1983), four essential fatty acids including linolenic acid (18:3n-3, LNA), linoleic acid (18:2n-6, LOA), eicosapentaenoic acid (20:5n-3, EPA), and docosahexaenoic acid (22:6n-3, DHA)

must be supplied from the diet of shrimp (Kanazawa *et al.* 1977; Tacon 1990a; Akiyama *et al.* 1992; Glencross & Smith 1999; Glencross & Smith 2001) and mud crab, *S. serrata* (Sheen & Wu 2002; Suprayudi *et al.* 2004). Further, several studies have indicated that crustacean species have a limited ability to biosynthesize n-3 and n-6 HUFAs from n-3 and n-6 PUFAs, respectively (D'Abramo 1997), and then HUFAs should be supplied in their diets (Anderson & De Silva 2003).

There appears to be some interaction between essential fatty acids in diets. The requirement for a single essential fatty acid (EFA) is high if it is the only one EFA in the diet, but the requirement for individual EFAs appears to be lower when the diet contains a mixture of EFAs. For example, in the absence of LNA the optimal requirement of LOA for *P. monodon* was 16% total fatty acids, but when both LOA and LNA were present in the diet, the optimal LOA level was 14% of total fatty acids (Glencross & Smith 1999). Similarly, when DHA was absent, the optimal requirement of EPA was 12% total fatty acids, but when both were present in the diet, then the best growth of prawn was achieved at EPA 4% of the total fatty acids (Glencross & Smith 2001).

1.2.5 Essential nutrient requirements

1.2.5.1 Protein requirement

Protein synthesis is a complex process involving deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and ribosomes in the genetic code of the animal which regulate the synthesis of particular proteins (De Silva & Anderson 1995) (Figure 1.2). When the rate of the protein synthesis is greater than the rate of protein degradation, the animal is able to grow. If there is insufficient energy or essential

amino acids, the rate of protein degradation is higher than that of protein synthesis and the animal loses weight**.**

Figure 1.2 Protein turnover modified after De Silva and Anderson (1995)

The optimal protein requirement has been defined as the minimum amount of dietary protein needed to produce maximum growth in a certain period (Tacon 1990a). In crustaceans, optimal protein requirements varied greatly among species and within species (Guillaume 1997). For example, juveniles of *P. japonicus* required 52-57% dietary protein with casein and albumin as the protein source (Deshimaru & Yone 1978), but they need only 42% dietary protein with crab meal as the protein source (Koshio *et al.* 1993).

According to Guillaume (1997), the reasons for the differential protein requirements of crustaceans are:

- The different metabolic energy or gross energy values
- The different compositions of amino acids from the dietary protein sources
- The different digestibility or biological values of the dietary proteins
- The different experimental conditions, different feeding habits, and different age.

Although crude protein requirement has been determined for most crustacean species, in practice the concept of "crude protein" is of little value for nutritionists (Houser & Akiyama 1997), because the "crude protein" value is generally an over-estimate of the true protein content of feed. True protein or a total amount of amino acids should be used to calculate the nutrient value of the diet. The analysis of true

protein is expensive (Houser & Akiyama 1997), so Cuzon and Guilliaume (1997) recommended that digestible protein should be used in crustacean species.

There is little information on the digestible protein requirement of crustacean species, but a number of studies have been conducted in fish. For example, digestible protein has been used to estimate optimum protein requirements in sea bass, *Dicentrarchus labrax,* (Alvarez *et al.* 1998), silver perch, *Bidyanus bidyanus,* (Allan *et al.* 2001), tilapia, *Oreochromis niloticus* (Hayashi *et al.* 2002), juvenile rockfish, *Sebastes schlegeli,* (Lee *et al.* 2002), and rainbow trout, *Oncorhynchus mykiss,* (Encarnacao *et al.* 2004). Nevertheless, the establishment of the digestible protein requirement is only possible when the protein digestibility of a range of ingredients is known for the target species.

Since the requirement for protein is a sum of the requirements for individual EAAs (Parker 2000), then the requirement of protein should be related to the balance of EAAs in diets. As a result, it is of little value to determine the optimal dietary protein requirement without taking into account the essential amino acid balance (Cho *et al.* 1985). Consequently, a low protein diet with a properly-balanced essential amino acids is better than one with higher protein diet but poorly-balanced essential amino acids (Houser & Akiyama 1997). This is an important aspect of feed formulation because protein is the most expensive component of a diet.

1.2.5.2 Essential amino acid requirements

As described in Sections 1.2.4.1 and 1.2.4.2, EAA requirements are influenced by interactions between essential amino acids, between essential and non-essential amino acids, and between amino acids and energy. For instance, if cystine is deficient in the diet, it can be synthesised by fish from methionine, and this

relationship also exists between phenylalanine and tyrosine (Tacon & Cowey 1985). Further, the level of one EAA affects the requirement for other EAAs. If a diet lacks one or more EAAs, then the animal cannot fully utilise other EAAs (Batterham 1992). In this case, these excess EAAs are broken down and used as energy.

According to Houser and Akiyama (1997), five of the ten essential amino acids are commonly deficient in feed ingredients, namely lysine, methionine, tryptophan, isoleucine, and threonine. If the feed contains enough of these then the other five are usually also present in sufficient quantity to meet dietary requirements.

To date, determining the essential amino acid requirements of fish and crustacean is still considered controversially (Encarnacao & Bureau 2004). There are available different methods to determine essential amino acid requirement such as determination of individual essential amino acid requirement, radio-labelled substrate technique, predictable method based on one single EAA requirement, and rapid method based on a ratio of EAA in body target tissue.

1.2.5.2.1 Individual essential amino acid requirement

To determine the requirement for individual essential amino acids, target animals are usually fed with diets containing graded levels of a single amino acid. The diet with the lowest level of the essential amino acid that produces maximum growth is used to estimate the requirement for that particular essential amino acid. This methodology has been conducted in *P. monodon* to which are summarised in Table 1.1.

Table 1.1 A summary of the optimal requirement of essential amino acid requirements for post-larvae black tiger shrimp, *P. monodon*

EAAs	Optimal level (g kg^{-1})	References
Arginine	18.5	Millamena *et al.* (1998)
Histidine	8	Millamena *et al.* (1999)
Isoleucine	10.1	Millamena *et al.* (1999)
Leucine	17	Millamena *et al.* (1999)
Lysine	20.8	Millamena *et al.* (1998)
Methionine	8.9	Millamena *et al.* (1996)
Phenylalanine	14	Millamena *et al.* (1999)
Threonine	14	Millamena *et al.* (1997)
Tryptophan	2	Millamena *et al.* (1999)
Cystine	4.1	Millamena *et al.* (1996)
Valine	13.5	Millamena *et al.* (1996)

1.2.5.2.2 Radio-labelled substrate technique

Another method used to determine which amino acids are essential is the use of ^{14}C labelling. In this method, a ^{14}C-labelled substrate such as glucose is injected into the animal. The ^{14}C is only incorporated into those non-essential amino acids that animals can synthesize leaving essential amino acids unlabelled. However, this method is more qualitative than quantitative (De Silva & Anderson 1995).

1.2.5.2.3 Prediction method based on only one single EAA requirement

Recently, a new method has been used, based on the idea that there should be a correlation between whole body amino acid composition and their dietary amino acid requirement. By determining the requirement for one essential amino acid from growth data, the other nine essential amino acids can be estimated from their ratio being proportional to the whole body amino acid composition. This method was used to estimate the quantitative dietary essential amino acid requirements of the fry of Catla fish, *Catla catla* (Ravi & Devaraj 1991) and silver perch *Bidyanus bidyanus* (Ngamsnae *et al.* 1999), and it has been used to predict the essential amino acid

requirement of hybrid striped bass (Twibell *et al.* 2003). However, there appears to be some problems with this method. Oohara *et al.* (1998) indicated that the essential amino acid requirement profiles based on fish growth assay appear to be different among fish species rather than those of body amino acid compositions.

1.2.5.2.4 Rapid method of determination of essential amino acid requirements

This method is based on the proportion of each essential amino acid in the composition of the whole body tissue of the species in question, and then essential amino acids in diets can be formulated relative to their ratio in the body of the animal (Wilson & Poe 1985). Tacon (1990a) used this approach to calculate different protein levels having different ratios of EAAs to be similar to those in the whole body composition of fish (for fish feed) and short neck-clam (for shrimp feed). The same approach was taken by Small and Soares (1998) to determine the EAA requirement of the striped bass *Morone saxatilis*. The EAA requirement (g kg^{-1}) was obtained at an equal digestible energy of 13.39 MJ kg^{-1} and included arginine, 14; histidine, 6; isoleucine, 9; leucine, 19; lysine, 22; methionine + cysteine, 10; phenylalanine + tyrosine, 17; threonine, 11; tryptophan, 3; and valine, 10.

1.2.5.3 Essential fatty acid requirements

There have been a number of studies investigating the effect of essential fatty acids on the growth of crustaceans. Kanazawa *et al.* (1977) found that the weight gain of *P. japonicus* improved with 1% of either 18:2n-6 and 18:3n-3 in diet. More recently, optimal LOA and LNA in *P. monodon* were determined to be 14 and 21% of total fatty acids, respectively as long chain polyunsaturated fatty acids were not present in the diet (Glencross & Smith 1999). Furthermore, the best growth of *P. monodon* was achieved at EPA 4% and DHA 4% of total fatty acids (Glencross & Smith 2001).

1.2.6 Introduction to the biology and aquaculture of mud crabs

1.2.6.1 Taxonomy

Based on morphology, Estampador (1949) determined that there were three main species of the genus Scylla, namely *Scylla serrata*, *S. tranquebarica*, and *S. olivacea*. This classification also showed that one existing species in question was morphologically described similar to *S. serrata* and named *S. paramamosain.* Keenan *et al.* (1998) reviewed the genus recently and confirmed that there were four species in the genus Scylla namely *Scylla serrata* (Forskal, 1775), *S. olivacea* (Herbst, 1796), *S. tranquebarica* (Fabricius, 1798), and *S. paramamosain* (Estampador, 1949). The two species in this study are shown in Figures 1.3-1.4 sourced from Keenan (1999b).

Figure 1.3 *Scylla serrata* (Forskal 1775) sourced from Keenan (1999b)

Figure 1.4 *Scylla paramamosain* (Estampador 1949) sourced from Keenan (1999b)

1.2.6.2 Distribution

Mud crabs, *Scylla* spp., the largest species of the Family Portunidae, are widely distributed in tropical and sub-tropical marine and estuary waters (Shokita 1991). Most species of Scylla occur in shallow coastal areas and are usually associated with mangroves and estuaries. Female crabs migrate to deeper water to spawn, and the floating zoea and megalops then return to the coastal areas where juveniles develop into adults (Wright 1989). The life cycle of mud crab is described in Figure 1.5.

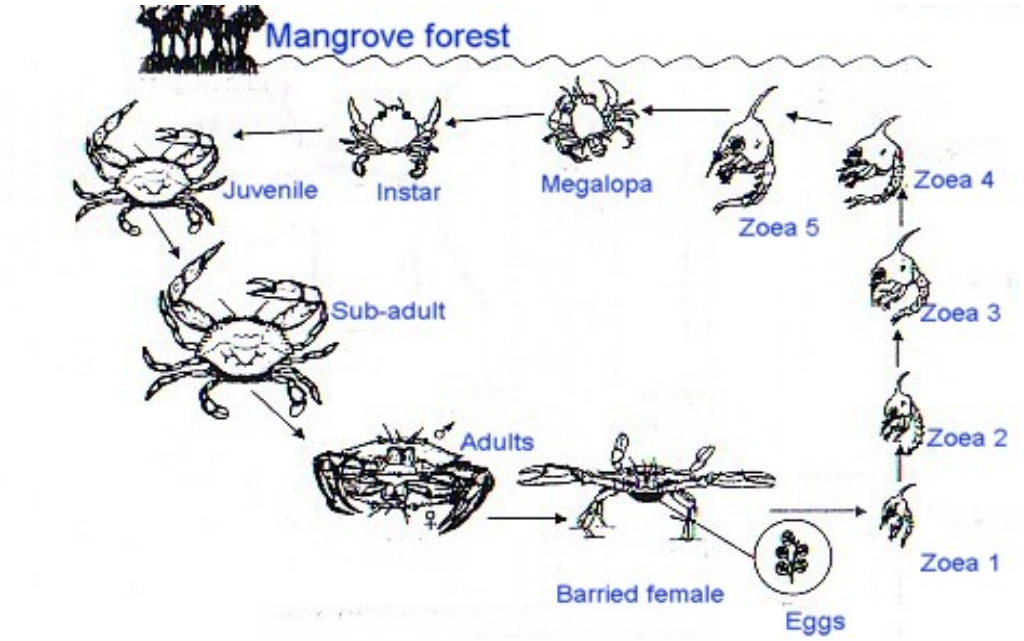

Figure 1.5 The life cycle of the mud crab was described by Quinitio and Estepa (2003)

The distribution of the four mud crab species ranges from East African Coast through to Eastern Pacific Ocean and South China Sea (Keenan 1999a). The mud crab, *S. serrata*, the dominant species in Australia (Keenan 1999b) and *S. paramamosain* the most common in the south of Vietnam (Macintosh *et al.* 2002), were the two species on which this study focused.

1.2.6.3 Physio-chemical factors

1.2.6.3.1 Water temperature and salinity

Mud crabs, *S. serrata,* tolerate a wide range of temperature from 13 ^{o}C to 40 ^{o}C, but they are likely to eat less when temperatures fall below 20 ^{o}C (Shokita 1991). Similarly, laboratory studies have shown that whilst water temperatures of 18-30 ^{o}C were most suitable for *S. serrata*, they rested at 12-14 ^{o}C, and they died at temperatures lower than 11 ^{o}C (Luo & Wei 1986). Similarly, the mud crab, *S. serrata* did not feed at 12 ^{o}C and only 65 % of them fed at 25 ^{o}C (Hill 1980).

The optimum salinity for *S. paramamosain* has been reported to be 15-25 ‰ (Ut & Vay 2001). However, mud crabs can tolerate very extreme salinities. *S. serrata* has been found in one South African lagoon with salinity fluctuations from 2 to 89 ‰, and they survived a four-month period of low salinity in the order of 2 ‰ (Hill 1979b). Similarly, it has been found that the mud crabs, *S. paramamosain,* in Vietnam could withstand in a large fluctuation of salinity from 5 to 3 6 ‰ (1999). In a recent experiment, it was found that production of juvenile *S. serrata* was maximal at approximately 30 ^{o}C and in salinities of 10-25 ‰ in northern Australia (Ruscoe *et al.* 2004).

1.2.6.3.2 Dissolved oxygen and pH

Mud crabs can survive in water with DO levels ranging from zero to over-saturation (Shokita 1991). In earth pond, mud crabs can live for a long time in mud bottom layer where oxygen level was approximately zero. In addition, mud crabs are well adapted to living for long periods out of water (Wright 1989). Furthermore, mud crabs have a wide pH tolerance. According to Dat (1999), they can tolerate pH

ranging from 6.5 to 9.2. In pond conditions, pH levels of 7.95-8.25 have been found to be the most suitable for mud crab culture (Mwaluma 2002).

1.2.6.4 Feeding habits

While chiefly carnivorous, mud crabs can feed on a variety of materials. In nature, their diet includes molluscs, crustaceans, fish, plant material, and detritus (Hill 1976; Prasad & Neelakantan 1988). However, mud crabs appear to prefer small crabs as prey. It was hypothesised that small crabs have a higher energy content compared with other prey organisms (Hill 1979a). Furthermore, feeding habits are influenced by size and age. Juvenile crabs with a carapace width of less than 70 mm appear to feed mainly on detritus while sub-adults and adults preferred eating crustaceans and fish (Prasad & Neelakantan 1988).

1.2.6.5 Mud crab aquaculture

1.2.6.5.1 Mud crab aquaculture production

Statistical data from FAO show that mud crab production from both sectors (capture and aquaculture) increased gradually from 1984 to 2002 (Figure 1.6). During this period, the annual quantity of mud crab captured was higher than that of mud crab aquaculture. However, aquaculture production has increased dramatically since 2002. The production in 2002 was approximately 14,500 tons but increased rapidly to 120,000 tons in 2003, and remained stable in 2004.

In Vietnam, the total crab aquaculture production (mud crabs and swimming crabs) in 2004 was around 10,026 tons (Lindner 2005). Mud crab are stocked in many coastal provinces including Quang Ninh, Hai Phong, Thanh Hoa, Nghe An, Thua Thien - Hue, Ba ria- Vung Tau, Ho Chi Minh City, Ben Tre, Tra Vinh, Soc Trang, Ca Mau, Kien Giang, and Bac Lieu.

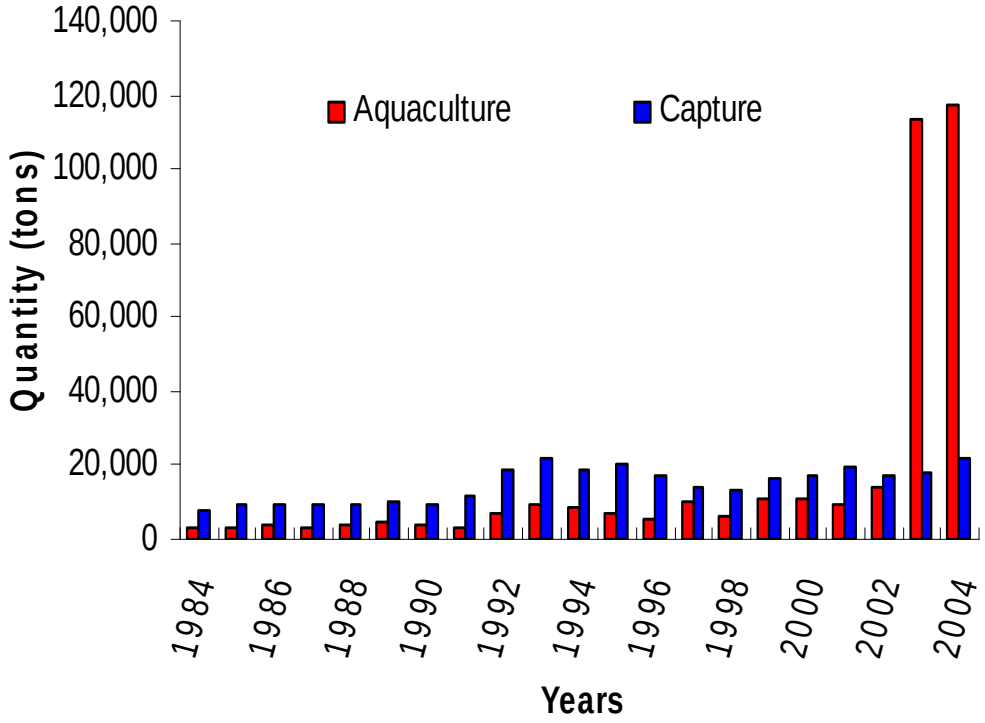

Figure 1.6 The global mud crab production from 1984 to 2004 (data source: http://www.fao.org/figis/servlet/species?fid=2637)

Attempts to establish a large commercial mud crab culture industry in Australia have so far not been very successful, mainly because of low yields associated with high levels of cannibalism, an expensive live food supply, and expensive labour harvesting operations (Lindner 2005).

1.2.6.5.2 Mud crab seed

Hatchery techniques for mud crabs have now improved significantly. In Vietnam, Nghia *et al.* (1998) reported 26% survival of larvae from zoea 1 to megalop stages, and 72% survival from megalop to the first crab stages. In Australia, hatchery production of mud crab seed has achieved a survival rate of 40% from zoea to megalop, and 50% from megalop to crab 1 (Cann & Shelley 1999). Success in producing artificial seed has also been reported in India (Marichamy & Rajapackiam 1992) and in Malaysia (Jamari 1992). It appears therefore that in the future the supply of hatchery-produced juveniles will not be a limiting factor for mud crab aquaculture development.

1.2.6.5.3 Mud crab culture system

Mud crabs have been cultured since the late nineteenth century in China (Yalin & Qingsheng 1994) and for about the last three decades in most South East Asian countries (Baliao *et al.* 1998), using seed mostly caught from the wild. Now that ample supplies of hatchery seed can be produced it is likely that mud crab culture will expand very rapidly.

Mud crabs are cultured using a variety of techniques including pond culture (Dat 1999; Johnston & Keenan 1999), pen culture (William & Abdullah 1999; Mwaluma 2002; Trino & Rodriguez 2002), and cage culture (Chandrasekaran & Perumal 1993; Dat 1999). The culture of mud crabs is generally divided in to two categories: (1)

grow-out and (2) fattening. For grow-out, the small juveniles are often cultured in an earthen pond or pen for up to one year till they reach to marketable sizes (Chandrasekaran & Perumal 1993). In contrast, the fattening method involves keeping "empty crabs" either from the wild or farms, and feeding them well for a short period (Dat 1999).

Mud crab aquaculture has a potential to produce a significant profit for growers. An economic analysis of mud crab monoculture showed that even if the value of mud crabs were to decrease by 28%, mud crab would still be economically viable (Agbayani *et al.* 1990). A comparison of brackish prawn and crab culture with saline-tolerant tilapia and prawn culture, freshwater carp culture and traditional rice systems suggested that mud crab farming is lower in capital investment and risk than shrimp farms (Salam *et al.* 2003). More recently, Sathiadhas and Najmudeen (2004) reported that the mud crab farming systems (fattening and culture) are more profitable than any other coastal aquaculture operations. Furthermore, during the last decade, outbreaks of white spot syndrome virus (WSSV) in shrimp have been the leading cause of production losses (Flegel 1997). The culture of mud crabs (*S. tranquebarica)* has been proven to be an alternative to prawn culture in areas affected by shrimp disease such as WSSV (Raghavulu *et al.* 1998).

1.2.6.6 Current constraints to mud crab culture

For mud crab aquaculture to progress more intensive research is needed on larval rearing techniques, nutrition, genetics and marketing (Macintosh & Tan 1999). This study focuses on nutritional aspects of the mud crabs *S. serrata* and *S. paramamosain.*

1.2.6.6.1 Use raw materials as feed for mud crab culture

Despite the rapid expansion of mud crab aquaculture throughout South East Asian countries, most culture systems are based on traditional methods which require unprocessed materials as feeds (Sugama & Yunus 1998): trash fish, clam meat, decayed vegetable and chicken offal (Baliao *et al.* 1998; Dau 1998; Marichamy & Rajapckkiam 1998; William & Fitzgerald 2002). Trash fish and clam meat have been shown to be suitable for mud crab culture but in some countries (e.g. Japan) the price of clam meat is higher than that of mud crab in the markets, so the practice is not economically viable (Shokita 1991). Similarly, trash fish may not always be available, particularly in the wet season, and there are extra costs such as electricity and a freezer necessary for storage (William & Abdullah 1999).

Using trash fish or undersized commercial species as feed could impact negatively on coastal fisheries and environmental sustainability (William & Fitzgerald 2002). Furthermore, uneaten raw materials like trash fish could cause water pollution and contribute to disease outbreaks (Thach 2003).

1.2.6.6.2 Using trash fish as feed in aquaculture in Vietnam

Although many countries ban or discourage the use of trash fish and invertebrates as a feed source (Tacon & Forster 2003), trash fish is still used widely as feed for carnivorous fish and crustacean species in Vietnam. A total of 0.93 million tons of trash fish from 100 marine species was used in 2001 (Edwards *et al.* 2004). The authors indicated that the quality of trash fish is usually poor because of inadequate preservation on board ship, in particular from offshore fisheries when vessels may be at sea for 1-6 weeks.

Although only a small part of the total catch of trash fish is used for mud crab culture in Vietnam, quality, availability, cost, and environmental impact should be considered. The development of a formulated feed would stimulate further development of mud crab aquaculture in both Vietnam and Australia.

1.2.7 The study of artificial feed development in mud crab

Research in Australia has shown that juveniles of *S. serrata* can utilise feeds formulated for *P. japonicus* (Vital and Ebi-star made by Higashimaru Company, Japan) (Shelley 2001; Ruscoe *et al.* 2004). However, the prices of Vital and Ebi-star feeds were considered to be too expensive (A$11 kg^{-1} and A$6 kg^{-1} respectively) to support commercial mud crab aquaculture development (Shelley 2001). Furthermore, Vital and Ebi-star feeds are too small in size for crabs to hold in their claws. Another study using two formulated diets and a mussel diet fed for juvenile mud crab (*S. serrata*) showed that the weight increase of crab fed the mussel diet was greater than that of crabs fed two other feeds (Cheong *et al.* 1991).

Specific requirements for a number of components in mud crab feed are now known. Dietary lipid levels for mud crab juveniles (*S. serrata)* have been found to range from 5.3% to 13.8% (Sheen & Wu 1999), while the dietary cholesterol requirement was approximately 0.51% (Sheen 2000). It has also been found that *S. serrata* grow well when fed with diets containing 32% or 40% protein, and 6% or 12 % lipid at energy levels between 14.7 MJ kg^{-1} and 17.6 MJ kg^{-1} (Catacutan 2002). As for essential fatty acids, Sheen and Wu (2002) reported that *S. serrata* juveniles showed significant improvement in weight gain when fed with a supplement of docosahexaenoic acid, arachidonic acid, and linolenic acid.

Apparent digestibility (AD) for nine ingredients, including fish, acetes spp., meat-bone, squid, copra, corn, and soy bean meals, bread flour, and rice bran have also been reported for *Scylla serrata* (Catacutan *et al.* 2003). This was the first step toward selecting ingredients for practical feed formulation.

Finally, there is some evidence that feeding young mud crabs with live feed is not beneficial in pond culture systems. Working in the Mekong Delta, Vietnam, Christensen *et al.* (2004) stocked 0.5 crab per m^2 of young *S. paramamosain* and *S. olivacea* in earthen ponds and fed with trash fish (mainly *Oreochromis mossambicus*) and mangrove sesarmid crabs (Ba khia in Vietnamese). They found no difference in growth between these two supplements and unfed controls after 115 days of feeding. This supports previous work which demonstrated that small crabs with a carapace width less than 70mm feed mainly on detritus (Prasad & Neelakantan 1988).

Chapter 2 General materials and methods

This chapter describes the general materials and methods (GMMs) for six experiments. There are three experiments on *S. serrata* conducted in Australia and three experiments on *S. paramamosain* carried out in Vietnam. Details of the analytical methods are placed in appendices for ease of reading.

2.1 LOCATIONS

2.1.1 Darwin Aquaculture Centre (DAC)

Three experiments were conducted at the Darwin Aquaculture Centre (Department of Primary Industries and Fisheries, Northern Territory, Australia) from February 2003 to July 2004.

The Darwin Aquaculture Centre (DAC) is located at Channel Island in Darwin Harbour (12 ^{0}.42$^{'}$ S and 130 ^{0}.88$^{'}$ E), Northern Territory, Australia (Figure 2.1).

2.1.2 Bac Lieu Experimental Station for Aquaculture (BLESA)

Three experiments were performed at the Bac Lieu Experimental Station for Aquaculture, a branch of the Research Institute for Aquaculture No 2 (Ministry of Fisheries, Vietnam) from October 2004 to May 2006.

The Bac Lieu Experimental Station for Aquaculture (BLESA) is located at Nha Mat Ward, Bac Lieu Town, Bac Lieu Province (105 ^{0}43' E and 09 ^{0}17' N), in the Southern Vietnam (Figure 2.2).

Figure 2.1 Location of Darwin Aquaculture Centre (DAC) in the top end of Australia

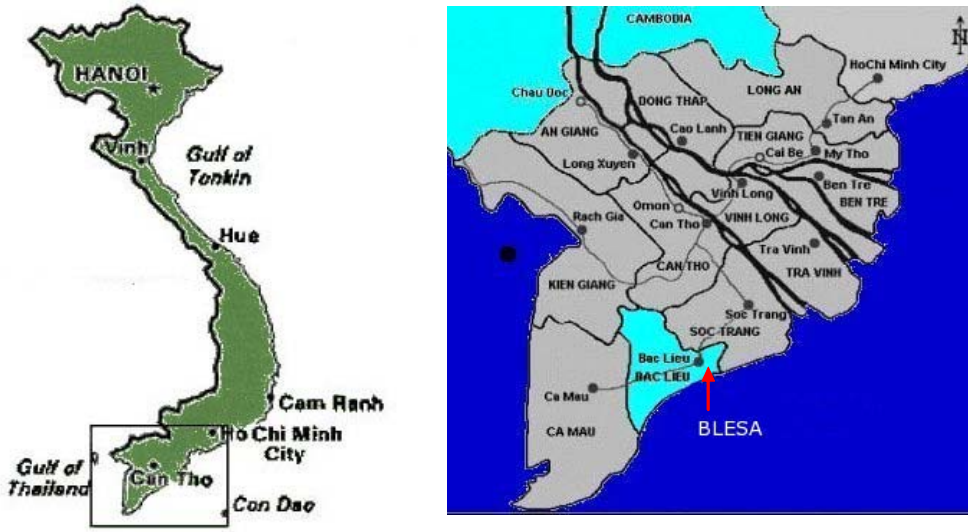

Figure 2.2 Location of Bac Lieu Experimental Station for Aquaculture (BLESA) in Mekong Delta, Vietnam

2.2 EXPERIMENTAL DESIGN

All experiments were carried out in indoor laboratories. The experiments were designed with a randomised complete design (RCD), except for one trial described in Chapter 5, which was set up with a randomised completed block design (RCB), according to Gomez and Gomez (1984) and Zar (1999).

2.3 CULTURE SYSTEM

All experiments were carried out at the indoor systems with no access to natural food.

A flow-through system was used for the experiments conducted at the DAC (Figure 2.3). Water flow through rate was set at 8 L $hour^{-1}tank^{-1}$ with one dripper (Irrigation Warehouse Group Pty Ltd., NSW, Australia). The detailed culture system of each experiment is given in each Chapter (Section 3.1 of Chapter 3, Chapter 4, and Chapter 5).

The experiments carried out at the BLESA used a recirculation culture system. The waste water was reused after passing through a bio-filter system (Figure 2.4). The recirculated water entered each tank via two drippers at 16 L $hour^{-1}tank^{-1}$. The detailed culture system of each experiment is presented in Section 2 of Chapter 3, Chapter 6, and Chapter 7.

2.4 WATER PREPARATION

For the experiments at the DAC, water supply was the same source as that used for finfish and crustacean hatcheries of the DAC. Water was pumped from the sea through a series of filters: a pro-filter (Spin Klin with filter disc of 200 μm), a sand filter system (Automatic Backwash- Netafim- with 5 μm in diameter). It was then

continuously filtered by a slow sand filter before being used in the experimental system.

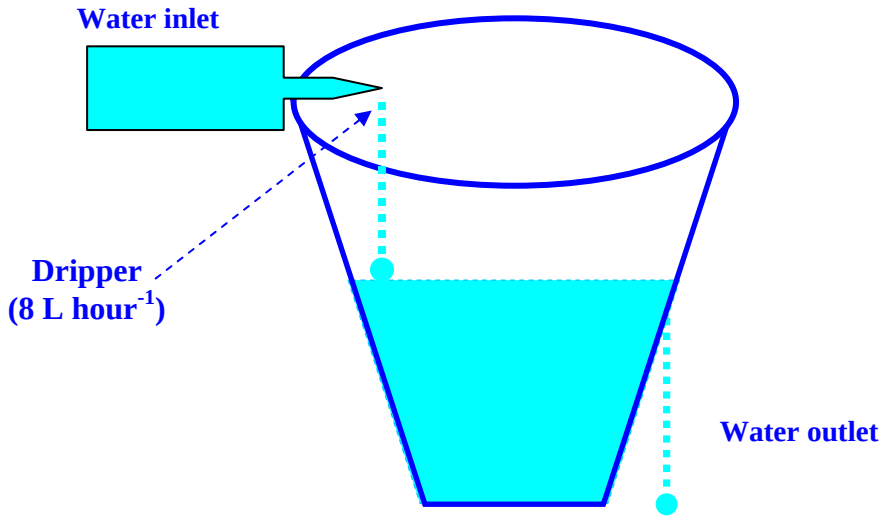

Figure 2.3 A flow through culture system at the DAC

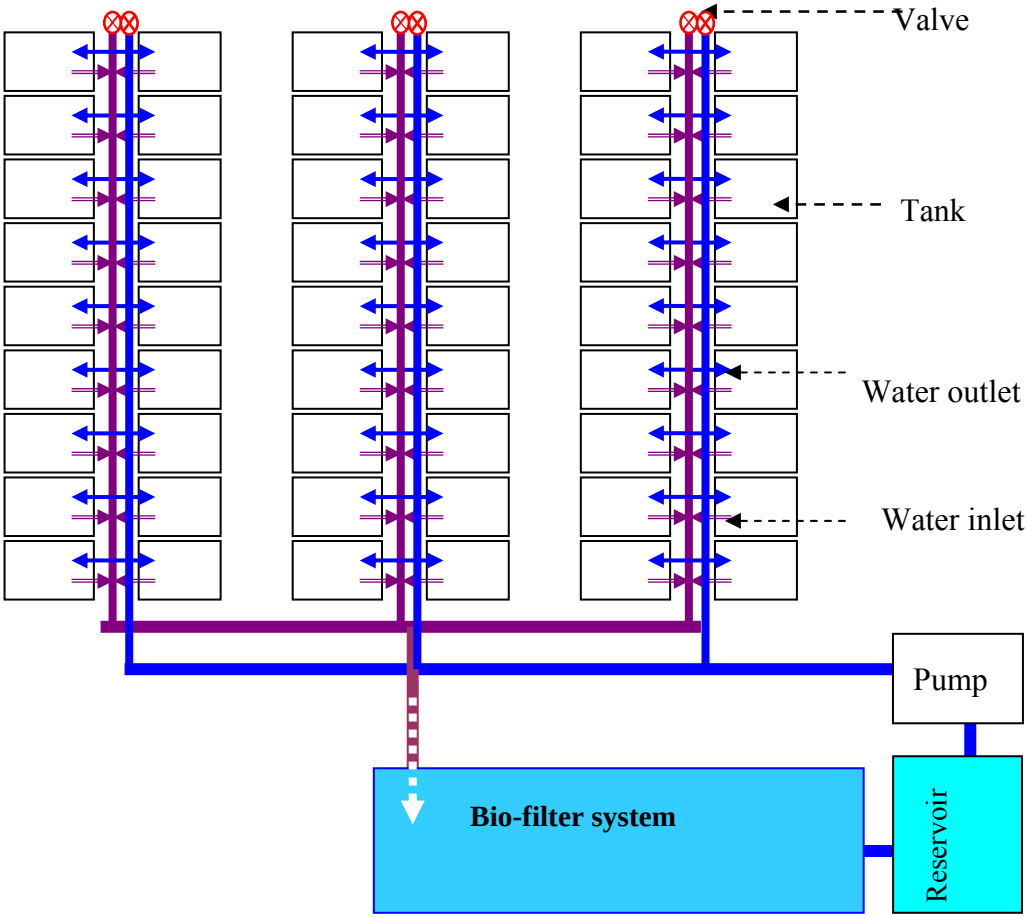

Figure 2.4 A recirculation culture system for three experiments at the BLESA

For the experiments at the BLESA, water was prepared using a similar procedure of that for prawn and mud crab hatcheries of the BLESA. Seawater was collected into a settling reservoir by tidal movements at high spring tide, and allowed to settle for 10 days before the water was pumped into 30 m^3-tank where particulate matter was allowed to settle for a further three days using 80 mg L^{-1} dolomite (CP group, Thailand). After that the water was treated with 1 mg L^{-1} of potassium permanganate ($KMnO_4$) for two days to oxidize organic matter, after which it was pumped to another tank and chlorinated with 35 ppm chlorine powder (70 % of pure chlorine) and aerated vigorously. Three days after being chlorinated, the water was filtered with a sand gradient filter and supplied to the experimental system.

2.5 WATER QUALITY MEASUREMENT

For the experiments conducted at the DAC, four water quality parameters were recorded daily at 9:00-10:00 hours. Dissolved oxygen (DO) was measured with an OxyGuard H01G-Handy Gamma (OxyGuard International, Birkerd, Denmark). Water temperature and pH were recorded with a WP-90 meter (TPS, Brisbane, Australia), and salinity was measured with a refractometer, Atago, model S10-E (Tokyo, Japan).

For the experiments carried out at the BLESA, three water quality parameters were measured daily at 8:00-9:00 hours. Dissolved oxygen was measured with a Hi-9142 D.O. meter (Hanna Instrument Ltd., Leighton Buzzard, UK). Temperature was measured with a hand-held thermometer (Yancheng Diling Medical Instruments Co. Ltd., China), and pH was measured with a Scan 2 pH meter (Eutech Instruments

Private Ltd, Singapore). Salinity measurements were made at weekly intervals with a refractometer (model S10-E, Atago, Tokyo, Japan).

2.6 MAJOR STEPS OF DIET PREPARATION

The main steps of diet preparation are outlined in Figure 2.5. First of all, some ingredients (fish meal, shrimp meal, acetes shrimp meal, shrimp head meal, peanut oil cake meal, coconut oil cake meal, and rice bran were passed through a 0.45 mm mesh sieve. Some parts of these ingredients can not pass through the sieve which were grinded and screened repeatedly using a meat grinder. A rest of hard and large particle were discarded. After that, all dry components were mixed together to form a dry pre-mixture. Then this dry pre-mixture was mixed with the liquid ingredients (such as fish oil) to form a pre-mixture. A binder (alginic acid or vital wheat gluten) was diluted in hot water (60-80 ^{o}C) to form a viscous solution which was mixed with the pre-mixture to form a pre-moist mixture. This mixture was mixed by extruded through the kitchen mincer (Meat grinder, model No: MG-80, Gali) three times. Then, the final moist-mixture was squeezed into moist pellets of 10-30 cm length and 1.0-3.0 mm diameter, and then dried at 35-40 ^{o}C in an oven. When moisture contents were less than 15 %, the pellets were stored at a -20 ^{o}C freezer until required.

2.7 SAMPLE PREPARATION FOR CHEMICAL ANALYSIS

The wet samples from the experiments conducted at the DAC were dried with a freeze-drier (RVT400-240, Savant Instrument Inc., Farmingdale, NY, USA). The procedures were similar to those steps of dry matter determination (see details in Section 2.8.1). The dried samples were prepared for further analysis, including chromic oxide, crude protein, gross energy, lipid, or carbohydrate.

Similarly, the wet samples from the experiments carried out at the BLESA were dried with a freeze-dryer (model TFD 5505, *ilShin* Lab Co. Ltd., Gyeonggi, South Korea). These samples were prepared for further analysis including chromic oxide, crude protein, crude fat, or ash.

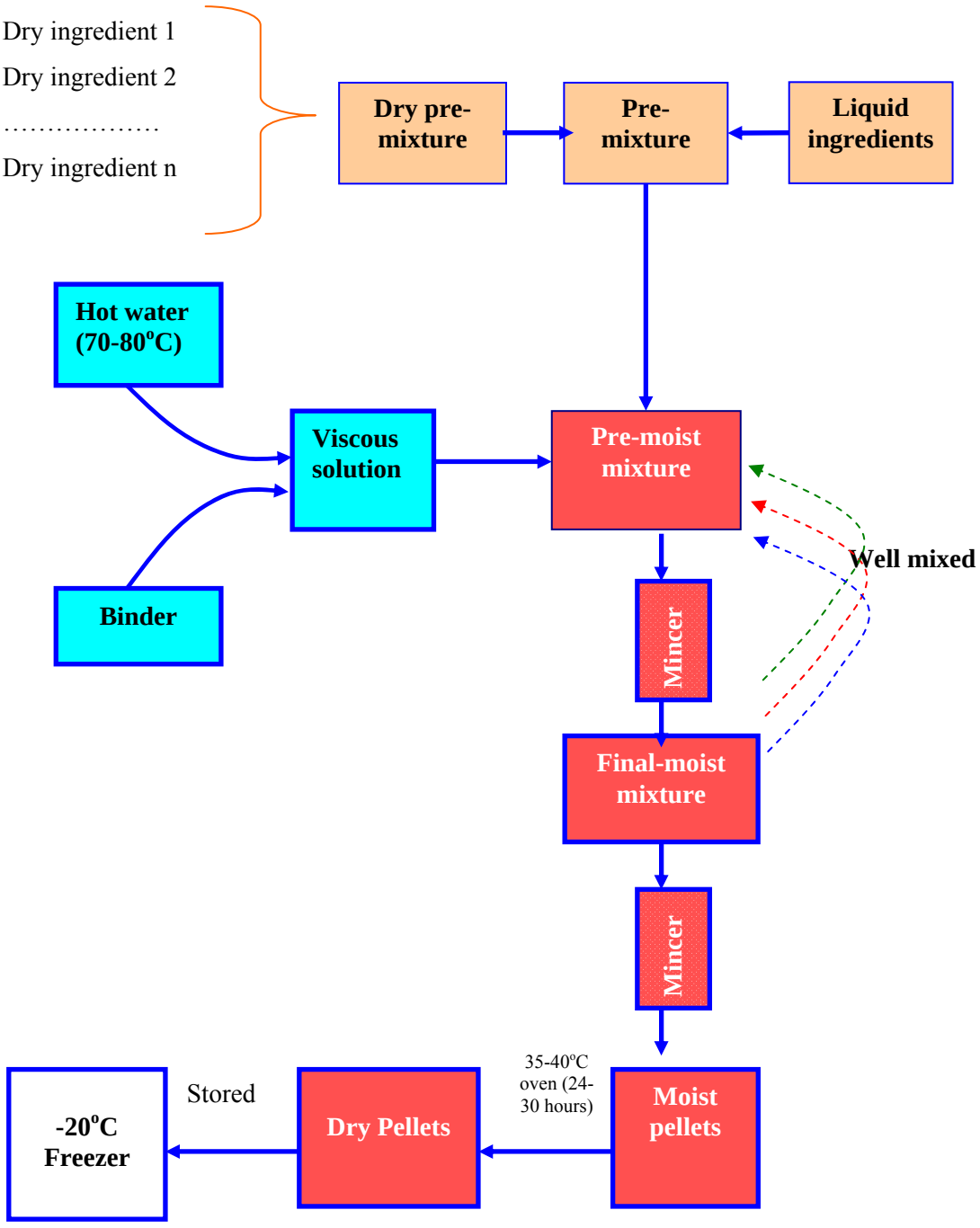

Figure 2.5 The main steps of the laboratory pellet feed preparation

2.8 CHEMICAL ANALYSIS

During a course of study, 997 samples were chemically analyzed for dry matter, gross energy, chromic oxide, crude protein, crude lipid, ash, carbohydrate, fatty acids, and amino acids. A number of analyses for dry matter, chromic oxide, gross energy, crude protein, crude lipid, carbohydrate, ash, fatty acids, and amino acids were 1392 (464 samples x triplicates), 300 (100 samples x triplicates), 88 (44 samples x duplicate), 478 (158 samples x triplicates), 348 (116 samples x triplicates), 108 (36 samples x triplicates), 207 (69 samples x triplicates), 24 (8 samples x triplicates), and 6 (2 samples x triplicates), respectively.

Since budget spent for chemical analysis is limited, some data values were taken from the published paper and some values were calculated basically on approximate analysis for individual ingredient.

2.8.1 Dry matter determination

The dry matter content of sample from experiments conducted at the DAC was determined using a freeze-drying method. The materials (feed ingredients, feed pellets, uneaten feeds, or faeces) were put into each vial and weighted with a Sartorius A200S electronic balance (accuracy to 0.0001 g). The vials including material were covered with ply of tissue paper to avoid loss of material and kept frozen in a -20 ^{o}C freezer. After being frozen, the vials were arranged in the freeze-drier, model number: RVT400-240 (Savant Instrument Inc., Farmingdale, NY, USA) for drying until constant weight. The samples were dried until constant weight (from 48 to 72 hours). Dry matter was determined as follows:

$$\text{Dry matter } (\%) = \frac{\text{Constant weight (g) x 100}}{\text{Final weight (g)} - \text{Vital weight (g)}}$$

The dry matter of the samples from the experiments conducted at the BLESA was determined by the Research Institute for Aquaculture No2 (RIA2), in Ho Chi Minh City (HCM City), Vietnam. The samples were dried with a 105 °C oven model: IM 200 (Memmert, Germany) until constant weight. The ratio between final and initial weight is the dry matter content (%).

$$\text{Dry matter } (\%) = \frac{\text{Final weight (mg)}}{\text{Initial weight (mg)}} \text{ x } 100$$

2.8.2 Crude protein determination

The nitrogen content of the sample from the experiments conducted at the DAC was determined using the Kjeldahl method with a Lachat 8000 (Lachat Instruments Milwaukee, WI, USA) as described by Diamond (1992).

Nitrogen content of the samples from the experiments carried out at the BLESA was determined by the RIA 2 (HCM, Vietnam) using the Kjeldahl method described by AOAC International (1996). The sample was digested with concentrated sulphuric acid and catalysts ($CuSO_4$ and K_2SO_4) for converting nitrogen to ammonia (NH_3) which is distilled and titrated with standard NaOH solution. Both distillation and titration were performed with a semi-automatic steam distilling unit (model UDK 132, Velp, Italy).

Crude protein content of the samples was estimated by multiplying its nitrogen content by 6.25. The details of above crude protein analytical methods are given in Appendix 2.1 and Appendix 2.2.

2.8.3 Lipid determination

Total lipid content of the samples from the experiments conducted at the DAC was determined gravimetrically using the method described by Mason and Nell (1995). Total lipid in the diets and crab tissues (in powder form) was extracted with chloroform: methanol (2:1) solvent. The lipid extract was collected in a beaker and after the solvent was evaporated completely, the remaining total lipid was dried and weighed. Details of the method are presented in Appendix 2.3.

Crude lipid content of the samples from the experiments conducted at the BLESA was analysed by the RIA2 using the method provided by AOAC International (1996). The crude fat was extracted using petroleum ether (Merk) and a Soxhlet extraction apparatus (model EV 14, Gerhardt, Germany). Solvent was passed through the sample to extract the crude fat. The crude fat was determined gravimetrically after the solvent was evaporated. The details of the method are presented in Appendix 2.4.

2.8.4 Ash determination

The samples were incinerated in a furnace (model L5/12/B170, Nabertherm, Germany) at 500 °C for 12 hours. Ash content was considered as the total inorganic content of the sample and calculated based on ratio between final weight and initial weight.

$$\text{Ash} \quad (\%) = \frac{\text{Ash weight (mg)}}{\text{Sample weight (mg)}} \text{ x } 100$$

2.8.5 Nitrogen Free Extract (NFE)

Nitrogen free extract (carbohydrate and fibre) was calculated (on dry matter basis) as follows:

NFE (%) = 100 – (Protein + crude fat + ash)

with protein, crude fat and ash expressed as percentages.

2.8.6 Carbohydrate determination

Soluble carbohydrates were extracted with the method described by Kochert (1978). The samples were extracted with H_2SO_4, and then the carbohydrate was determined with the phenol-sulfuric acid spectrophotometric method. The sample, standard, and blank solutions were measured at 485nm with a ultra-violet visible spectrophotometer (model U-1100, Hitachi, Tokyo, Japan) and calculated based on the calibration graph of the standard glucose concentrations. Details of this method are presented in Appendix 2.5.

2.8.7 Gross energy determination

Gross energy of feed pellets and faeces was determined by the South Australian Research and Development Institute (SARDI) using a Parr 1281 bomb calorimeter (Parr Instrument Co., Illinois, USA). When completed, the calorific value was displayed in MJ kg^{-1} on the screen. Details of this method are provided in Appendix 2.6.

2.8.8 Chromic oxide determination

Chromic oxide (Cr_2O_3) content of the samples from the experiment carried out at the DAC was determined by the Queensland University of Technology (QUT) using the method described by Furukawa and Tsukahara (1966). The chromic oxide was digested with nitric and perchloric acids, and the concentrations of chromium in the extract were estimated using a Liberty 200 ICP spectrometer (Varian, Inc., CA, USA) at 350 nm after adjusting to zero for the blank solution. The blank were

prepared with acids and distilled water at the same time as the samples. Details procedures are presented in Appendix 2.7.

Chromic oxide content in the sample from the experiment conducted at the BLESA was analysed by the Can Tho University (Can Tho City, Vietnam) using the method described by Temminghoff (2000). The concentrations of chromium were measured with an atomic absorption spectrophotometer (model: Z5000, Hitachi, Tokyo, Japan) at 359.3 nm. Details of this method are provided in Appendix 2.8.

2.8.9 Fatty acid analysis

The fatty acid profiles of dietary ingredients were quantitatively determined by the Can Tho University (Can Tho City, Vietnam) using the method described by AOAC International (2000) with a gas chromatograph (model: GC-14, Shimadzu Co., Kyoto, Japan).

2.8.10 Amino acid analysis

The amino acid profiles of dietary ingredients were quantitatively determined by the Can Tho University (Can Tho City, Vietnam) using the method described by AOAC International (2000) with a Biochrom 20 Plus amino acid analyzer (Pharmacia LKB Biochrom Ltd., Cambridge, England).

2.9 CALCULATIONS OF APPARENT DIGESTIBILITY (AD)

The AD for dry matter, nutrients or energy of test and reference diets was calculated according to De Silva and Anderson (1995). The data was on a dry matter basis.

- AD for dry matter (ADD)

$$\text{ADCD}\,(\%) = 100 - 100 \text{ x } \frac{\%\,\text{Marker in diet}}{\%\,\text{Marker in faeces}}$$

- AD for nutrients or energy

$$ADC_{Nutrients\ or\ energy}\ (\%) = 100 - 100\ x\ \frac{\%\ Marker\ in\ diet}{\%\ Marker\ in\ faeces}\ x\ \frac{F}{D}$$

where F is the percent of nutrient or energy in faeces, D is the percent of nutrient or energy in diet,

- The apparent digestibility of test ingredient (AD_{ingr}) was determined using the equation was recommended by Forster (1999).

$$ADC_{ingr}\ (\%) = \frac{(a + b)\ x\ ADC_{com} - a\ x\ ADC_{ref}}{b}$$

where a = nutrient contribution of reference diet to nutrient content of combined diet, = (level of nutrient in reference diet) x (100 – i); i is the level of test ingredient in combined diet (%); b = nutrient contribution of test ingredient to nutrient content of combined diet, = (level of nutrient in test ingredient) x i; (a + b) is the level of nutrient in combined diet (%). AD_{ingr} is the apparent digestibility for the test ingredient, AD_{cm} is the apparent digestibility for the combined diet, and AD_{ref} is the apparent digestibility for the reference diet.

2.10 GROWTH AND FEED UTILISATION

Weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio were calculated according to Tacon (1990a)

Weight gain (WG, %)

$$WG\ (\%) = \frac{W_f - W_i}{W_i}\ x\ 100$$

Specific growth rate (SGR, % day^{-1})

$$SGR\ (\%\ day^{-1}) = 100 * \frac{LnW_f - LnW_i}{T}$$

Feed conversion ratio (FCR)

$$FCR = \frac{D_f}{WG}$$

Protein efficiency ratio (PER)

$$PER = \frac{\text{Wet weight gain (g)}}{\text{Amount of dry protein in diets (g)}}$$

where W_i is the initial wet weight (g), W_f is the final wet weight (g), T is the time in days between measurements of W_i and W_f, and D_f is the real dry feed intake (g) determined as total feed intake (g) minus uneaten feed (g).

The carapace width increase (CWI) was calculated as below

$$CWI\,(\%) = \frac{CW_f - CW_i}{CW_i} \text{ x } 100$$

where CW_i is initial carapace width, CW_f is the final carapace width,

Inter-moult period (IMP) is a total period (days) between initial moult and final moult.

Chapter 3 Apparent digestibility of *Scylla* spp. for common feed ingredients

The determination of apparent digestibility (AD) by animals for specific ingredients is important because it is fundamental to evaluate the quality of ingredients for the formulation of feeds with low cost and minimum wastage (see details in Section 1.2.3 of Chapter 1). This chapter describes two experiments, one to determine AD for 7 ingredients by *S. serrata* in Australia (Section 3.1), and a second to obtain the AD for 13 ingredients by *S. paramamosain* in Vietnam (Section 3.2). The findings in the first section have been published in the journal Aquaculture Research Vol. 37, pp. 359-365, 2006 (reprint attached).

3.1 AD OF SOME FEED INGREDIENTS BY *S. serrata* IN AUSTRALIA

3.1.1 Introduction

Determining nutrient digestibility is an important element for diet formulation (De Silva & Anderson 1995). Recently, the AD for dry matter and protein of nine feedstuffs for the mud crab *Scylla serrata* were determined (Catacutan *et al.* 2003) but AD for energy (ADE) were not reported. In addition, cellulose has been used as a filler in a number of mud crab diets (Sheen & Wu 1999; Sheen 2000; Catacutan 2002), although Rutledge (1999) and Pavasovic *et al.* (2004) demonstrated that mud crabs possess cellulase, indicating that they may be able to use cellulose as a feed source. This study was undertaken to investigate the AD of a range of feedstuffs, including cellulose, fish meal, shrimp meal, blood meal, defatted soybean meal, wheat flour, and cod liver oil to further refine the diet of *S. serrata*. The specific aims of this study are:

- To determine apparent digestibility (AD) for dry matter (ADD) and for energy (ADE) of Australian fish meal, shrimp meal, blood meal, defatted soybean meal, wheat flour, cellulose, and cod liver oil.
- To determine AD for protein (ADP) of Australian fish meal, shrimp meal, blood meal, defatted soybean meal, and wheat flour.

3.1.2 Materials and methods

The experiment was carried out at Darwin Aquaculture Centre, Northern Territory, Australia (Section 2.1.1 of Chapter 2) from 11th February 2003 to 25th March 2003 with juvenile mud crabs (crablets) of 95.65 ± 2.17 g, sourced from one batch of hatchery-reared individuals. Forty juveniles were fed Vital Feed® for ten days to acclimate to experimental conditions. Vital Feed® is a commercial feed for *Penaeus japonicus*, appears to be assimilated well by mud crabs, *S. serrata* (Shelley 2001; Ruscoe *et al.* 2004). Vital Feed®contained 15.5 % lipid and 52.7 % protein (analysed values).

3.1.2.1 Culture system

The general description of culture system, water preparation, and water managements are presented in Sections 2.3, 2.4, and 2.5 of Chapter 2. Forty round tanks of 60 L each were used to hold animals individually. A flow-through system supplied filtered seawater at a rate of 8 L hour^{-1}tank^{-1}, with a water depth of 300 mm in all tanks. During the experimental period, temperature, salinity, pH and dissolved oxygen of the water were 28.75 ± 0.72 ^{o}C, 26.00 ± 1.26 g L^{-1}, 7.50 ± 0.06, and 4.10 ± 0.29 mg L^{-1} respectively.

3.1.2.2 Source of ingredients

The sources of all ingredients are listed in Table 3.1.

Table 3.1 The source of test ingredients, reference diet, and inert marker

Items	Source
Vital feed®	Higashimaru Company, Kagoshima, Japan
Alginic acid	Sigma-Aldrich, Inc., MO, USA
Chromic oxide (Purity of 99.8%)	Ace chemical company, South Australia, Australia
Australian fish meal (fish meal)	Skretting Company, Tasmania, Australia.
Danish whole shrimp meal (shrimp meal)	Ridley AgriProducts, Victoria, Australia
Australian ring dried blood meal (blood meal)	As above
Alpha cellulose (cellulose)	As above
Defatted dehulled soybean meal (soy bean meal)	Lowan Whole Foods, Victoria, Australia
Bread wheat flour (wheat flour)	Goodman Fielder, North Ryde, New South Wales, Australia
Cod liver oil	Healtheries of New Zealand, Auckland, New Zealand

3.1.2.3 Experimental diets

Eight diets were formulated (Table 3.2), including one reference diet (T1) and seven test diets (T2-T8). Each of the six test diets (T2-T7) contained approximately 70 % reference diet and 30 % test ingredients (fish meal, shrimp meal, blood meal, soybean meal, wheat flour, and cellulose), the recommended ratio reported by De Silva and Anderson (1995). The small deviations from the ratio of 70: 30 in test diets T2-T7 (Table 3.2) were due to the difference in moisture contents of the ingredients. The last test diet (T8) contained approximately 78.2 % reference diet and 21.8 % test ingredient (cod liver oil). The deviation of T8 from the recommended 70: 30 ratio was due to the high lipid content of cod liver oil, which had to be limited in the diet (Allan *et al.* 1999a; Gunasekera *et al.* 2002). Chromic oxide was added at 5 g kg^{-1} to both reference and test diets as an inert marker. The reference diet was prepared using Vital Feed® (965 g kg^{-1}) and alginic acid (30 g kg^{-1}).

Table 3.2 Composition of reference diet (T1) and test diets (T2 - T8) in dry matter basis (g kg^{-1} dry weight)

Ingredients	Experimental diets							
	T1	T2	T3	T4	T5	T6	T7	T8
Vital feed	965.0	687.9	688.6	688.1	680.6	691.6	685.3	754.5
Alginic acid	30.0	21.4	21.4	21.4	21.2	21.5	21.3	23.5
Chromic oxide	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Fish meal	-	285.7	-	-	-	-	-	-
Shrimp meal	-	-	285.0	-	-	-	-	-
Blood meal	-	-	-	285.5	-	-	-	-
Soybean meal	-	-	-	-	293.2	-	-	-
Wheat flour	-	-	-	-	-	281.9	-	-
Alpha cellulose	-	-	-	-	-	-	288.4	-
Cod liver oil	-	-	-	-	-	-	-	217.0

Dash (-): zero

3.1.2.4 Diet preparation

Diet preparation was carried out as described in Section 2.6 of Chapter 2. After being mixed with water, the moist mixture was pelleted into 3 mm diameter and 30 mm length. The pellets were then dried at 40 ^{o}C until the moisture content was less than 15 %. Dried diets were stored at a -20 ^{o}C freezer until required.

3.1.2.5 Feeding and faecal collection

Experimental animals were fed the experimental diets for one week prior to the first faecal collection as recommended by Lee and Lawrence (1997). They were fed twice a day (at 08:00 and 16:00 hours on working days) and once a day (at 08:00 on the weekend). Two 2 hours after feeding at 8:00 hours, all uneaten feed were removed. Tweezers were used for faecal collection (Catacutan *et al.* 2003) (Figure 3.1). The faeces of each replicate were collected daily between 10:00 and 16:00, filtered through a 30 μm mesh, rinsed gently with distilled water, pooled and stored at a -20 ^{o}C freezer until having enough for analysis required.

Figure 3.1 Using tweezers to collect mud crab faeces

3.1.2.6 Chemical analyses

The methods of chemical analysis are described in Section 2.8 of Chapter 2. Dry matter was determined using the freeze-drying method. Gross energy analyses were performed by the South Australian Research and Development Institute (SARDI) using a Parr 1281 bomb calorimeter (Parr Instrument Co., Illinois, 2002) in duplicate. Nitrogen was determined using the Kjeldahl method with Lachat Instruments (Diamond 1992), and crude protein was calculated by multiplying nitrogen content by 6.25. Chromic oxide (Cr_2O_3) was determined by the Queensland University of Technology (QUT) using the method described by Furukawa and Tsukahara (1966). Because cellulose and cod liver oil do not contain protein, the determination of ADP for both of these ingredients was not needed. Therefore, two experimental diets (T7 and T8) were not analysed for protein. The compositions of diets and test ingredients including dry matter, energy, and protein are shown in Table 3.3. The chromic oxide

contents of diets and faeces are presented in Appendix 3.1. All samples were analysed in triplicate for dry matter and crude protein.

Table 3.3 Dry matter (DM), gross energy (GE) and crude protein (CP) of experimental diets and test ingredients used in the formulation of the diets (data are expressed as means ± standard error (n=3), except GE value from n=2)

Sources	DM (%)	GE* (MJ/kg)	CP* (%)
Experimental diets			
T1	92.06 ± 0.52	18.85 ± 0.00	50.39 ± 0.39
T2	89.11 ± 0.48	19.65 ± 0.03	57.94 ± 1.03
T3	87.34 ± 0.64	19.45 ± 0.04	56.14 ± 0.32
T4	90.55 ± 0.46	20.07 ± 0.03	61.05 ± 1.48
T5	95.16 ± 0.45	19.27 ± 0.01	47.47 ± 0.13
T6	91.07 ± 0.69	18.60 ± 0.02	39.37 ± 0.43
T7	94.47 ± 0.01	17.86 ± 0.11	-
T8	94.47 ± 0.07	23.35 ± 0.00	-
Test ingredients			
Fish meal	92.82 ± 0.34	21.64 ± 0.12	76.66 ± 4.54
Shrimp meal	92.53 ± 0.45	20.93 ± 0.16	70.46 ± 0.14
Blood meal	92.76 ± 0.46	23.09 ± 0.11	87.52 ± 4.20
Soybean meal	96.29 ± 0.54	20.26 ± 0.05	40.49 ± 1.36
Wheat flour	91.12 ± 0.86	17.95 ± 0.06	11.48 ± 0.54
Cellulose	94.05 ± 0.30	15.44 ± 0.37	-
Cod live oil	100.00 ± 0.0	39.47 ± 0.03	-

*: the values were expressed in dry matter basis; -: not analysed

3.1.2.7 Calculations

The apparent digestibility (AD) for dry matter, protein or energy of test and reference diets was calculated with the equations reported by De Silva and Anderson (1995). The apparent digestibility of test ingredient (AD_{ingr}) was determined using the equation descibed by Forster (1999). These equations are presented in Section 2.9 of Chapter 2.

3.1.2.8 Experimental design

Eight treatments were initially set up in a randomised complete design (RCD) with 5 replicates assigned to each treatment (Gomez & Gomez 1984). Each replicate had one crab and faecal samples from each replicate were daily collected and pooled until having enough sample for analysis required (Castell 1989; D'Abramo & Castell 1997). During the course of the experiment, a few replicates were lost because some mud crabs died whilst moulting, but the treatment was not affected by the loss of these replicates. The lowest number of replicates was 3 in treatment T6 (see Table 3.4). Therefore the experiment data were analysed as an unequal replication design.

3.1.2.9 Statistical analysis

Data expressed as a percentage were arcsine transformed prior to performing a one-way ANOVA. Tukey HSD test was applied to determine significant differences among treatments *(P*< 0.05). The data were processed using *STATISTICA 7* (StatSoft, Inc., OK, USA, 2004).

3.1.3 Results

The apparent digestibility for dry matter (ADD), for energy (ADE), and for protein (ADP) of test ingredients were in the range of 70.0-95.7%, 77.4-97.1%, and 57.7-97.9% respectively (Table 3.4). For ease of reading, the values of AD for experimental diets are given in Appendix 3.2.

Table 3.4 Apparent digestibility for dry matter (ADD), energy (ADE) and protein (ADP) in juvenile mud crab for test ingredients (data was expressed in mean ± SE with n = replicates in parentheses)*

Test ingredients	ADD (%)	ADE (%)	ADP (%)
Fish meal	85.7 ± 1.2^{d} (4)	91.5 ± 1.3^{de} (4)	95.0 ± 0.9^{bc} (4)
Shrimp meal	78.9 ± 1.9^{bcd} (4)	84.4 ± 0.2^{bc} (4)	91.9 ± 0.6^{b} (4)
Blood meal	84.8 ± 0.6^{cd} (5)	85.8 ± 1.3^{bcd} (4)	93.5 ± 0.6^{b} (5)
Defatted soybean meal	95.7 ± 1.3^{e} (5)	97.1 ± 1.0^{e} (5)	97.9 ± 0.9^{c} (5)
Wheat flour	70.0 ± 0.6^{a} (3)	79.9 ± 1.1^{ab} (3)	57.7 ± 2.3^{a} (3)
Alpha cellulose	78.0 ± 1.5^{b} (5)	77.4 ± 2.2^{a} (5)	nd
Cod live oil	78.4 ± 2.4^{bc} (4)	89.2 ± 1.1^{cd} (4)	nd

* Means within the same column having a similar superscript letter are not significantly different at 5% of significance (P>0.05); nd = not determined

3.1.3.1 Apparent digestibility for dry matter

Table 3.4 shows that soybean meal had the highest ADD (95.7 %) while wheat flour had the lowest value (70.0%) ($P < 0.05$). The ADD values of cellulose (78.0 %), cod liver oil (78.4 %), and shrimp meal (78.9 %) were not significantly different ($P \geq 0.05$). In addition, the ADD of blood meal (84.8 %) did not differ significantly from that of cod liver oil (78.4 %) and shrimp meal (79 %) ($P \geq 0.05$). By contrast, the ADD of blood meal was higher than that of cellulose (78.0 %) or wheat flour (P<0.05). The ADD of fish meal (85.7 %) was not different from that of shrimp or blood meal. Otherwise, fish meal digestibility was significantly greater than those of cod liver oil, cellulose or wheat flour (P<0.05).

3.1.3.2 Apparent digestibility for energy

In Table 3.4, although defatted soybean meal and fish meal had similar ADE, only defatted soybean meal ADE (97.1 %) was significantly higher than all the other ingredients ($P < 0.05$). The ADE of fish meal (91.5 %) was not different from blood meal (86.9 %) or cod liver oil (89.2 %), but significantly higher than those of shrimp

meal, wheat flour, and cellulose ($P < 0.05$). Similarly, the ADE of blood meal, cod liver oil, and shrimp meal (84.5 %), did not differ significantly ($P \geq 0.05$). The ADE of wheat flour and cellulose was significantly lower than that of cod liver oil ($P < 0.05$), whereas the ADE of blood meal, shrimp meal, and wheat flour were similar ($P \geq 0.05$). In particular, wheat flour and cellulose had similar ADE values of 79.9 % and 77.4 % ($P \geq 0.05$) respectively.

3.1.3.3 Apparent digestibility for protein

Whilst high ADP values for defatted soybean meal (97.9 %), fish meal (95.0 %), blood meal (93.5 %), and shrimp meal (91.9 %) were measured, wheat flour (57.7 %) was significantly lower ($P < 0.05$). The ADP of defatted soybean meal and fish meal were not significantly different ($P \geq 0.05$), but the ADP of soybean meal was significantly higher that that of shrimp and blood meals ($P < 0.05$). There was no significant difference in the ADP of fish, shrimp, or blood meal ($P \geq 0.05$).

3.1.4 Discussion

Cellulose is thought to be indigestible and therefore of little nutritional value in formulated fish or crustacean diets. As a result, cellulose has been used as a filler in a number of crab diets (Mu *et al.* 1998; Sheen & Wu 1999; Sheen 2000; Catacutan 2002). However, this study showed that crablets could digest cellulose. A number of studies have shown that crustacean species possess an endogenous cellulase. The endogenous cellulase activities have been recorded from crayfish, *Cherax quadricarinatus*, (Xue *et al.* 1999), in mud crab *S. serrata*, (Rutledge 1999; Pavasovic *et al.* 2004), and in white shrimp *L. vannamei* (Moss *et al.* 2001). Secretions exhibiting cellulase activity from several crustacean species explain how they can digest natural cellulose sources. For example, white shrimp *Penaeus*

indicus, can assimilate decomposing mangrove leaves (Athithan & Ramadhas 2000), the freshwater prawn *Macrobrachium rosenbergii* can digest approximately 80 % of the cellulose in its feed (Gonzalez-Pena *et al.* 2002), and mud crabs can digest 90-97 % of the fibre in their diets (Catacutan *et al.* 2003). However, cellulose reduces protein digestibility in *Macrobrachium rosenbergii* (Gonzalez-Pena *et al.* 2002). Therefore, cellulose should be considered to include in a formulated diet for *S. serrata*.

High ADE values for fish, soybean and blood meals, as well as cod liver oil, were demonstrated in this study. The high ADE values of these ingredients showed that they can be a good nutritional source for mud crabs. Fish, soybean, and blood meals are known to be good sources of essential amino acids, energy, and fatty acid (Lovell 1998). Cod liver oil, investigated in this study, is another excellent source of essential fatty acids (Merican & Shim 1994; Glencross & Smith 2001). Similar results have been found for other crustaceans. The ADE for fish meal and soybean meal were found to be 82.5 % and 83.1 % respectively for the crayfish *Procambarus clarkii* (Reigh *et al.* 1990), whilst the ADE for soybean meal was found to vary from 84% to 88% in *L. vannamei* (Divakaran *et al.* 2000), and to be around 76.09 % and 74.59% for soybean meal and fish meal, respectively, in the diet of *P. setiferus* (Brunson *et al.* 1997). Allan *et al.* (2000) reported that the apparent digestibility of blood meal for energy was 99.9 % in silver perch, *Bidyanus bidyanus*. Although the present study showed that shrimp meal was also highly digestible (84.5 %) by mud crabs, the ADE of shrimp meal has not been investigated in most farmed species. Similarly, although the ADE of cod liver oil (89.2 %) was determined in this study, there is little information regarding its ADE for many commonly farmed aquatic

animal species. Recently, ADE of cod liver oil was reported to be around 100 % in silver perch (Allan *et al.* 1999a).

In this study, wheat flour was found to be highly digestible for energy (79.9 %) and dry matter (70 %) by *S. serrata*. Catacutan *et al.* (2003) similarly found that the ADD of bread flour was 90.6 % for *S. serrata*. This suggests that mud crabs can digest carbohydrate sources more effectively than many fish. Growers and juveniles of the Rockfish, *Sebastes schlegeli,* metabolised only 39 % and 46 % of the energy in wheat flour, respectively (Lee 2002a). Allan *et al.* (2000) found that the ADE and ADD of wheat flour was 53.2 % and 52.9 %, respectively, in silver perch. A similarly low ADD (35.57 %) for wheat flour was also reported for red drum *Sciaenops ocellatus* (McGoogan & Reigh 1996).

Dry matter in fish meal, shrimp meal, blood meal, defatted soy bean meal, and cod liver oil were was found to be highly digestible by mud crabs (Table 3.4). These findings were similar to the results of Catacutan *et al.* (2003), who found high ADD for Peruvian fish meal (89.9 %), defatted soybean meal (90.9 %), and acetes meal (88.3%) in *S. serrata*. A high ADD for these ingredients has also been reported for other fish and crustacean species. For example, Divakaran *et al.* (2000) found that the ADD for soybean meal was in the range 79-83 % for *L. vannamei,* the ADD values of about 100 % for blood meal and cod liver oil was determined in silver perch, *B. bidyanus*, by Allan *et al.* (2000) and Allan *et al.* (1999a), respectively. McGoogan and Reigh (1996) reported an ADD of about 71.6 % for blood meal in the diet of red drum, The lipid digestibility of cod liver oil in tiger prawn, *P. monodon,* diets was 90.9% (Merican & Shim 1994).

With respect to the protein content used in this study, fish, shrimp, blood, and soybean meal were all highly digestible by crablets. These results support the

findings of Catacutan *et al.* (2003), working in the Philippines, who reported ADP values for Peruvian fish meal (94.8 %), defatted soybean (95.2 %), and Acetes meal (94.9 %) for *S. serrata*. Slightly lower values for the ADP of fish meal (83.6 %), shrimp meal (88.2 %), and soybean meal (85.9 %) in the diet of the Chinese hairy crab, *Eriocheir sinensis*, were reported by Mu *et al.* (2000). Similar values for ADP by other crustaceans have been reported for Australian fish meal (Smith *et al.* 2000), and soybean meal (Divakaran *et al.* 2000), and by fish for blood meal (Lee 2002a).

Although the ADP of most ingredients in this study was quite high, protein in wheat flour was relatively poorly digested (57.7 %). This result contrasts with those of McGoogan and Reigh (1996) and Allan *et al.* (2000) who found the protein from wheat was highly digestible for red drum and silver perch respectively. The ADP of wheat flour in this study was also lower than that of bread flour (95.2 %) reported by Catacutan *et al.* (2003). This much lower protein digestibility in our study suggests protein damage to the batch of wheat flour we tested. This is supported to some extent by the lower ADE and ADD values compared with other ingredients. If protein was damaged it would not have contributed to energy. However, this ingredient should be re-examined with increasing a number of replicates.

This study has demonstrated that mud crabs are capable of very efficient digestion of both animal and plant ingredients. The high ADD and ADE values recorded in this study for cellulose demonstrate that mud crabs are capable of digesting cellulose.

3.2 AD OF SOME FEED INGREDIENTS BY *S. paramamosain* IN VIETNAM

3.2.1 Introduction

In Vietnam, there are several potential sources of protein, lipid and carbohydrate with which to formulate diets for aquatic animals. The common ingredients include local Vietnamese fish meal, Vietnamese shrimp head meal, Vietnamese dried acetes shrimp meal, defatted hulled soybean meal, Vietnamese expelled peanut oil cake, whole rice bran, rice flour, corn meal, potato meal, coconut meal and cassava meals (Luu 1993; Edwards *et al.* 2004). As a result of the shrimp farming industry in coastal areas of Vietnam, a range of imported sources of lipid, such as fish oil and squid oil, are also available in the local aquatic feed market. Other cheap sources of lipids that may have potential for use in mud crab diets include pig fat oil and vegetable oil. Despite the availability of various ingredients, there is little information on how to best use these products to produce feed for aquatic animals.

The production of the mud crab, *S. paramamosain,* is expanding rapidly as a result of advances in hatchery and nursery culture technology in Vietnam (Lindner 2005). However, the lack of availability of a cost-effective formulated feed designed specifically for them is becoming a major limitation to the expansion of this industry. Little is known of the nutritional requirements of mud crabs or of their ability to digest different ingredients. Such information is required for successful formulation of cost-effective diets for crabs. To provide some of the information required, this study investigated the apparent digestibility of a range of ingredients including Vietnamese fish meal (VINA fish meal), Vietnamese dried acetes shrimp meal (VINA acetes meal), Indian defatted soybean meal, full fat soybean meal, coconut oil cake meal, peanut oil cake dried meal, whole rice bran, ground cassava meal, bread wheat flour, Korean fish oil, Korean squid oil, local pig fat oil, and vegetable oil (a

mixture of soybean oil, canola oil, and sunflower oil) by juvenile mud crabs *(S. paramamosain)*, with the following specific aims:

- To determine apparent digestibility (AD) for dry matter, protein, ash, and nitrogen free extract of Vietnamese fish meal, Vietnamese dried acetes shrimp meal, defatted soybean meal, soybean oilcake dried meal, coconut oil cake dried meal, expelled peanut oil cake, whole rice bran, ground cassava meal, and bread wheat flour.
- To determine AD for crude fat of thirteen ingredients including Vietnamese local fish meal, Vietnamese dried acetes shrimp meal, defatted soybean meal, full fat soybean meal, expelled coconut oil cake, expelled peanut oil cake, whole rice bran, ground cassava meal, bread wheat flour, Korean fish oil, Korean squid oil, local pig fat oil, and vegetable oil.

3.2.2 Materials and methods

The experiment was carried out at the Bac Lieu Experimental Station for Aquaculture (Section 2.1.2 of Chapter 2) from 18th December 2004 to 28th January 2005.

3.2.2.1 Juvenile mud crab preparation

One hundred and sixty eight (168) juvenile mud crabs of 60.2 ± 5.3 g animal^{-1} were used. Six thousand hatchery juveniles had been stocked in earthen ponds at the BLESA for 50 days prior to the experiment. The crabs were collected from the earthen ponds using a hand net and bathed with 100 mg L^{-1} of formalin for 30 minutes. For the experiment, only crabs with similar size and health (e.g., no damaged signage or missing appendages) were selected. A group of four crablets

with similar weight and carapace width was held in one tank. Crabs were acclimated to trial conditions for 7 days.

During the acclimation period, crabs were fed a modified Concord feed for black tiger prawn (Concord feed No.3, made by Asian Aquaculture Co., Ltd. Thailand) four times a day at 06:00, 11:00, 17:00, and 22:00 hours. The original Concord feed was modified by grinding it into powder, to which 5 % gluten binder was added. The mixture was then put through a mincer to produce larger pellets of about 3 mm diameter and 30 mm length. This feed contained 40.6 % crude protein and 7.1 % crude fat. Additionally, the likelihood of cannibalism was monitored and, where necessary, the risk reduced by removing particularly aggressive crabs. After seven days of acclimation, the crablets were fed experimental diets for one week prior to collecting faeces.

3.2.2.2 Culture system, water supply and water measurement

The general culture system, water preparation, and water measurement are described in Sections 2.3-2.5 of Chapter 2. Forty two tanks of 60 L each were used with a seawater recirculation system. Recycled water was supplied and water level was kept at 300 mm depth for all tanks. During the experimental period, water temperature, salinity, pH and dissolved oxygen of the water were in the ranges of 26.06 ± 0.37 ^{o}C, 29.20 ± 0.65 g L^{-1}, 8.12 ± 0.10, and 4.88 ± 0.55 mg L^{-1} respectively.

3.2.2.3 Ingredient sources

The sources of test ingredients, reference feed, and chromic oxide are listed in Table 3.5.

Table 3.5 Sources of reference diet, inert maker, and a range of test ingredients

Items	Sources
Vietnamese local fish meal (VINA fish meal)	Local product was supplied the RIA2, HCM, Vietnam
Vietnamese dried acetes shrimp meal (VINA acetes shrimp meal)	As above
Vietnamese full fat soybean meal (VINA full fat soybean meal)	As above
Vietnamese expelled coconut oil cake (VINA coconut oil cake)	As above
Vietnamese expelled peanut oil cake (VINA peanut oil cake)	As above
Vietnamese ground cassava meal (VINA cassava meal)	As above
Vietnamese whole rice bran (VINA rice bran)	As above
Local pig fat oil	Purchased locally, Bac Lieu, Vietnam
Tuong An vegetable oil (a mixture of soybean oil, canola oil, and sunflower oil –vegetable oil)	Local product, Tuong An Co. Ltd., HCM, Vietnam
Bread wheat flour	Imported product was provided from the RIA2, HCM, Vietnam
French vital wheat gluten	Imported product was provided from the RIA2, HCM, Vietnam
Indian defatted dehulled soybean meal (Indian defatted soybean meal)	As above
Korean fish oil	As above
Korean squid oil	As above
Chromic oxide (Purity of 99.9 %)	Tianjin Jinchuan Chemials Co., Tianjin, China
Concord feed®	Asian Aquaculture Co. Ltd., Nonthaburi, Thailand

Eleven (11) test ingredients were provided by the Research Institute for Aquaculture No. 2 (RIA2) in HCM City, Vietnam. These ingredients included Vietnamese fish meal, Vietnamese acetes shrimp meal, defatted dehulled soybean meal, dried full fat soybean meal, dried coconut oil cake meal, dried peanut oil cake meal, ground cassava flour, whole rice bran, bread wheat flour, Korean fish oil, and Korean squid oil. The other ingredients, pig fat oil and soya oil, were purchased locally. The

reference feed was based on Concord feed for *P. monodon* and added 5 % vital wheat gluten.

3.2.2.4 Experimental diets

Fourteen diets were prepared (Table 3.6) which included one reference diet (V1) and thirteen test diets (V2-V14). Nine of the test diets (V2-V10) contained 70 % reference diet and 30 % test ingredients (VINA fish meal, VINA acetes shrimp meal, Indian defatted soybean meal, VINA full fat soybean meal, VINA coconut oilcake, VINA peanut oilcake, VINA cassava meal, VINA rice bran, and wheat flour). The four last test diets (V11-V14) contained 85 % reference diet and 15 % test ingredients (Korean fish oil, Korean squid oil, local pig oil and Tuong An soy oil). Chromic oxide was added as an inert marker at 5 g kg^{-1} to both reference and test diets. The reference diet was prepared using Concord feed® (945 g kg^{-1}) and vital wheat gluten (50 g kg^{-1}).

3.2.2.5 Diet preparation

The main steps of diet preparation were followed as described in Section 2.6 of Chapter 2. The specific steps were similar to Section 3.1.2.4 of this Chapter.

3.2.2.6 Feeding and faecal collection

To reduce cannibalism, the crabs were fed four times a day at 6:00, 11:00, 17:00 and 22:00 hours (seven days a week). All uneaten feed was removed at the end of a two-hour feeding period. The faeces were collected daily during the periods of 8:00-11:00, 13:00-17:00, 19:00-22:00, and 24:00-6:00. The faeces of each replicate were collected daily between 10:00 and 16:00, filtered through a 30 μm mesh, rinsed gently with distilled water, pooled and stored at a -20 ^{o}C freezer until having enough for analysis required.

Table 3.6 Composition of reference diet (V1) and test diets (V2-V14) on a dry weight basis (g kg^{-1})

Ingredients	Experimental diets													
	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V13	V13	V14
Prawn feed	945.0	661.5	661.5	661.5	661.5	661.5	661.5	661.5	661.5	661.5	803.3	803.3	803.3	803.3
Vital wheat gluten	50.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	42.5	42.5	42.5	42.5
Chromic oxide	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Fish meal	-	298.5	-	-	-	-	-	-	-	-	-	-	-	-
ASM	-	-	298.5	-	-	-	-	-	-	-	-	-	-	-
DFSBM	-	-	-	298.5	-	-	-	-	-	-	-	-	-	-
Soybean oilcake	-	-	-	-	298.5	-	-	-	-	-	-	-	-	-
Coconut oilcake	-	-	-	-	-	298.5	-	-	-	-	-	-	-	-
Peanut oilcake	-	-	-	-	-	-	298.5	-	-	-	-	-	-	-
Cassava meal	-	-	-	-	-	-	-	298.5	-	-	-	-	-	-
Rice bran	-	-	-	-	-	-	-	-	298.5	-	-	-	-	-
Bread wheat flour	-	-	-	-	-	-	-	-	-	298.5	-	-	-	-
Fish oil	-	-	-	-	-	-	-	-	-	-	149.3	-	-	-
Korean squid oil	-	-	-	-	-	-	-	-	-	-	-	149.3	-	-
Pig fat oil	-	-	-	-	-	-	-	-	-	-	-	-	149.3	-
Vegetable oil	-	-	-	-	-	-	-	-	-	-	-	-		149.3

Notes:

Dash (-): zero

ASM: acetes shrimp meal

DFSBM: defatted soybean meal

3.2.2.7 Chemical analysis

Details of chemical analysis are given in Section 2.8 of Chapter 2. The dry matter, protein, crude fat, and ash (Table 3.7) were determined using the methods described by AOAC International (1996). The chromic oxide were analysed using the method reported by Temminghoff (2000). The concentration of chromic oxide in diets and faeces are presented in Appendix 3.3.

Table 3.7 Composition of experimental diets and test ingredients for dry matter (DM), crude fat (CF), crude protein (CP), ash, and nitrogen free extract (NFE) (values are means ± standard error; n=3)

Sources	DM (%)	CF (%)	CP (%)	Ash (%)	NFE (%)
Experimental diets					
V1	92.2 ± 0.8	7.1 ± 0.1	40.6 ± 0.1	15.8 ± 0.1	36.5 ± 0.1
V2	85.9 ± 0.1	6.7 ± 0.1	47.2 ± 0.2	17.6 ± 0.1	28.7 ± 0.4
V3	87.9 ± 0.0	5.2 ± 0.0	38.7 ± 0.0	26.5 ± 0.2	29.7 ± 0.2
V4	85.0 ± 0.0	6.0 ± 0.1	40.8 ± 0.2	13.1 ± 0.1	40.2 ± 0.2
V5	86.8 ± 0.0	10.9 ± 0.1	34.7 ± 0.3	15.1 ± 0.1	39.4 ± 0.3
V6	88.2 ± 0.0	7.78 ± 0.5	34.8 ± 0.1	17.8 ± 0.1	39.7 ± 0.6
V7	86.7 ± 0.1	10.1 ± 0.3	38.0 ± 0.3	18.0 ± 0.1	33.9 ± 0.4
V8	87.9 ± 0.0	5.3 ± 0.2	31.5 ± 0.5	12.0 ± 0.0	51.3 ± 0.6
V9	88.4 ± 0.1	6.2 ± 0.3	31.1 ± 0.6	18.7 ± 0.2	44.1 ± 0.6
V10	86.8 ± 0.1	5.3 ± 0.2	33.1 ± 0.3	11.45 ± 0.1	50.2 ± 0.6
V11	91.0 ± 0.0	20.3 ± 0.7	-	-	-
V12	90.1 ± 0.2	20.4 ± 0.2	-	-	-
V13	89.0 ± 0.1	20.9 ± 0.1	-	-	-
V14	88.8 ± 0.1	20.8 ± 0.2	-	-	-
Test ingredients					
Fish meal	93.2 ± 0.9	5.7 ± 0.0	62.1 ± 1.0	21.9 ± 0.0	10.4 ± 1.1
Acetes shrimp meal	88.5 ± 0.8	0.6 ± 0.3	34.6 ± 0.3	51.5 ± 0.7	13.7 ± 0.7
DFSBM	92.8 ± 0.7	3.2 ± 0.4	41.4 ± 0.5	6.7 ± 0.0	48.7 ± 0.9
FFSBM	91.9 ± 0.4	19.5 ± 0.1	20.9 ± 1.3	13.5 ± 0.4	46.1 ± 1.0
Coconut oil cake	91.4 ± 1.5	9.0 ± 1.5	21.0 ± 0.6	22.6 ± 0.2	47.3 ± 1.9
Peanut oil cake	90.6 ± 0.1	17.2 ± 0.6	31.8 ± 1.1	23.2 ± 0.5	27.8 ± 1.2
Cassava meal	94.0 ± 0.3	0.9 ± 0.8	10.3 ± 1.4	3.2 ± 0.1	85.7 ± 2.3
Rice bran	95.6 ± 0.7	4.1 ± 1.1	8.8 ± 1.5	25.4 ± 0.5	61.7 ± 2.2
Wheat flour	93.7 ± 0.3	1.0 ± 0.4	15.4 ± 1.2	1.5 ± 0.2	82.1 ± 1.8
Fish oil	-	96.0 ± 0.3	-	-	-
Korean squid oil	-	96.8 ± 2.0	-	-	-
Pig fat oil	-	100.0 ± 0.0	-	-	-
Vegetable oil	-	99.4 ± 1.2	-	-	-

Note: Dash (-): not determined; DFSBM: defatted soybean meal; FFSBM: full fat soybean meal

3.2.2.8 Calculations

Apparent digestibility (AD) for reference and test diets were calculated using equations described by De Silva and Anderson (1995). The apparent digestibility of test ingredients were determined using the equation described by Forster (1999). These equations are described in Section 2.9 of Chapter 2.

3.2.2.9 Experimental design

The experiment had fourteen treatments which were set up using a randomized complete design (RCD) (Figure 3.2) with 3 replicates each. Each replicate had 4 crabs.

3.2.2.10 Statistical analyses

Data in percentage were arcsine transformed prior to performing a one-way ANOVA. When the mean had significant difference, Tukey HSD test were used to determine significant differences between treatments. The software used was *STATISTICA 7* (StatSoft, Inc., OK, USA, 2004).

Figure 3.2 The system used in AD determination for *S. paramamosain* at the BLESA

3.2.3 Results

Apparent digestibility (AD) of *S. paramamosain* for dry matter (ADD), protein (ADP), crude fat (ADF), ash (ADA), and nitrogen free extract (ADN) of test ingredients are presented in Table 3.8. For ease of reading, the values of AD for experimental diets are given in Appendix 3.4.

3.2.3.1 Apparent digestibility for dry matter

Apparent digestibility for dry matter were above 75 % for a number of local ingredients, notably defatted soybean meal, fish meal, acetes meal, cassava meal, wheat flour and rice bran (Table 3.8). The ADDs for defatted soybean meal (85.3 %) and cassava meal (85.0 %) were the highest whilst the ADDs for coconut oilcake (60.2 %) and full fat soybean meal (69.4 %) were significantly lowest ($P < 0.05$).

3.2.3.2 Apparent digestibility for protein

In terms of apparent digestibility of protein, the ingredients (Table 3.8) fell into three broad groups. Defatted soy bean meal (95.9 %), cassava meal (95.4 %), and rice bran (94.2 %) had significantly highest ADP values. The second group, with intermediate ADPs, included wheat flour (90.1 %), acetes shrimp meal (86.3 %), fish meal (86.1 %), soy bean waste meal (84.9 %), and peanut oilcake (79.9%0, which ADPs were not significantly different ($P < 0.05$). Finally, coconut oil cake had the lowest ADP (64.5 %).

Table 3.8 Apparent digestibility for dry matter (ADD), crude fat (ADF), protein (ADP), ash (ADA), and nitrogen free extract (ADN) in juvenile mud crab, *S. paramamosain*, for thirteen feed ingredients (data was expressed in mean ± SE with n = 3)*

Test ingredients	ADD (%)	ADP (%)	ADA (%)	ADN (%)	ADF (%)
VINA fish meal	78.5 ± 2.6^{bcd}	86.1 ± 2.5^{bc}	64.2 ± 4.6^{abc}	63.2 ± 5.1^{bc}	74.6 ± 4.1^{bc}
VINA acetes shrimp meal	76.4 ± 3.0^{bcd}	86.3 ± 3.3^{bc}	83.0 ± 2.0^{c}	27.1 ± 11.3^{a}	51.5 ± 8.0^{a}
Indian defatted soybean meal	85.3 ± 1.5^{d}	95.9 ± 1.2^{c}	67.2 ± 9.4^{abc}	78.8 ± 1.0^{de}	83.1 ± 2.6^{bcd}
VINA full fat soybean meal	69.4 ± 2.1^{ab}	84.9 ± 2.2^{bc}	46.6 ± 8.5^{a}	60.4 ± 3.4^{bcd}	88.4 ± 1.1^{cd}
VINA coconut oilcake	60.3 ± 2.5^{a}	64.5 ± 0.8^{a}	57.0 ± 2.1^{ab}	52.8 ± 4.0^{bc}	94.4 ± 1.0^{d}
VINA peanut oilcake	71.0 ± 0.4^{ab}	79.9 ± 0.8^{b}	74.7 ± 2.9^{bc}	40.3 ± 1.2^{ab}	98.1 ± 1.6^{d}
VINA cassava meal	85.0 ± 0.8^{d}	95.4 ± 1.6^{c}	62.8 ± 2.6^{abc}	84.7 ± 0.8^{e}	67.2 ± 5.4^{ab}
VINA rice bran	78.5 ± 0.7^{bcd}	94.2 ± 4.0^{c}	55.6 ± 2.3^{ab}	84.8 ± 0.6^{e}	88.2 ± 1.8^{cd}
Bread wheat flour	79.7 ± 1.5^{cd}	90.1 ± 3.4^{bc}	61.8 ± 4.7^{abc}	78.0 ± 1.3^{de}	74.2 ± 4.3^{bc}
Korean fish oil	nd	nd	nd	nd	90.1 ± 0.6^{cd}
Korean squid oil	nd	nd	nd	nd	91.3 ± 1.0^{cd}
Local pig fat oil	nd	nd	nd	nd	92.4 ± 0.3^{d}
Vegetable oil	nd	nd	nd	nd	89.1 ± 1.6^{cd}

*Means within the same column having a similar superscript letter are not significantly different at 5% of significance (P>0.05); nd = not determined

3.2.3.3 Apparent digestibility for crude fat

In Table 3.8, although the ADD and ADP for coconut and peanut oil cake were generally lower than those for other ingredients, their ADF values were very high, 98.0 % for coconut oil cake and 94.4 % for peanut oil cake. These ADF values were similar to those of fish oil, Korean squid oil, pig fat oil, and vegetable oil ($P > 0.05$). Lower ADF values were found for crabs fed with cassava meal and acetes shrimp meal in diets, both of which have a low intrinsic crude fat content of less than 1 % (Table 3.7).

3.2.3.4 Apparent digestibility for ash

Acetes shrimp meal, which contains over 50 % ash (Table 3.7), had the highest ash AD of 83 % (Table 3.8). High ADA values were also found for peanut oil cake, defatted soy bean meal, fish meal, rice cassava meal, and wheat flour. The lowest ADA value of 46.7 % was found in full fat soybean meal. Other ingredients such as rice bran (55.6 %), coconut oil cake (57.0 %), wheat flour (61.8 %), cassava meal (62.8 %), fish meal (64.2 %), and defatted soy bean meal (67.2 %) had intermediate ADA values.

3.2.3.5 Apparent digestibility for nitrogen free extract

In Table 3.8, almost all plant ingredients had very high ADN values, which were in range of 63.2-84.8 % while acetes shrimp meal containing a low NFE content (13.7 %) was not well digested by mud crab in this study (27.1 %).

3.2.4 Discussion

The local fish meal had less crude protein (62 %) than Peruvian fish meal of 68.3 % (Catacutan *et al.* 2003) and Australian fish meal of 77.0 % (Section 3.1 of this Chapter). The local fish meal also had slightly lower apparent digestibility (AD) for

dry matter (79 %) and for protein (86.1 %) than those reported for Peruvian fish meal (Catacutan *et al.* 2003) and Australian fish meal (Section 3.1 of this Chapter). Other studies show that AD for the same component varied considerably from fish meal to fish meal and from crustacean to crustacean. For example, the Malaysian fish meal had ADP of 84 % in Chinese hairy crab, *Eriocheir sinensis,* (Mu *et al.* 2000); and the menhaden fish meal had ADP of 76 % in white shrimp, *Penaeus setiferus,* (Brunson *et al.* 1997).

Although the Acetes shrimp meal contained only 34 % crude protein (Table 3.7), it nevertheless had moderately high AD for dry matter (76%), crude protein (86 %), and for ash (83 %), but low AD for crude fat (51 %) and for nitrogen free extract (27 %). However, these AD are lower than those reported by Catacutan *et al.* (2003), where the acetes meal contained 69 % crude protein. Acetes shrimp meal has a high ash level and given its high ash digestibility coefficient (83 %), it may be a very good source of inorganic minerals for crabs. However, further analysis of its mineral composition is needed to identify its mineral profile.

Of the plant ingredients tested, defatted soybean meal appears to be particularly promising as an ingredient for mud crab diets. It had moderate to high AD for dry matter (85 %), protein (95 %), crude fat (83 %), for ash (67 %), and for nitrogen free extract (79 %). Other studies have also demonstrated the high AD values of defatted soybean meal in other crustacean species such as in white shrimp, *P. setiferus* (Brunson *et al.* 1997), in *L. vanname* (Divakaran *et al.* 2000), Chinese hairy crab, *Eriocheir sinensis,* (Mu *et al.* 2000), in mud crabs, *S. serrata* (Catacutan *et al.* 2003) and Section 3.1 of this Chapter.

In addition to defatted soybean meal, full fat soybean meal (FFSBM) had intermediate AD for dry matter (69.4 %), protein (84.9 %), and crude fat (88.4 %).

There have been no previous reports of AD for full fat soybean meal in crustaceans but this ingredient has been found to have AD of greater than 70 % for dry matter and greater than 90 % for nitrogen in several species of fish (Allan *et al.* 2000; Cheng & Hardy 2003). Full fat soybean meal therefore appears to be a promising alternative protein source for mud crab diets.

Coconut oil cake has been used in several mammal diets (Hammond & Wildeus 1993) but its potential as an ingredient for fish and crustacean diets has not be investigated previously. In this study, coconut oil cake was found to have very high crude fat AD (94.4 %) with lower AD for other dietary components. Consequently, coconut oil cake may be used in diet formulation with low level.

Peanut oil cake, an inexpensive ingredient available in the local market in Vietnam, also appears to be suitable for use in mud crab diets. It had high apparent digestibility for dry matter (71 %), protein (80 %), ash (75 %) and crude fat (98 %), and AD for nitrogen free extract of around 40 % (Table 3.8). These values are similar to those reported on fish species (Allan *et al.* 2000; Zhou *et al.* 2004).

Like coconut oil cake and peanut oil cake, cassava meal appears to have potential as an inexpensive ingredient in crab diets in Vietnam. It was found in this study to have high apparent digestibility for dry matter (85 %), protein (95 %) and nitrogen free extract (85 %), with lower AD for crude fat (67 %) and ash (63 %) (Table 3.8). This finding contrasted to the results of Gomes and Pena (1997) who revealed that the cassava meal appears to be less digestible by the freshwater prawn, *Macrobrachium rosenbergii*.

Rice bran appears to be digested poorly by fish species (Laining *et al.* 2003) and to have a low AD for dry matter in the diet of the white shrimp, *P. setiferus* (Brunson *et*

al. 1997). However, in this study with *S. paramamosain*, rice bran had apparent digestibility greater than 79 % for all constituents. High AD for all constituents except the nitrogen free extract were also found when rice bran was an ingredient in the diet of the mud crab *S. serrata* (Catacutan *et al.* 2003).

Wheat flour had high AD values for dry matter (80 %) and protein (90 %) in the current trial. Catacutan *et al.* (2003) also found similarly high AD for dry matter and protein in *S. serrata* in the Philippines. These findings are in contrast to those of Section 3.1 of this Chapter which found somewhat lower AD for dry matter (70%) and protein (58 %) in the diet of *S. serrata* in Australia.

Presently, most aquafeeds use fish oil or squid oil in feed formulation because they are excellent sources of both essential and non-essential fatty acids (Tacon 1990b; Merican & Shim 1994; Glencross & Smith 2001), although little known of AD for commercial fish and squid oils in the diets of fish and crustacean species. High crude fat AD for fish oil (90 %) and squid oil (91 %) were found in the present study (Table 3.8), consistent with those found in the diet of *P. monodon* (Merican & Shim 1994).

The present work also evaluated the ADF for vegetable oil containing a mixture of soybean oil, canola oil, and sunflower oil, which has been reported to be a good source of energy and fatty acids, particularly polyunsaturated fatty acids. In this study, vegetable oil was found to have a high crude fat AD (89 %).

Although pig fat (Pork lard) is a cheap ingredient at most local market in Vietnam, it has been little used in formulating feeds for fish and crustaceans. Pig fat was formerly used for cooking, but now it has been replaced by vegetable oil. As a result, it has become cheaper in the local market. Pig fat also contains important

fatty acids such as oleic (33.0-45 % of total lipid) and linoleic acids (9.8-28.4 %) (Maw *et al.* 2003) and it has been reported as a good source of linoleic acid (23.3 % of total fatty acid) in diets for Chinese mitten-handed crab (*Eriocheir sinensis*) (Wen *et al.* 2002). Clearly, its cheap price, good nutrition value (in energy and fatty acids), and high digestibility (92 %, this study) makes pig fat oil a promising ingredient for the formulation of mud crab diets. However, pig fat lacks the key essential fatty acids for mud crabs such as EPA, DHA, and arachidonic acid. Hence, it could be considered as a supplementary lipid source that provides fat to a diet that already has adequate levels of the essential fatty acids.

3.3 CONCLUSION

All of the ingredients evaluated in these two trials can be used to select the suitable ingredients for each target culture system of mud crabs. The apparent digestibility determined in this chapter provide a basis for estimating optimal digestible levels for digestible proteins (Chapters 4) and digestible energy (Chapter 5), and for selecting the most suitable ingredients (Chapter 7) in this thesis.

Chapter 4 Optimal dietary digestible protein specification for juvenile mud crab, *Scylla serrata* (Forskal 1775)

4.1 INTRODUCTION

With the availability of data on the digestibility of different feed ingredients (see Chapter 3), it is now possible to determine the requirement for each nutrient that is important for growth and reproduction of mud crabs. Protein is usually the most expensive component of feeds. It is therefore important to work out the optimum level of dietary protein for mud crabs, so that the feed formulated can be low in cost, without compromising the growth of the animal.

Catacutan (2002) found that the mud crabs, *Scylla serrata*, grew well when fed diets containing 32-40 % dietary protein with either 6 % or 12 % lipid at dietary energies ranging from 14.7-18.6 MJ kg^{-1}. However, this data was not based on digestible protein, and optimum levels were not determined.

This chapter describes an experiment undertaken to find the optimum digestible protein requirement of the juvenile mud crabs. Specifically, it aimed to:

- Determine optimum digestible protein level required to maximise weight gain, carapace width increase, specific growth rate, and protein efficiency ratio.
- Obtain the optimal level of digestible protein to minimise feed conversion ratio and inter-moult period.

4.2 MATERIALS AND METHODS

The experiment was conducted at the Darwin Aquaculture Centre (DAC), Northern Territory, Australia (see Section 2.1 of Chapter 2), from 1st September to 11th December 2003 with juvenile mud crabs (crab 6, after 6 moults from megalops

stage) sourced from the same batch reared in a hatchery. Two hundred and thirty crabs at stage 5 provided by the hatchery of the DAC were acclimatised to experimental conditions for 2-5 days and fed using Vital feed® (Higashimaru Company, Kagoshima, Japan), a feed designed for *P. japonicus,* which has been used successfully in juvenile mud crab growth trials (Shelley 2001; Ruscoe *et al.* 2004). The Vital feed® contained 15.5 % lipid and 52.7 % protein. The times when the crabs first moulted were recorded as moult zero (M0) and feeding with experimental diets started. A total of 90 crablets at stage 6 with a similar carapace width and weight (0.66 ± 0.05 g) were selected and used to test ten diets, with nine crabs for each diet.

4.2.1 Culture system

The general culture system, water preparation and water measurement are described in Sections 2.3, 2.4, and 2.5 of Chapter 2. Ninety round plastic containers of 2 L each were used in this experiment, with one crablet per container. The containers were placed in one of three tanks (dimensions 80 cm x 200 cm x 30 cm), containing water to buffer against changing temperature. There were thirty containers in each tank, three for each experimental diet. A flow-through system was used to supply seawater to each container with a dripper (Irrigation Warehouse Group Pty Ltd, NSW, Australia) of 8 L $hour^{-1}$. The water levels were maintaining at 150 mm in depth. During the experimental period, water temperature, salinity, pH and dissolved oxygen were 30.7 ± 1.1 ^{0}C, 31.3 ± 2.3 ‰, 8.0 ± 0.2, and 4.9 ± 0.3 mg L^{-1} respectively.

4.2.2 Experimental diets

Ten levels of digestible protein ranging from 203 g kg^{-1} to 635 g kg^{-1} were prepared, all with the same digestible energy level of 18.0 MJ kg^{-1} (Table 4.1).

Table 4.1 Composition of the experimental diets (on a dry matter basis) with ten levels of dietary digestible protein (units for digestible energy and gross energy are MJ kg^{-1}; all other ingredients have units of g kg^{-1})

Ingredients	Experimental diets									
	T20	**T25**	**T30**	**T35**	**T40**	**T45**	**T50**	**T55**	**T60**	**T65**
Fish meal	149	190	232	273	315	356	398	439	480	523
Shrimp meal	50	63	77	91	105	119	133	146	160	174
Blood meal	50	63	77	91	105	119	133	146	160	174
Wheat flour	324	297	265	230	191	154	123	88	63	0
Cod liver oil	186	163	142	122	102	82	60	39	17	0
Cellulose	202	184	167	153	143	130	114	101	80	89
Vitamin premix[1]	10	10	10	10	10	10	10	10	10	10
Mineral premix[1]	10	10	10	10	10	10	10	10	10	10
Alginic acid	20	20	20	20	20	20	20	20	20	20
DP(Calculated)[2]	203	251	299	347	395	443	491	540	588	635
DE(Calculated)[2]	18	18	18	18	18	18	18	18	18	18
CP(Calculated)[2]	230	280	330	380	429	478	528	578	628	676
GE(Calculated)[2]	22	21	21	21	21	21	21	21	20	20

Notes:
DP: Digestible protein DE: Digestible energy
CP: Crude protein GE: Gross energy
[1] Souced from Skretting Company, Tasmania, Australia.
[2]Due to budget limitation, levels of DP, CP, DE, and GE of each diet were calculated using previous analytical result of each ingredient

The values of DP, DE, CP, and GE were calculated from previous data of the same ingredients (Australian fish meal, Danish shrimp meal, Australian ring blood meal, bread wheat flour, alpha cellulose, cod liver oil) in Section 3.1 of Chapter 3. Those data are summarised in Table 4.2.

Table 4.2 A summary of crude protein (CP), gross energy (GE), and apparent digestibility for protein (ADP) and energy (ADE)

Ingredients	CP (%)	GE (MJ kg^{-1})	ADP (%)	ADE (%)
Fish meal	76.7	21.6	95.0	91.5
Shrimp meal	70.5	20.9	91.9	84.4
Blood meal	87.5	23.1	93.5	85.8
Wheat flour	11.5	18.0	57.7	79.9
Cellulose	nd	15.4	nd	77.4
Cod liver oil	nd	39.5	nd	89.2

nd=not determined

4.2.3 Diet preparation

The main steps of diet preparation are described in Section 2.6 of Chapter 2. In this study, a final moist mixture was squeezed into pellets of about 1.5 mm in diameter and 100 mm in length. The pellets were then dried at 35 ^{o}C until its moisture content was less than 10%. The long pellets were broken up into shorter ones of around 10 mm in length and stored at -20 ^{o}C until required.

4.2.4 Feeding and collection of uneaten feed

Initially, feed for each replicate was separately weighted and kept in a small plastic jar. Animals were fed ad-libitum daily at 09:00 hours each morning. Six hours after feeding, uneaten feed was siphoned from each tank into a mesh of 30 μm, gently rinsed with distilled water, and then stored at a -20 ^{o}C freezer until the experiment was completed. Uneaten feed and fed feed was dried and weighted. The feed intake was calculated is to be a difference between fed feed and uneaten feed.

Feeding only one per day due to feed was adjusted to satisfaction of crab (ad libitum). Furthermore, the experiment was only carried during working time (from 8:00 to 16:30) due to NT government does not allow staff spend overnight at the DAC.

4.2.5 Chemical analysis

Dry matter was determined using a freeze-drying method which is described in Section 2.8.1 of Chapter 2. The values for energy and crude protein for experimental diets were based on analyses of the same ingredients and their estimated digestible protein and digestible energy from a previous experiment (Section 3.1 of Chapter 3).

4.2.6 Measurements and calculations

The experiment was carried out for three moult intervals of mud crabs (from M0 to M3) with an experimental period of 92 days. To avoid the possibility of death after moulting (soft shell), crabs were weighed four days after moulting had been completed. Weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio, carapace width increase, and inter-moult period were calculated as described in Section 2.10 of Chapter 2.

4.2.7 Experimental design

The experiment was a randomised complete block design (RCB) with 2 fixed factors. Factor 1 was the amount of digestible protein (10 levels) and Factor 2 was the holding tank (with 3 tanks). Each tank holds 30 containers of 2 L each with a single crab in each container. There were 90 individual containers used in the experiment (3 replicate x 3 tanks x 10 diets = 90 total) (Figure 4.1).

Figure 4.1 Culture system designed to determine optimal dietary digestible protein content of *S. serrata*

4.2.8 Statistical analyses

Data were analysed using factorial ANOVA (factor 1 was DP level and factor 2 was holding tank) (Appendix 4.1). The analyses showed that there was no significant effect of tank and no significant interaction between diet and tank (P>0.05) but there were significant differences between DP levels (P < 0.05). Tukey HSD tests were used for multiple comparisons between means. The optimum dietary digestible protein content was estimated using the quadratic model : $y = \beta + \alpha*x + \gamma*x^2$ for the weight gain and feed conversion ratio, where β, α, and γ are the slopes (Shearer 2000). Curve fitting was established by means of treatments and standard errors. The maximum weight gain (Y_{max}) and minimum feed conversion ratio (Y_{min}) were achieved at X (digestible protein) = - β/2γ. The data were processed using *STATISTICA 7* (StatSoft Inc., Tulsa, OK, USA, 2004) and SigmaPlot 8.0 (Systat Software Inc., California, USA, 2002).

4.3 RESULTS

4.3.1 Weight gain, carapace width increase, specific growth rate, and inter-moult period

Weight gain (WG), carapace width increase (CWI) and specific growth rate (SGR) all increased with increasing digestible protein (DP) up to about 540 g kg^{-1} (T55) (Table 4.3). However, when the DP was in the range of 588 and 635 g DP kg^{-1}, the values of WG, CWI and SGR tended to be declined. The lowest values of WG and SGR were recorded for crabs fed diets with 203 g DP kg^{-1} (T20) while the highest WG and SGR values were obtained in crabs fed diet containing DP levels of 491 (T50) and 540 g kg^{-1}(T55), respectively (P < 0.05).

Table 4.3 The final weight (FW), weight gain (WG), carapace width increase (CWI), specific growth rate (SGR), and inter-moult period (IMP) of juvenile mud crabs fed diets containing ten digestible protein levels (g kg^{-1}) (the data were expressed in means ± SE, N= replicates)*

Diets	FW (g)	WG (%)	CWI (%)	SGR(%day^{-1})	IMP (days)	N
T20	2.1±0.1^{a}	223.8 ± 19.5^{a}	58.2 ± 2.94^{a}	1.5 ± 0.1^{a}	78.7 ± 2.7^{b}	9
T25	2.2±0.1^{a}	234.4 ± 16.4^{ab}	61.4 ± 3.88^{ab}	1.7 ± 0.1^{ab}	71.1 ± 4.1^{ab}	8
T30	2.5±0.2^{ab}	273.8 ± 31.2^{abc}	68.3 ± 4.47^{abc}	1.7 ± 0.1^{ab}	76.4 ± 2.5^{ab}	8
T35	2.5±0.2^{ab}	281.1 ± 23.7^{abc}	66.9 ± 4.64^{abc}	1.8 ± 0.1^{abc}	74.3 ± 5.1^{ab}	7
T40	3.2±0.3^{bcd}	401.2 ± 49.3^{cd}	87.5 ± 4.72^{cd}	2.5 ± 0.1^{bcd}	63.6 ± 3.3^{ab}	7
T45	3.5±0.2^{cd}	423.8 ± 29.1^{cd}	91.7 ± 3.28^{d}	2.6 ± 0.2^{cd}	64.6 ± 4.3^{ab}	8
T50	3.8±0.1^{d}	453.3 ± 27.9^{d}	95.7 ± 2.38^{d}	2.7 ± 0.2^{cd}	64.4 ± 4.5^{ab}	7
T55	3.8±0.3^{d}	485.5 ± 38.3^{d}	97.9 ± 5.63^{d}	2.9 ± 0.3^{d}	63.0 ± 4.2^{ab}	9
T60	2.8±0.2^{abc}	343.6 ± 36.7^{abcd}	77.9 ± 4.58^{bcd}	2.2 ± 0.2^{abcd}	67.3 ± 2.5^{ab}	7
T65	3.3±0.2^{bcd}	398.8 ± 30.1^{bcd}	86.7 ± 2.96^{cd}	2.8 ± 0.2^{cd}	58.8 ± 3.6^{a}	6

* Means within the same column having a similar superscript letter are not significantly different at 5 % of significance ($P > 0.05$)

The relationship between the weight gain and digestible protein levels of diets is described in Figure 4.2. Using quadratic regression, it was found that the maximum WG (422 %) occurred at 53 % digestible protein (530 g DP kg^{-1}). This value supports the ANOVA and Tukey's test in the present study. In the figure and analysis, it is also important not only determine the point at which maximum growth occurs, but also apply the method of Zeitoun (1976) and calculate the point at which most optimal growth occurs.

Inter-moult period (IMP) decreased gradually in crabs fed with higher dietary digestible protein levels. The shortest IMP was 58.8 days in crabs fed diet with diet consisting of 635 g digestible protein kg^{-1} while the longest period (78.7 days) was the group fed diet containing 203 g kg^{-1}DP, although IMP values were not significantly different among crab groups fed with T25, T35, T40, T45, T50, T55, and T60 (Table 4.3)

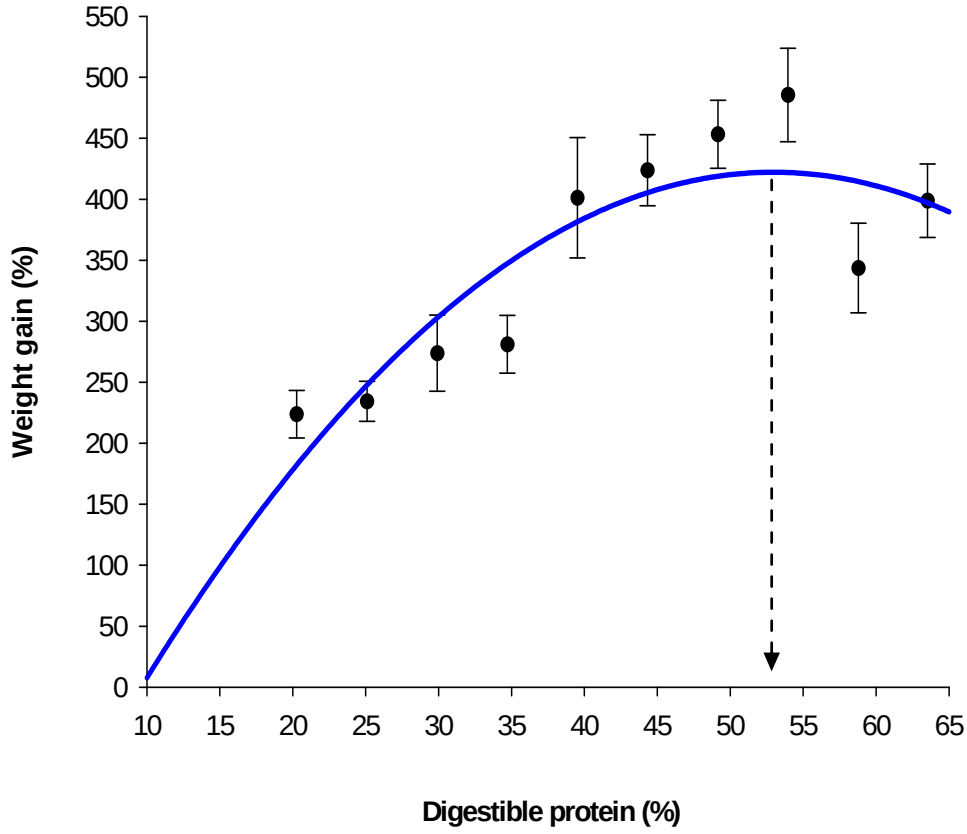

Figure 4.2 Weight gain of mud crabs fed diets containing different digestible protein (DP) levels. The curve was fitted using the quadratic model: y= - 207.536 + 23.775*x - 0.224421*x^2 with R = 0.88. The maximum weight gain was estimated at 53 % digestible protein (arrow)

4.3.2 Feed conversion ratio and protein efficiency ratio

Table 4.4 The feed conversion ratio (FCR), protein efficiency ratio (PER) of juvenile mud crab fed diets containing ten digestible protein levels at one digestible energy (18 MJ kg^{-1}) (the data were expressed in means ± SE, N= replicates)*

Diets	FCR	PER	N
T20	2.9 ± 0.1^{d}	1.7 ± 0.1^{d}	9
T25	2.4 ± 0.1^{bcd}	1.7 ± 0.1^{d}	8
T30	2.4 ± 0.2^{bcd}	1.6 ± 0.3^{cd}	8
T35	2.0 ± 0.1^{abc}	1.4 ± 0.1^{cd}	7
T40	2.0 ± 0.0^{abc}	1.3 ± 0.0^{bcd}	7
T45	1.7 ± 0.1^{ab}	1.3 ± 0.1^{bcd}	8
T50	1.5 ± 0.1^{a}	1.4 ± 0.1^{bcd}	7
T55	1.7 ± 0.2^{ab}	1.2 ± 0.1^{abc}	9
T60	2.8 ± 0.2^{cd}	0.6 ± 0.1^{a}	7
T65	2.0 ± 0.1^{abc}	0.8 ± 0.0^{ab}	6

* Means within the same column having a similar superscript letter are not significantly different at 5 % of significance ($P > 0.05$)

The feed conversion ratio (FCR) decreased significantly as dietary DP level increased from 203 g kg^{-1} to 491 g k^{-1} DP, and thereafter, FCR increased as dietary DP level rose up to 635 g kg^{-1} (Table 4.4, Figure 4.3). However, FCRs were not significantly different ($P > 0.05$) for diets T35, T40, T45, T50, T55, and T65 (Table 4.4). A quadratic model estimated the minimum FCR to be 1.8 at 45.8 % DP, or 458 g kg^{-1} DP (Figure 4.3).

Unlike the FCRs, the protein efficiency ratio (PER) showed an overall decreasing trend with increasing digestible protein levels (Table 4). Although PER values were highest in crabs fed T20 and T25 and lowest in crabs fed T60 and T65, the PER values were similar in groups fed T20, T25, T30, T35, T40, T45, and T50 (Table 4.4).

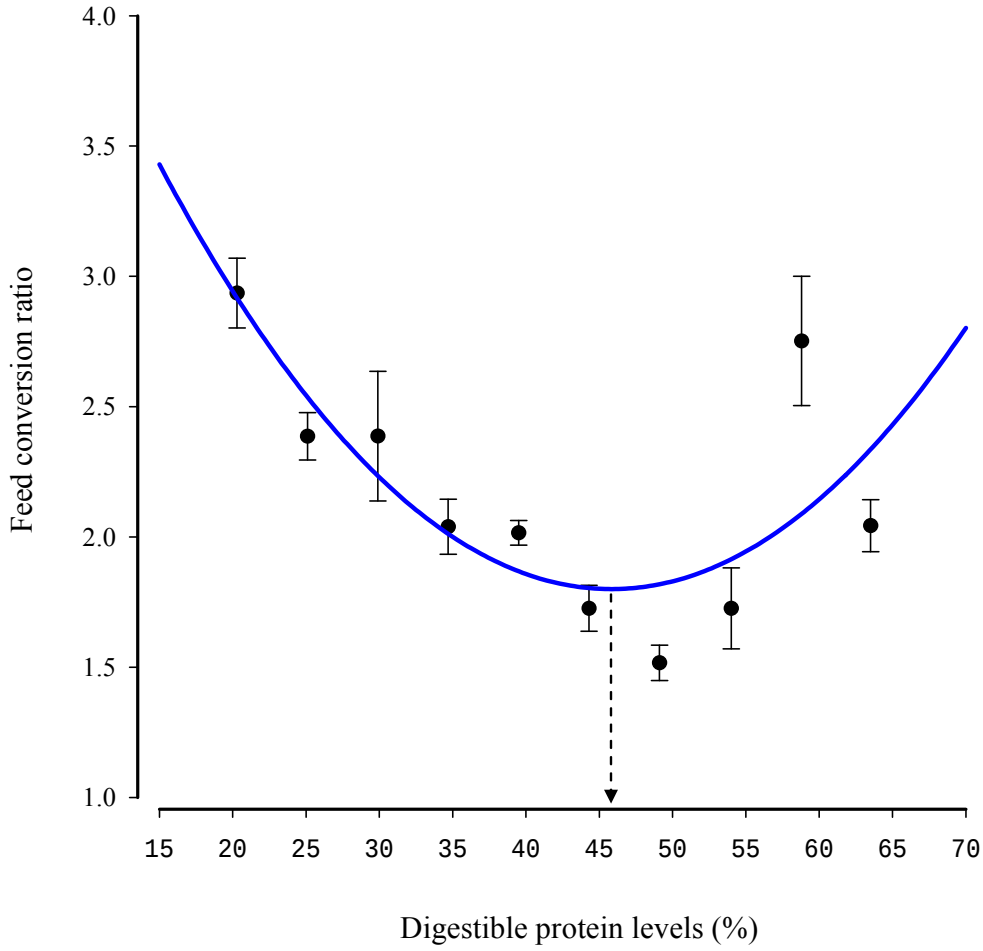

Figure 4.3 The relationship between the feed conversion ratio (FCR) and digestible protein levels. The curve was fitted using the quadratic model: $y = 5.38798 - 0.156509*x + 0.00170713*x^2$ with R=0.78 and *P*=0.0354. The minimum FCR was found at 45.8 % digestible protein (arrow)

4.4 DISCUSSION

4.4.1 Weight gain and carapace width increase

The optimum dietary protein content for maximum weight gain (WG) for mud crab in this study was 530 g DP kg^{-1}, which was similar to that of other crustaceans. For example, optimal requirement of 52 % crude protein was reported for the Kuruma prawn, *P. japonicus* (Deshimaru & Yone 1978). At 10 % lipid, the optimum protein content to maximise weight gain was 53 % for the spiny lobster, *Panulirus ornatus*, (Smith *et al.* 2003). This study showed that WG values were not significantly different in crabs fed T40, T45, T50, T55, T60, and T65. These results support the findings of Catacutan *et al.* (2002), using mud crabs grown in the Philippines, who reported that there was no significant difference in weight gain when juvenile mud crabs, *S. serrata,* were fed diets containing 32, 40, and 48 % crude protein at 6 % or 12 % lipid. In addition, although 42 % dietary protein diet maximised growth of kuruma prawns, *P. japonicus*, there was no significantly difference in WG among prawns fed the 41.6, 50.3, and 60.7 % dietary protein diets (Koshio *et al.* 1993). The high protein requirement of mud crab in this study relates to their feeding habit. In nature, mud crab often eat small fish, mollusca, and small other crabs (Hill 1976; Hill 1979a).

Although the protein requirements of juvenile mud crab determined in this study were similar to those reported for the more carnivorous crustaceans, it was higher than that for other omnivorous species. For example, the maximum weight gain in juvenile freshwater cray fish, *Cherax quadricarinarus*, was estimated to be 34.2 % crude protein (Jacinto *et al.* 2003). Furthermore, the maximum weight gain of Chinese hairy crab, *Eriocheir sinensis,* was revealed at 39 % crude protein (Mu *et al.*

1998). Similarly, *P. monodon* required protein levels of 36-44 % for maximising weight gain (Shiau & Chou 1991).

In principle, weight gain and carapace width increase can both be used for estimating optimum level of digestible protein in the diet. For digestible protein levels between 395 g kg^{-1} and 540 g kg^{-1} maximum weight gain (WG) increased by more than four-fold (400%) over the three inter-moult periods. However, maximum carapace width increase (CWI) increased by only 98 % over the same period. Thus, weight gain appears to be a more sensitive indicator for estimating digestible protein requirements. In contrast to the view of Catacutan (2002), who suggested that CWI was a better indicator of performance than WG values because the graded protein levels did not affect to the WG of those mud crabs.

4.4.2 Inter-moult period and specific growth rate

There was no significant difference in the IMP between diets containing DP levels ranging from 251 g kg^{-1} to 588 g kg^{-1} (Table 4.3). These results are consist with those of Catacutan (2002) who found that crab fed with diets containing crude protein in the range 32-48 % at either 6 or 12 % lipid did not have significantly IMP difference. However, the present study found that a significant decrease of IMP values was only detected in crabs fed the lowest DP level (T20) and the highest DP level (T65). The effect of high or low protein levels on IMP was reported on the Australian fresh water crayfish, *Cherax albidus* and *Cherax destructor* Clark. The higher dietary protein levels, the shorter inter-moult period was observed (Jones *et al.* 1996).

Although the shortest IMP was found in crabs fed the highest protein content of 635 g kg^{-1}, the maximum specific growth rate was found at the lower protein content of 540 g kg^{-1}DP. This result was similar to the finding in juvenile tropical spiny lobster,

P. ornatus, (Smith *et al.* 2003). The authors found that the best specific growth rate of lobster was obtained at protein level of 530 g kg^{-1} at 100 g kg^{-1} lipid. Furthermore, when spiny lobster (*Panulirus ornatus*) was fed one of five isolipidic feeds (approximately 130 g kg^{-1}) in which the crude protein was serially incremented between 330 and 610 g kg^{-1}, the higher growth rate was obtained in group fed with the highest protein diet (Smith *et al.* 2005b). By contrast, the maximum specific growth rate was revealed at lower protein requirement in many crustacean species such as 39 % crude protein in the Chinese hairy crab, *Eriocheir sinensis,* (Mu *et al.* 1998), 350-400 g kg^{-1} crude protein in *P.monodon* (Burford *et al.* 2004), and 310 g kg^{-1} crude protein in Australian redclaw crayfish, *Cherax quadricarrinatus*, (Jacinto *et al.* 2005).

4.4.3 Feed conversion ratio and protein efficiency

This study indicated that the minimum FCR value of 1.8 was obtained with a DP level of 458 g kg^{-1}. A decrease or increase on either side of this optimum DP value resulted in an increase in FCR (Figure 4.3). Similar trends have been reported in brown trout, *Salmo trutta,* (Arzel *et al.* 1995), in snakehead, *Channa striata* (Mohanty & Samantaray 1996), and crayfish, *Cherax quadricarinatus,* (Jacinto *et al.* 2003; Jacinto *et al.* 2004). The FCR values (1.5-2.9) in the present study were higher than those in crayfish, *Cherax quadricarinatus,* (Jacinto *et al.* 2003) where it was reported that the FCR of the crayfish were in the ranges of 1.1-1.3 and 1.3-1.5 for males and females, respectively. However, the present FCR values were lower than those in mud crab, *S. serrata* studied by results differ from those reported by Catacutan (2002), who found no significant difference in FCR ranging from 3.37 to 4.21 in the mud crab *S. serrata* fed protein levels ranging from 32 % to 48 % with lipid levels of 6 or 12 %.

The protein efficiency ratio (PER) of the present study decreased from 1.7 to 0.8 with increasing dietary digestible protein contents from 203 to 635 g kg^{-1}. Similar trend of PER was reported for several crustacean species. For example, the PER decreased from 1.4 to 1.1 with increasing crude protein from 31.4 to 60.7 g kg^{-1} in kuruma prawns, *P. japonicus* (Koshio *et al.* 1993); from 0.80 to 0.3 with graded crude protein from 390 to 548 in Chinese hairy crab, *Eriocheir sinensis*, (Mu *et al.* 1998); from 1.04 to 0.44 for females and from 1.44 to 0.49 for males of the crayfish, *Cherax quadricarinatus* with increasing crude protein from 270 to 450 g kg^{-1} (Jacinto *et al.* 2004). Clearly, the PER values of the present study were higher than those reported for other crustacean species. As a result, the nitrogen *(P*rotein) in the lower DP diets was utilised better by the mud crabs in this study.

4.5 Conclusion and recommendation

This study found that the mud crab, *S. serrata* needed approximately 530 g kg^{-1}DP diet to maximise weight gain but they required 458 g kg^{-1}DP to minimise feed conversion ratio. The high DP requirements of mud crabs may be because the present study used a mixture of fish meal, blood meal, and shrimp meal as protein sources, however, no attempt was made to match amino acids to those in crab tissue. The next chapter will describe the modification of the essential amino acid profiles of the diets in an attempt to improve the protein requirement.

Chapter 5 Optimal dietary specifications for total balanced essential amino acid and digestible energy by juvenile mud crab, *Scylla serrata* (Forskal 1775),

5.1 INTRODUCTION

There are 20 standard amino acids in crustacean proteins (Deshimaru & Shigheno 1972; Tacon 1990a; Akiyama *et al.* 1992) which are divided into two groups, essential amino acids and non-essential amino acids. The essential amino acids (EAAs) are those cannot be synthesized by an animal and must be present in the diet, while the non-essential amino acids can be synthesized from other components in the animal's body. The 10 essential amino acids are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Two additional amino acids, tyrosine and cystine, are usually added to diets (Guillaume 1997) for the reason explained below.

Clearly, essential amino acid requirements are influenced by interactions between essential amino acids, between essential and non-essential amino acids, and between essential amino acids and energy. For instance, if cystine is deficient in a fish diet, fish can synthesise it from methionine, and this relationship also exists between phenylalanine and tyrosine (Tacon & Cowey 1985). Further, the level of one EAA affects the requirement of other EAAs. Batterham (1992) reported that if a diet lacks one or more EAAs, then the animal cannot fully utilise other EAAs. In this case, these excess EAAs are broken down and used as energy. Furthermore, the energy level influences significantly the requirement for EAAs in a diet. If the energy in a diet is insufficient, surplus EAAs cannot be utilised for protein synthesis but are broken down and used as an energy source. In contrast, if EAAs are deficient then

surplus energy is converted to fat (Batterham 1992). Therefore, the requirement for essential amino acids needs to be evaluated at different energy levels.

The aim of this study was to determine the optimal dietary specifications for total balanced essential amino acid (TB-EAA) at three levels of digestible energy (DE) by juvenile mud crabs, *Scylla serrata*, with the following specific aims.

- To estimate an optimal level of total balanced essential amino acid and digestible energy to maximise weight gain, carapace width increase, specific growth rate, protein efficiency ratio, and apparent net protein utilisation.
- To determine the optimal levels of TB-EAA and DE to minimise feed conversion ratio and inter-moult period.

5.2 MATERIALS AND METHODS

This investigation was conducted at the Darwin Aquaculture Centre (DAC), Northern Territory, Australia (see Section 2.1.1 of Chapter 2) from 15th March to 3rd July 2004 with juvenile mud crabs of 0.39 ± 0.01 g (crab 5).

5.2.1 Juvenile mud crab preparation

Preparation of juvenile mud crabs (crablets) was similar to the steps described in Section 4.2 of Chapter 4. A total of 2,000 juvenile mud crabs at crab 4 stage (four moults from megalops) were obtained from one batch hatched at the DAC hatchery. When crabs moulted to crab 5 (5 moults from megalops stage), they were recorded as moult zero (M0), then one hundred and eight (108) M0-crablets were selected for the feeding trial.

5.2.2 Culture system

One hundred and eight plastic containers of 2 L each were used (Figure 5). Each container held one crablet. A flow through system supplied seawater to each container at rate 8 L hour^{-1} container^{-1} and maintained a depth of 150 mm in all containers. During the experimental period, water temperature, salinity, pH and dissolved oxygen were 29.0 ± 2.6 ^{o}C, 31.0 ± 1.8 ‰, 7.7 ± 0.3, and 5.6 ± 0.3 mg L^{-1}, respectively. Details of water preparation and water measurement are described in Sections 2.3-2.5 of Chapter 2.

5.2.3 Dietary ingredients

The ingredients used in this study are listed in Table 5.1. Fish meal, wheat flour, cellulose, cod liver oil, shrimp meal were taken from Section 3.1, Chapter 3.

Table 5.1 Sources of used ingredients

Ingredients	Sources
Australian fish meal	Skretting Co., Cambridge, Tasmania, Australia
Bread wheat flour	Goodman Fielder, North Ryde, NSW, Australia)
Cod liver oil	Healtheries of New Zealand, Auckland, New
Alpha cellulose	RidleyAgriProducts (Pakenham, Vic., Australia)
Vitamin premix	Skretting Co., Cambridge, Tasmania, Australia
Mineral premix	Skretting Co., Cambridge, Tasmania, Australia
Danish dried shrimp meal	RidleyAgriProducts (Pakenham, Vic., Australia)
Astaxanthin (10 %)	RidleyAgriProducts (Pakenham, Vic., Australia)
Mono sodium glutamate	Ajinomoto, Kuala Lumpur, Malaysia
Alginic acid	Sigma-Aldrich, Inc., MO, USA
L-Arginine	As above
L-Histidine	As above
L-Isoleucine	As above
L-Leucine	As above
L-Lysine HCl	As above
L-Methionine	As above
L-Phenylalanine	As above
L-Threonine	As above
L-Valine	As above
L-Tyrosine	As above
L-Cystine	As above
L-Tryptophan	As above

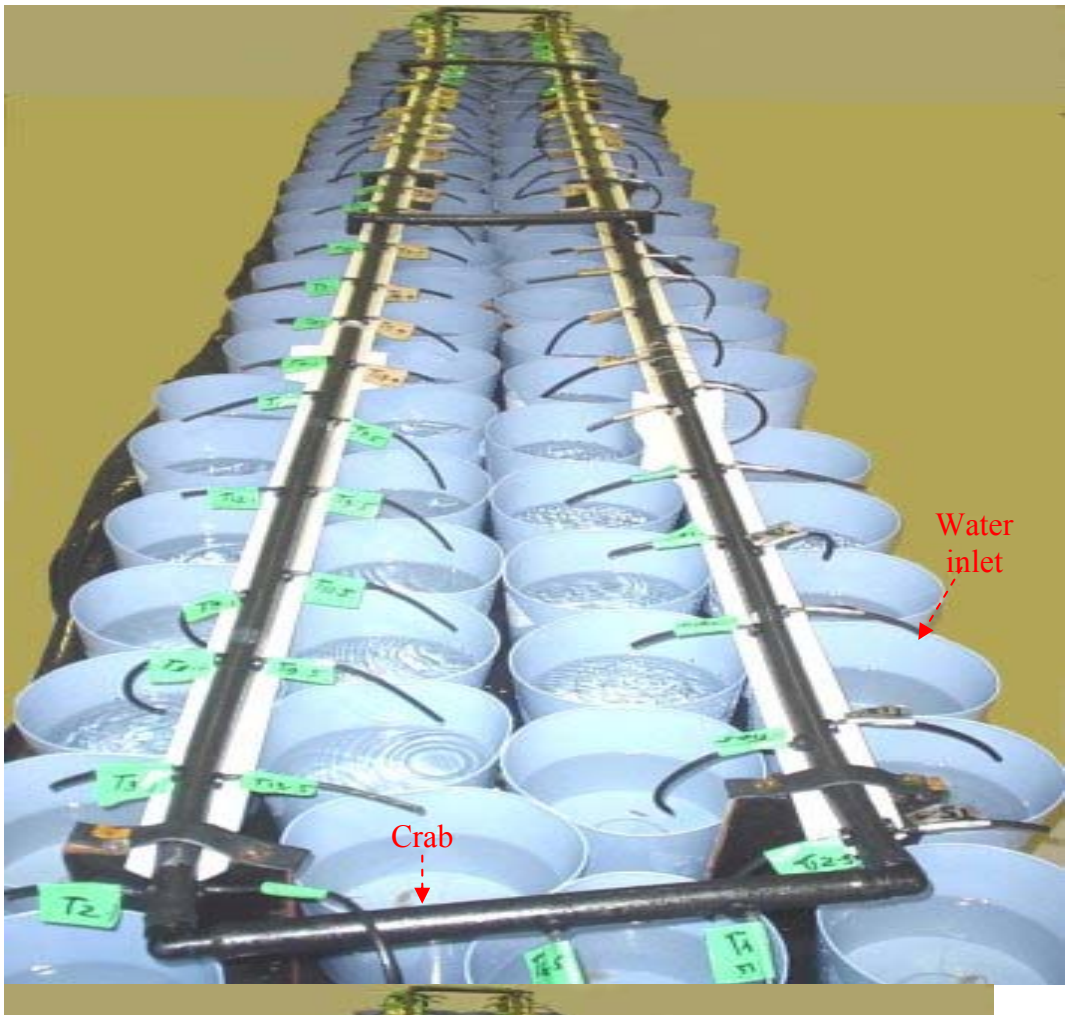

Figure 5.1 Experimental system used to determine optimal specifications of *S. serrata* for total essential amino acid and digestible energy

5.2.4 Composition of ingredients and apparent digestibility for energy

The compositions of these ingredients are detailed in Section 3.1 of Chapter 3. The crude protein (CP), gross energy (GE), and AD for energy (ADE) values of these ingredients are summarised in Table 5.2.

Table 5.2 Crude protein (CP) and gross energy (GE) (based on dry weight), and apparent digestibility for energy (ADE) of the ingredients used in feed formulation for this trial.

Ingredients	CP (g kg^{-1})	GE (MJ kg^{-1})	ADE (%)
Australian fish meal	766.6	21.6	91.5
Danish dried shrimp meal	704.6	20.9	84.4
Bread wheat flour	114.8	18.0	79.9
Cod liver oil	nd	39.5	89.2
Alpha cellulose	nd	15.4	77.4

nd = not determined

5.2.5 Essential amino acid profiles

The EAA compositions of the dietary ingredients are shown in Table 5.3.

Table 5.3 EAA compositions of the dietary ingredients expressed on a dry matter basis

EAAs	Fish meal (g kg^{-1})	Shrimp meal (g kg^{-1})	Wheat flour (g kg^{-1})	Mud crab tissue (g/100g protein)
Arginine	60.0	42.6	4.3	4.8
Histidine	27.0	23.4	2.5	3.4
Isoleucine	36.0	46.2	4.7	5.1
Leucine	59.0	56.8	8.7	6.5
Lysine	69.0	47.6	2.5	6.8
Methionine	23.0	17.8	1.8	4.8
Cystine	10.0	5.7	3.0	1.2
Phenylalanine	33.0	70.3	6.0	4.5
Tyrosine	27.0	29.8	3.4	4.9
Threonine	39.0	32.0	3.3	5.7
Valine	40.0	34.1	5.0	4.5
Tryptophan	8.0	7.1	1.2	1.0

EAAs: essential amino acids

The EAA profiles of shrimp meal and tryptophan of fish meal were taken from Mu *et al.* (2000). The EAA profile of fish meal was taken from Allan *et al.* (2000) except for tryptophan. The EAA profile of wheat flour was taken from Tacon (1990b). The EAA profile of mud crab tissue was taken from Mukundan *et al.* (1981).

5.2.6 Experimental diets

The experimental diets, consisting of basal ingredients and supplemental EAAs, were prepared with 4 levels of total balanced essential amino acid (195, 258, 327, and 398 g kg^{-1} diet) and 3 digestible energy levels (14.7, 15.7, and 16.7 MJ kg^{-1} diet). The total essential amino acids (TB-EAA) are the sum of the total essential amino acids of the basal ingredients and the supplementary crystalline essential amino acids. The several steps balanced total essential amino acid for each diet are presented at Section 5.2.7.

The fish meal, shrimp meal, wheat flour, cod liver oil, cellulose, and alginic acid in diets were the same as those described in Section 3.1 of Chapter 3. The composition of the 12 experimental diets is presented in Tables 5.4-5.6.

Table 5.4 Composition of 12 diets (expressed on dry matter basis) with 4 levels of total balanced essential amino acid (TB-EAA) and 3 levels of digestible energy (DE) (g kg^{-1})

Diet code	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Fish meal	186.0	186.0	186.0	252.0	252.0	252.0	324.0	324.0	324.0	402.0	402.0	402.0
Shrimp meal	124.0	124.0	124.0	168.0	168.0	168.0	216.0	216.0	216.0	268.0	268.0	268.0
Wheat flour	246.0	246.0	246.0	211.0	211.0	211.0	170.0	170.0	170.0	50.0	50.0	49.0
Cod liver oil	60.0	102.0	145.0	40.0	83.0	126.0	19.0	61.0	103.0	1.0	46.0	90.0
Alpha cellulose	254.0	212.0	169.0	180.0	137.0	94.0	102.0	60.0	18.0	88.0	43.0	0.0
Vitamin mixture	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Mineral mixture	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Astaxanthin (10 %)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Alginic acid	41.0	41.0	41.0	41.0	41.0	41.0	41.0	41.0	41.0	41.0	41.0	41.0
MSG	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
C-EAA mixture	53.7	53.7	53.7	72.4	72.4	72.4	92.8	92.8	92.8	114.7	114.7	114.7
DP (calculated)	232.0	232.0	232.0	306.3	306.3	306.3	387.1	387.1	387.1	469.6	469.6	469.5
CP1 (calculated)*	258.2	258.2	258.2	335.8	335.8	335.8	420.1	420.1	420.1	502.7	502.7	502.6
CP2 (analysed)*	310.5	318.4	322.8	422.0	415.0	415.7	496.2	514.3	502.3	627.3	635.0	631.7
DE (MJ kg^{-1})	14.7	15.7	16.7	14.7	15.7	16.7	14.7	15.7	16.6	14.6	15.7	16.7
TB-EAA	194.9	194.9	194.9	258.2	258.2	258.2	327.1	327.1	327.1	398.1	398.1	398.1

Notes: MSG: Mono sodium glutamate; C-EAA: Crystalline essential amino acid; DP: Digestible protein; CP1: Calculated crude protein; CP2: Analysed crude protein; TB-EAA: Total balanced essential amino acids; DE: Digestible energy

* Calculated crude protein and analyzed crude protein are significantly different because the calculated crude protein (CP1) was computed without including amount of crystalline essential amino acid (C-EAA mixture) while analyzed crude protein (CP2) were determined for whole diet.

Table 5.5 Calculated composition of total balanced essential amino acid (TB-EAA) for each diet (g kg^{-1})*

Diet code	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Arginine	17.5	17.5	17.5	23.2	23.2	23.2	29.4	29.4	29.4	35.8	35.8	35.7
Histidine	12.3	12.3	12.3	16.3	16.3	16.3	20.6	20.6	20.6	25.1	25.1	25.1
Isoleucine	18.6	18.6	18.6	24.6	24.6	24.6	31.2	31.2	31.2	38.0	38.0	38.0
Leucine	23.8	23.8	23.8	31.5	31.5	31.5	39.9	39.9	39.9	48.5	48.5	48.5
Lysine	24.9	24.9	24.9	33.0	33.0	33.0	41.8	41.8	41.8	50.9	50.9	50.9
Methionine	17.6	17.6	17.6	23.3	23.3	23.3	29.6	29.6	29.6	36.0	36.0	36.0
Cystine	4.5	4.5	4.5	6.0	6.0	6.0	7.6	7.6	7.6	9.2	9.2	9.2
Phenylalanine	16.6	16.6	16.6	22.0	22.0	22.0	27.8	27.8	27.8	33.9	33.9	33.9
Tyrosine	17.9	17.9	17.9	23.7	23.7	23.7	30.0	30.0	30.0	36.6	36.6	36.6
Threonine	20.9	20.9	20.9	27.6	27.6	27.6	35.0	35.0	35.0	42.6	42.6	42.6
Valine	16.6	16.6	16.6	22.0	22.0	22.0	27.8	27.8	27.8	33.9	33.9	33.9
Tryptophan	3.7	3.7	3.7	4.9	4.9	4.9	6.3	6.3	6.3	7.6	7.6	7.6

* Data of each EAA was calculated from EAA profile of each ingredient in diet.

Table 5.6 Crystalline essential amino acid supplements added to each diet (g kg^{-1})

Diet code	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
L-Arginine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L-Histidine	3.8	3.8	3.8	5.0	5.0	5.0	6.4	6.4	6.4	7.9	7.9	7.9
L-Isoleucine	5.0	5.0	5.0	6.8	6.8	6.8	8.8	8.8	8.8	10.9	10.9	10.9
L-Leucine	3.6	3.6	3.6	5.2	5.2	5.2	7.0	7.0	7.0	9.2	9.2	9.2
L-Lysine HCL	7.2	7.2	7.2	9.1	9.1	9.1	11.3	11.3	11.3	13.3	13.3	13.3
L-Methionine	10.7	10.7	10.7	14.2	14.2	14.2	18.0	18.0	18.0	21.9	21.9	21.9
L-Cystine	1.2	1.2	1.2	1.9	1.9	1.9	2.6	2.6	2.6	3.5	3.5	3.5
L-Phenylalanine	0.3	0.3	0.3	0.6	0.6	0.6	0.9	0.9	0.9	1.5	1.5	1.5
L-Tyrosine	8.3	8.3	8.3	11.2	11.2	11.2	14.3	14.3	14.3	17.6	17.6	17.6
L-Threonine	8.8	8.8	8.8	11.8	11.8	11.8	14.9	14.9	14.9	18.2	18.2	18.2
L-Valine	3.7	3.7	3.7	5.1	5.1	5.1	6.7	6.7	6.7	8.4	8.4	8.4
L-Tryptophan	1.1	1.1	1.1	1.5	1.5	1.5	1.9	1.9	1.9	2.5	2.5	2.5
Total	53.7	53.7	53.7	72.4	72.4	72.4	92.8	92.8	92.8	114.7	114.7	114.7

Note: Lysine HCL contains 77.84 % lysine

5.2.7 Calculation of total balanced essential amino acids

The essential amino acid balance in a diet was made by matching its A/E ratio with that of EAAs of mud crab's tissue. A/E ratio is a ratio between the weight of that EAA divided by the weight of the total EAAs and multiplied by 1000 (Arai 1981). There are 6 main steps to be followed to obtain a matching balance of EAAs of a diet having the same A/E ratio of mud crab's tissue. These steps are as follows:

- Step 1: Design a series of diets with 4 levels of crude protein: 260, 340, 420, and 500 g kg^{-1} and 3 levels of digestible energy: 14.7, 15.7, and 16.7 MJ kg^{-1} (see Table 5.4)
- Step 2: Calculate EAA profile of each diet (see in Appendix 5.1)
- Step 3. Compute A/E of EAAs in the diets and that of EAAs in mud crab's tissue (see Appendix 5.1).
- Step 4. For each diet, determine the difference between the A/E values of EAAs in the diet and those of EAAs in the mud crab tissue. The EAA having the highest positive value is the one that does not need to change (see Appendix 5.1). The negative difference in the A/E ratio of a particular EAA indicates that that EAA should be supplemented with the crystalline form.
- Step 5. Set up the EAA balance (use the highest difference from Step 4 as ZERO supplied value)

  The following shows an example of the calculation. For diet T1, arginine was set at zero supplied value because arginine had the highest positive difference. All other EAAs in the diet would follow the ratio of arginine in mud crab tissue. For example, the amount of histidine in diet T1 was calculated as follows: the level of arginine in the T1 diet was 17.5 g kg^{-1}, and the A/E of arginine in the mud crab's

tissue was 89.8. The A/E ratio of histidine in the mud crab's tissue is 63.1. In order to make histidine in the diet T1 balanced (relative to arginine), the amount of histidine in T1 was to be 17.5 * 63.1/89.8=12.3 g kg^{-1}. This value is amount of histidine in the total balanced EAAs of diet T1 (as in Table 5.5).

- Step 6: To determine the amount of each crystalline EAA needs to supply (X). X is the difference between the amount of particular EAA in balance and the amount of that EAA in the diet. For example, the value of histidine in balance is 12.3 g kg^{-1} (step 5) and the value of histidine in the basal ingredients that made up the T1 diet is 8.5 g kg^{-1}, thus the amount of crystalline histidine needed is 12.3-8.5=3.8 g kg^{-1} (as in Table 5.6).

5.2.8 Diet preparation

Alginic acid was used to coat crystalline amino acids before mixing them with other ingredients (Millamena *et al.* 1999; Alam *et al.* 2004b). A portion of alginic acid (10 g kg^{-1}diet) was dissolved in approximate 20 mL of hot tap water (70-80 ^{o}C), into which the C-EAA mixture was added, mixed together until dough-like consistency. These coated EAA substances were mixed with other dry ingredients and cod liver oil to form a pre-mixture. The remaining alginic acid (31 g kg^{-1}diet) was dissolved thoroughly in hot tap water (70-80 ^{o}C) at rate of 500 mL kg^{-1} to form a viscous solution into which the pre-mixture was added and thoroughly mixed to produce a moist mixture. The moist mixture was then made into pellets by following the steps described in Section 2.6 of Chapter 2.

Finally, pellets of 1.5 mm in diameter and 100 mm in length were dried by placing in a 40 ^{o}C oven for until the moisture content of dry pellets were less than 10 %. The

long pellets were broken down into shorter pellets of approximate 10-15 mm, and stored in a -20 ^{o}C freezer until required.

5.2.9 Feeding and collection of uneaten feed

The feed for each replicate was weighted and kept separately in a small plastic jar. Animals were fed to apparent satiation twice a day at 09:00 and 16:00 hours. Crabs accepted all experimental diets very quickly after being fed. At the end of the experiment, the unused feed in the plastic jar of each replicate was weighed. The feed sample was dried to determine an exact amount of feed which were used on dry matter basis.

The uneaten feed from the 9:00 hours feeding was collected between 3:00 and 15:00 hours; the uneaten feed from that at 16:00 hours was collected between 8:00 and 9:00 hours the following morning. Uneaten feed was siphoned from each tank into a mesh of 30 μm mesh size, gently rinsed with distilled water, and then stored at a -20 ^{0}C freezer for later analysis. All uneaten feed were dried before being weighted.

The weight difference between the feed at the beginning and the unused feed at the end of the feeding trial and the uneaten feed gave the amount of feed intake in each treatment.

5.2.10 Chemical analysis

The diets and crabs (whole initial and final crabs) were dried in a freeze-drier and ground into powder using a mortar and pestle. Total nitrogen was determined by Kjeldahl digestion followed by measurement with Lachat 8000 (Lachat Instruments Milwaukee, WI, USA) as described by Diamond (1992). Total lipid was determined using the method described by Mason and Nell (1995) which used chloroform : methanol (2:1) to extract lipids. Soluble carbohydrates were estimated using the

method described by Kochert (1978). Details of chemical analysis methods are described in Section 2.8 of Chapter 2.

5.2.11 Measurements

Wet weight, carapace width, feed consumption, and date of moulting were recorded for three consecutive moulting intervals. To avoid the possibility of death after moulting (soft shell), crabs were only weighed 4 days after moulting. Weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio, carapace width increase, and inter-moult period were calculated using the equations given in Section 2.9 of Chapter 2. The apparent net protein utilization (ANPU) was calculated using an equation described by Tacon (1990a),

$$\text{ANPU}(\%) = \frac{\text{Pb} - \text{Pa}}{\text{Pi}} \text{x} 100$$

where Pb is the total body protein at the end of the feeding trial, Pa is the total body protein at the beginning of the feeding trial, and Pi is the amount of protein consumed over the period of the feeding trial.

5.2.12 Experimental design

The trial was designed as a two-factor experiment with four levels of total balanced essential amino acid (Factor 1) and 3 levels of digestible energy (Factor 2), giving a total of 12 treatments. There were 9 replicates per treatment (9 replicates x 4 TB-EAA levels x 3 DE levels = 108 crabs). During the course of the experiment, a few crabs died at moulting and thus the statistical analysis was based on unequal replications (Table 5.7).

5.2.13 Statistical analysis

Factorial ANOVA was used to detect the differences between treatments. When the mean of each response variable was significantly different, the Tukey HSD test was used for multiple comparisons between means. A distance weighted least square model was used to examine the interaction between two factors. All analyses were processed using *STATISTICA 7* (StatSoft, Inc., OK, USA, 2004).

5.3 RESULTS

5.3.1 Weight gain, carapace width increase, specific growth rate, and inter-moult period

Factorial ANOVA (Appendix 5.2) shows that increasing levels of TB-EAA has significant effect on weight gain, carapace width increase, specific growth rate, and inter-moult period ($P < 0.05$) but the means of these variables were not significantly different when varying digestible energy values was included ($P > 0.05$).

The highest WG (1095 %) and the highest CWI (130 %) were obtained in crabs fed diets containing TB-EAA of 258 g kg^{-1} and DE of 15.7 MJ kg^{-1} (Table 5.7) whereas the highest SGR (4.5 % day^{-1}) and the shortest IMP (55 days) were observed in crabs fed a diet composing of 327 g kg^{-1} TB-EAA and 14.7 MJ kg^{-1} DE.

Interaction between two factors (TB-EAA and DE) resulted in maximum weight gain over a range of TB-EAA and DE levels, as shown in Figure 5.2. Maximum weight gain was obtained when TB-EAA ranged from 250 g kg^{-1} to 275 g kg^{-1} and DE ranged from 15.4 MJ kg^{-1} to 16.0 MJ kg^{-1} (Figure 5.2). Clearly, any value of TB-EAA and DE in the smallest ring of Figure 5.2 could be optimal for weight gain in juvenile mud crab, as is also shown by the significance of differences between means in Table 5.7.

Table 5.7 Weight gain (WG), carapace width increase (CWI), specific growth rate (SGR), and inter-moult period (IMP) of crablets fed diets containing 4 levels of total balanced essential amino acid (TB-EAA) and 3 levels of digestible energy (DE) from M0 to M3 (the data were expressed as means ± Standard error, N= number of replicates)*

TB-EAA ($g\ kg^{-1}$)	DE ($MJ\ kg^{-1}$)	TB-EAA/DE ($g\ MJ^{-1}kg^{-1}$)	WG (%)	CWI (%)	SGR (% day^{-1})	IMP (days)	N
195	14.7	13	753.5 ± 50.3^{ab}	108.7 ± 5.6^{ab}	3.0 ± 0.2^{a}	72.8 ± 5.8^{a}	9
	15.7	12	852.8 ± 40.7^{abc}	114.5 ± 3.9^{ab}	3.0 ± 0.1^{a}	77.1 ± 3.9^{a}	9
	16.7	12	832.7 ± 78.5^{abc}	117.6 ± 6.4^{ab}	2.9 ± 0.1^{a}	77.9 ± 4.1^{a}	8
258	14.7	18	895.3 ± 55.3^{abc}	118.4 ± 4.3^{ab}	3.5 ± 0.3^{ab}	69.8 ± 5.9^{ab}	9
	15.7	16	1095.1 ± 83.2^{c}	130.1 ± 5.2^{b}	3.6 ± 0.2^{ab}	69.1 ± 4.3^{ab}	9
	16.7	15	903.4 ± 71.3^{abc}	115.7 ± 6.0^{ab}	3.3 ± 0.3^{ab}	72.7 ± 6.5^{ab}	7
327	14.7	22	998.5 ± 52.2^{bc}	121.5 ± 4.1^{ab}	4.5 ± 0.3^{b}	54.9 ± 3.7^{b}	7
	15.7	21	873.5 ± 66.2^{abc}	116.8 ± 5.5^{ab}	3.7 ± 0.2^{ab}	63.4 ± 4.1^{b}	8
	16.7	20	789.4 ± 93.0^{abc}	107.4 ± 8.5^{ab}	3.4 ± 0.3^{ab}	67.3 ± 7.8^{b}	8
398	14.7	27	632.5 ± 81.8^{a}	98.4 ± 8.2^{a}	3.4 ± 0.1^{ab}	57.6 ± 3.3^{b}	8
	15.7	25	664.2 ± 66.9^{ab}	104.4 ± 6.9^{ab}	3.4 ± 0.3^{ab}	60.2 ± 3.8^{b}	6
	16.7	24	781.9 ± 58.7^{abc}	108.2 ± 6.1^{ab}	3.6 ± 0.3^{ab}	62.9 ± 5.3^{b}	7

* Means within the same column having a similar superscript letter are not significantly different at 5 % of significance ($P > 0.05$)

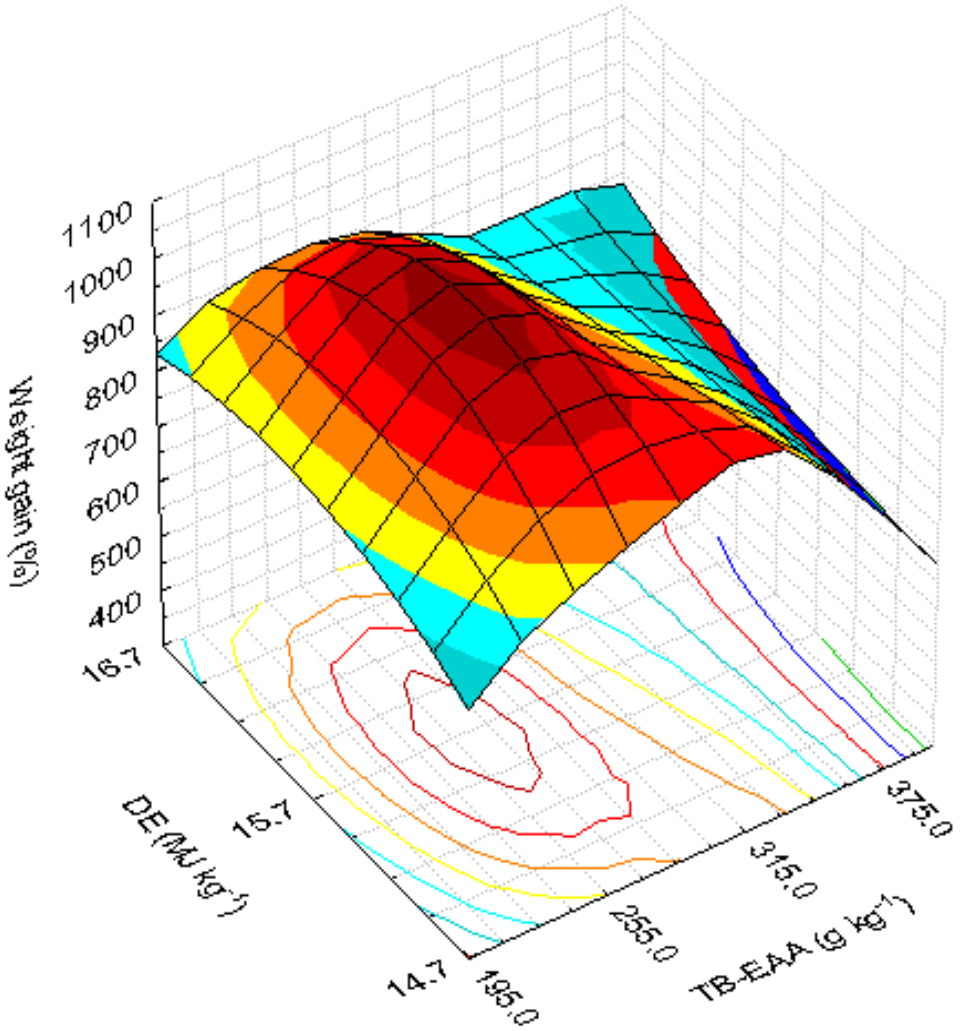

Figure 5.2 A model of distance weighted least squares describes the relationship of the weight gain of mud crabs fed diets consisting of 4 levels of total balanced essential amino acid (TB-EAA) and 3 levels of digestible energy (DE)

5.3.2 Feed conversion ratio, protein efficiency ratio, and apparent net protein utilisation

Table 5.8 Feed conversion ratio (FCR), protein efficiency ratio (PER), and apparent net protein utilisation (ANPU) of crabs fed diets containing 4 levels of TB-EAA and 3 levels of DE (the data were expressed as means ± SE, N= number of replicates)*

TB-EAA (g kg^{-1})	DE (MJ kg^{-1})	TB-EAA/DE (g $MJ^{-1}kg^{-1}$)	FCR	PER	ANPU (%)	N
195	14.7	13	1.3 ± 0.1^{abc}	2.5 ± 0.1^{de}	21.5 ± 0.8^{bc}	9
	15.7	12	1.1 ± 0.1^{ab}	3.0 ± 0.2^{e}	26.6 ± 2.2^{cd}	9
	16.7	12	1.3 ± 0.1^{abc}	2.5 ± 0.2^{de}	25.6 ± 1.8^{cd}	8
258	14.7	18	1.2 ± 0.0^{abc}	2.1 ± 0.1^{cd}	28.5 ± 0.8^{de}	9
	15.7	16	1.0 ± 0.0^{a}	2.3 ± 0.1^{d}	34.7 ± 1.4^{e}	9
	16.7	15	1.3 ± 0.1^{abc}	1.9 ± 0.1^{bcd}	26.1 ± 1.9^{cd}	7
327	14.7	22	1.4 ± 0.1^{abc}	1.5 ± 0.1^{abc}	21.3 ± 1.1^{bc}	7
	15.7	21	1.2 ± 0.1^{abc}	1.6 ± 0.1^{abc}	18.4 ± 0.9^{ab}	8
	16.7	20	1.6 ± 0.2^{bc}	1.4 ± 0.1^{ab}	18.4 ± 2.0^{ab}	8
398	14.7	27	1.7 ± 0.1^{c}	1.0 ± 0.1^{a}	12.7 ± 1.2^{a}	8
	15.7	25	1.7 ± 0.2^{c}	1.0 ± 0.1^{a}	10.9 ± 1.2^{a}	6
	16.7	24	1.7 ± 0.2^{c}	1.0 ± 0.1^{a}	13.1 ± 1.4^{a}	7

* Means within the same column having a similar superscript letter are not significantly different at 5 % of significance ($P > 0.05$)

ANOVA shows that FCR values were influenced significantly by the various levels of TB-EAA but not by DE levels. By contrast, the PER values were affected by both levels of TB-EAA and DE levels (Appendix 5.2).

The lowest FCR (1.0) was found in crabs fed a diet consisting of 15.7 MJ kg^{-1}DE and 258 g kg^{-1} TB-EAA, and the highest FCR (1.7) was obtained in groups fed the highest TB-EAA (398 g kg^{-1}) in each level of DE (Table 5.8). However, in general means for the higher and lower FCR values were not significantly different.

ANOVA (Appendix 5.2) showed that ANPU values were significantly affected by an increase in TB-EAA levels and by an interaction between TB-EAA levels and DE levels ($P < 0.05$). This interaction is illustrated in Figure 5.3.

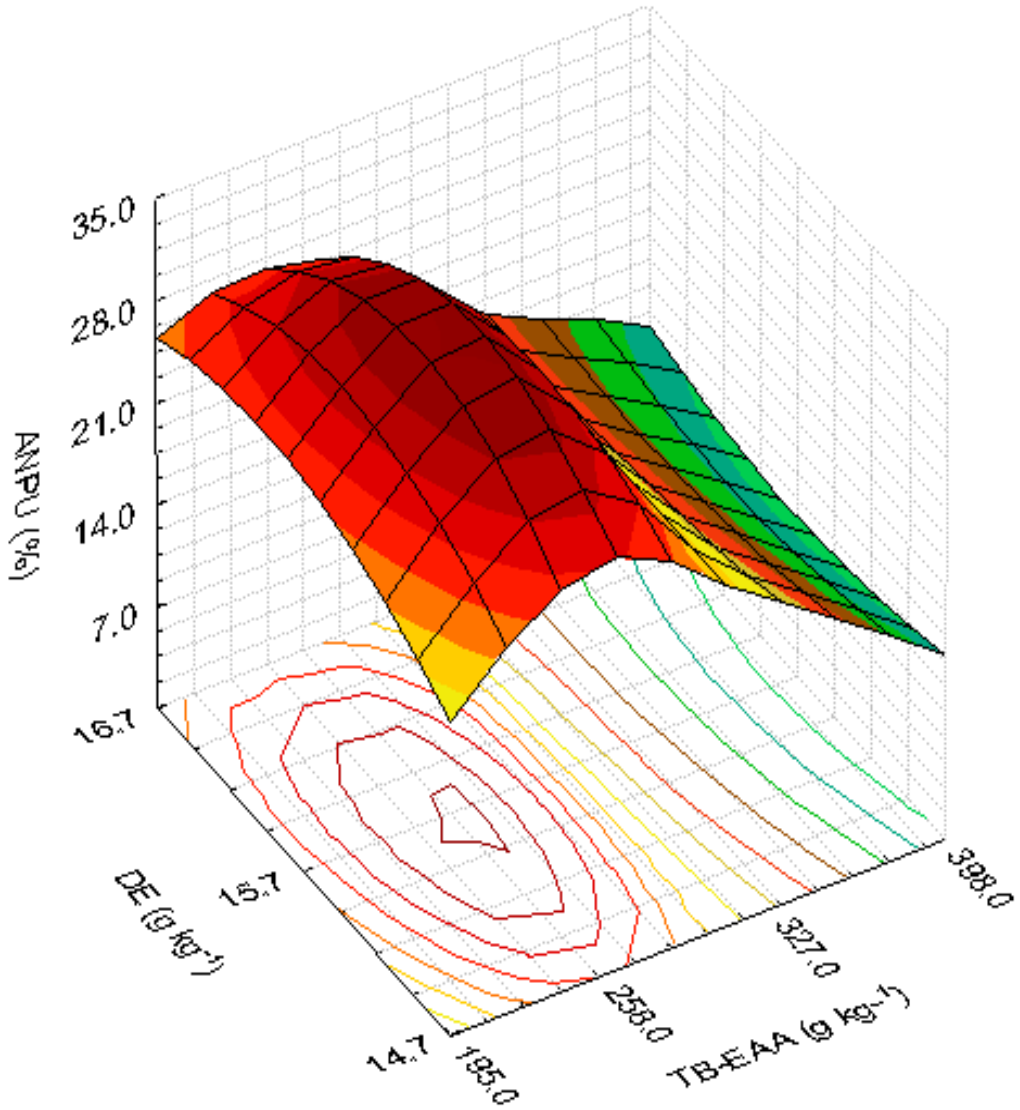

Figure 5.3 A model of distance weighted least squares describes a relationship between total balanced essential amino acid (TB-EAA) and digestible energy (DE) levels on apparent net protein utilization of mud crab.

The highest ANPU value was obtained in crabs fed the diet containing 258 g kg^{-1} TB-EAA and 15.7 MJ kg^{-1} DE; the lowest values were seen in the crabs fed diets containing 398 g kg^{-1} TB-EAA. The distance weighted least squares model shows that, as for weight gain (see Figure 5.2), a range of TB-EAA and DE levels could be optimal for maximum ANPU (Figure 5.3).

At the same DE level, there was a reduction in PER values as TB-EAA level increased. Nevertheless, at the same TB-EAA level, the crabs were fed the diet containing a medium DE level (15.7 MJ kg^{-1}) had better PER values.

5.3.3 Body chemical composition

Table 5.9 Dry matter (DM), crude protein (CP), total lipid (TL), and carbohydrate (CHO) of crabs whole body at the end of the feeding experiment and initial crabs (the data were expressed as means ± SE, n=3)*

TB-EAA	DE	Ratio	DM (%)	CP (%)	TL (%)	CHO (%)
	14.7	13	30.9 ± 0.1^{e}	9.6 ± 0.7^{a}	1.3 ± 0.1^{bcd}	1.2 ± 0.1^{bc}
195	15.7	12	29.8 ± 0.1^{c}	9.8 ± 0.5^{a}	1.9 ± 0.2^{e}	0.8 ± 0.0^{a}
	16.7	12	30.2 ± 0.0^{d}	10.8 ± 0.5^{ab}	1.6 ± 0.0^{de}	0.8 ± 0.0^{a}
	14.7	18	27.6 ± 0.1^{a}	14.2 ± 0.3^{de}	1.0 ± 0.1^{abc}	0.8 ± 0.0^{ab}
258	15.7	16	30.9 ± 0.1^{e}	15.0 ± 0.5^{e}	1.3 ± 0.0^{bcd}	0.9 ± 0.0^{ab}
	16.7	15	30.7 ± 0.0^{e}	14.0 ± 0.5^{cde}	1.4 ± 0.1^{cd}	0.8 ± 0.1^{a}
	14.7	22	32.7 ± 0.0^{f}	14.4 ± 0.4^{de}	1.2 ± 0.1^{abcd}	1.2 ± 0.1^{c}
327	15.7	21	29.0 ± 0.0^{b}	12.1 ± 0.8^{abc}	1.1 ± 0.0^{abc}	0.7 ± 0.1^{a}
	16.7	20	29.2 ± 0.0^{b}	13.8 ± 0.1^{cde}	0.9 ± 0.1^{abc}	0.9 ± 0.0^{ab}
	14.7	27	29.9 ± 0.1^{cd}	13.1 ± 0.2^{bcde}	0.7 ± 0.1^{a}	0.6 ± 0.1^{a}
398	15.7	25	27.7 ± 0.0^{a}	11.5 ± 0.6^{abc}	0.8 ± 0.1^{ab}	0.6 ± 0.1^{a}
	16.7	24	30.1 ± 0.1^{de}	13.4 ± 0.6^{bcde}	1.2 ± 0.1^{abcd}	0.6 ± 0.0^{a}
Initial crabs			31.7 ± 0.5	16.6 ± 3.1	1.4 ± 0.2	1.2±0.0

Notes:

TB-EAA: Total balanced essential amino acid (g kg^{-1})

DE: Digestible energy (MJ kg^{-1})

Ratio: ratio between TB-EAA and DE (g $MJ^{-1}kg^{-1}$)

* Means within the same column having a similar superscript letter are not significantly different at 5 % of significance ($P > 0.05$)

The ANOVA (Appendix 5.3) indicates that the value of dry matter, total lipid, and carbohydrate of final whole body crabs were significantly different by an increased levels of both TB-EAA and DE, and interaction between TB-EAA and DE (P<0.05).

5.3.3.1 Dry matter

The highest DM was found in the crab group fed the diet consisting of 327 g kg^{-1} TB-EAA and 14.7 MJ kg^{-1} DE, whilst the lowest values were revealed in crabs fed diet T4 (258 g kg^{-1} TB-EAA and 14.7 MJ kg^{-1} DE) and diet T11 (398 g kg^{-1} TB-EAA and 15.7 MJ kg^{-1}DE).

5.3.3.2 Total lipid

The highest total lipid content was found in crabs fed diet containing 195 g kg^{-1} TB-EAA and 15.7 MJ kg^{-1}DE (T2) while the lowest value was in crabs fed diet composing of TB-EAA of 398 g kg^{-1} and DE of 14.7 MJ kg^{-1}.

5.3.3.3 Carbohydrates

The highest CHO value was observed in crabs fed the diet having 327 g kg^{-1} TB-EAA and 14.7 MJ kg^{-1}DE, but this did not differ from that of crabs fed the diet consisting of 195 g kg^{-1} TB-EAA and 14.7 MJ kg^{-1}DE.

5.3.3.4 Crude protein

The factorial ANOVA shows that protein contents were significantly different with graded levels of TB-EAA and an interaction between TB-EAA and DE (P < 0.05), but did not differ significantly with increased level of digestible energy (P > 0.05). The highest protein values of mud crab body were found in crabs fed the diet containing TB-EAA of 258 g kg^{-1} and DE of 15.7 MJ kg^{-1}. The lowest protein level of whole body was in crab fed diet consisting of TB-EAA of 195 g kg^{-1} and DE of 14.7 MJ kg^{-1}.

5.4 DISCUSSION

5.4.1 Method for the determination of the EAA requirements

In the present study, the profile of essential amino acid (EAA) of mud crab, *S. serrata* was taken from Mukundan *et al.* (1981) and used to match the balance of EAAs in test diets. The EAA profiles of mud crab's tissues and other species are presented in Table 5.10. There were differences between A/E ratios in mud crab's tissues and those in *P. monodon* (Millamena *et al.* 1999), in short-necked clam (Tacon 1990a), in shrimp (Akiyama *et al.* 1992), in lobster, *Homarus americanus,* (Floreto *et al.* 2000), and in *P. japonicus* (Deshimaru & Shigheno 1972). However, overall there are not greatly different.

The crystalline essential amino acids were used in this study. Although Akiyama *et al.* (1992) indicated that the supplementation of crystalline essential amino acids (C-EAAs) was disadvantageous in crustaceans because of different absorption rates of synthetic amino acids and protein-bound amino acids, and high leaching, the C-EAAs supplementation improved significantly growth of crustaceans (Millamena *et al.* 1996; Millamena *et al.* 1997; Millamena *et al.* 1998; Millamena *et al.* 1999; Floreto *et al.* 2000; Alam *et al.* 2004a; Alam *et al.* 2004b; Alam *et al.* 2005; Michael *et al.* 2006). As a result, most recent investigators confirmed that the EAA supplementations have been beneficial in crustacean feeds, but they strongly recommended that C-EAAs should be pre-coated prior to adding into feed formulation. A number of binders have been found to be useful for pre-coating C-EAAs including carboxy-methyl cellulose, zein, k-carrageena, agar, casein, and gelatine (Alam *et al.* 2004b). In the present study, alginic acid was used as a binder.

Table 5.10 The EAA profiles and A/E ratios of *P. monodon*, short-necked clam, shrimp, *Homarus americanus, P. japonicus,* and *S. serrata*

EAAs	*P. monodon*		Short-necked clam		Shrimp		*Homarus americanus*		*P. japonicus*		*S. serrata*	
	% tissues[1]	A/E	% of carcass[2]	A/E	% of protein[3]	A/E	% of protein[4]	A/E	% of weight[5]	A/E	% protein[6]	A/E
Arginine	3.2	167.0	15.5	155.3	5.8	140.8	6.8	167.9	5.6	152.4	4.8	89.8
Histidine	1.0	51.8	4.4	44.1	2.1	51.0	2.1	51.9	1.6	44.9	3.4	63.1
Isoleucine	1.8	93.0	6.8	68.1	3.5	85.0	3.8	93.8	3.1	85.7	5.1	95.4
Leucine	2.9	153.8	14.0	140.3	5.4	131.1	6.4	158.0	5.5	150.2	6.5	121.9
Lysine	3.0	158.6	14.7	147.3	5.3	128.6	4.0	98.8	5.8	158.2	6.8	127.9
Methionine	1.0	52.9	5.4	54.1	2.4	58.3	2.8	69.1	2.0	54.2	4.8	90.4
Cystine	0.4	21.7	2.7	27.1	1.2	29.1	nd	nd	0.8	21.1	1.2	23.1
Phenylalanine	1.7	87.7	7.7	77.2	4.0	97.1	3.9	96.3	3.3	90.3	4.5	85.1
Tyrosine	nd	nd	7.8	78.2	3.1	75.2	nd	nd	2.9	78.5	4.9	91.9
Threonine	1.6	82.5	9.6	96.2	3.6	87.4	3.8	93.8	3.0	81.6	5.7	107.1
Valine	2.0	107.8	8.5	85.2	4.0	97.1	4.6	113.6	3.0	82.9	4.5	85.1
Tryptophan	0.4	23.3	2.7	27.1	0.8	19.4	2.3	56.8	nd	nd	1.0	19.2
Total	*18.9*	*1000*	*99.8*	*1000*	*41.2*	*1000*	*40.5*	*1000*	*36.5*	*1000*	*53.2*	*1000*

Notes: [1]Millamena *et al.* (1998); [2]Tacon (1990a); [3]Akiyama *et al.* (1992); [4]Floreto *et al.* (2000); [5]Deshimaru and Shigheno (1972) ; and [6]Mukundan et al (1981), nd=not determined.

5.4.2 Optimal dietary specification for total balanced essential amino acids

To maximise weight gain, carapace width increase, protein efficiency ratio, and apparent net protein utilisation, the optimal dietary specification of juvenile mud crab for TB-EAA was found to be 258 g kg^{-1} diet, with all EAAs in balance with one another. Table 5.11 shows a comparison between the current finding for mud crab and the optimal EAA specifications from determination of the optimal individual EAA specification in *P. monodon*, and the recommended specifications of EAAs in shrimp feeds.

Table 5.11 Optimal dietary EAA specifications (g kg^{-1}, dry matter basis) of present mud crab and *P.monodon* and dietary EAA specifications for shrimp feed of 45 % CP

EAAs	Optimal dietary specifications				Dietary EAA specifications of shrimp feed			
	P. monodon[1]		Present mud crab		Shrimp feed[2]		Shrimp feed[3]	
	A*	A/E	A*	A/E	A*	A/E	A*	A/E
Arginine	18.5	141.3	23.2	89.9	24.4	155.5	26.1	140.6
Histidine	8.0	61.1	16.3	63.2	6.9	44.0	9.5	51.2
Isoleucine	10.1	77.2	24.6	95.3	10.7	68.2	15.8	85.1
Leucine	17.0	129.9	31.5	122.0	22	140.2	24.3	130.9
Lysine	20.8	158.9	33	127.9	23.1	147.2	23.9	128.8
Methionine	8.9	68.0	23.3	90.3	8.5	54.2	10.8	58.2
Cystine	4.1	31.3	6	23.2	4.2	26.8	5.4	29.1
Phenylalanine	14.0	107.0	22	85.2	12.1	77.1	18	97.0
Tyrosine	nd	nd	23.7	91.8	12.3	78.4	14	75.4
Threonine	14.0	107.0	27.6	106.9	15.1	96.2	16.2	87.3
Valine	13.5	103.1	22	85.2	13.4	85.4	18	97.0
Tryptophan	2.0	15.3	4.9	19.0	4.2	26.8	3.6	19.4
Total	**130.9**	**1000**	**258.1**	**1000**	**156.9**	**1000**	**185.6**	**1000**

Notes: [1]valine from Millamena and Bautista-Teruel (1996), methionine and cystine from Millamena *et al.* (1996), threonine from Millamena et al (1997), arginine and lysine were from Millamena et al (1998), histidine, phenylalanine, isoleucine, leucine, and tryptophan from Millamena et al (1999), [2] Tacon (1990a). [3] Akiyama *et al.* (1992).

A*: amount (g kg^{-1}); nd=not determined; CP: crude protein; EAA: essential amino acid

Table 5.11 illustrates that the optimal requirement of mud crab for TB-EAA had the same A/E ratios of mud crab tissue (compared those values in Table 5.11 and Table 5.10). Similarly, the recommended EAA requirements at a protein level of 45 % in shrimp had similar A/E ratios to those in short-necked clam (Tacon 1990a) and whole shrimp (Akiyama *et al.* 1992) (compared those A/E ratios in Table 5.11 and Table 5.10). Nevertheless, the A/E ratios of the optimal individual essential amino acids which were determined in *P. monodon* (Table 5.11) were a little bit different from A/E in shrimp tissues in Table 5.10. It has been known that the best feed for certain species should have EAA patterns similar to those of their own body (Tacon & Akiyama 1997). Therefore, EAA patterns of diets in this study are suitable for growth of mud crabs.

5.4.3 Optimal specification of TB-EAA and protein specification

Although the concept of a totally balanced essential amino acids (TB-EAA) requirement has not been used widely, recommendations of dietary EAA specifications of shrimps at varying protein levels have been introduced by Tacon (1990a) and Akiyama *et al.* (1992). Basically, the TB-EAA specification relates closely to the protein specification. The TB-EAA is the total essential amino acids in a balanced diet, whilst the protein is the sum of both essential and non-essential amino acids. For example, in this study when the levels of TB-EAA increased from 195 to 398 g kg^{-1}, the crude protein levels (analysed values) also increased from 310 to 632 g kg^{-1}. At the optimal requirement of TB-EAA of 258 g kg^{-1}, the crude protein was approximately 420 g kg^{-1} (analysed value). The crude protein specification of 420 g kg^{-1} value for mud crabs in this study was lower than the optimal digestible protein specification of 530 g kg^{-1} in Chapter 4. This means that optimal requirement

of mud crab for protein may be reduced if they are fed with diet containing optimal TB-EAA level.

5.4.4 Optimal DE and TB-EAA specifications

The increase of DE levels from 14.7 to 16.7 MJ kg^{-1} did not affect significantly on the weight gain, carapace width increase, specific growth rate, inter-moult period, feed conversion ratio, and protein content in carcass ($P > 0.05$). This findings were similar to those of Catacutan (2002) who reported that varying gross energy levels from 14.7 to 18.7 MJ kg^{-1} did not have an effect on weight gain, carapace width increase, or feed conversion ratio for the mud crab, *S. serrata*. By contrast, in the present study, the various levels of TB-EAA and DE have a significant effect on protein efficiency ratio (PER), apparent net protein utilisation (ANPU), and body composition (dry matter, lipid, or carbohydrate).

5.4.4.1 Effects of TB-EAA and DE levels on PER

The highest PER was obtained in crabs fed the diet containing 195 g kg^{-1} TB-EAA (or 310 g kg^{-1} crude protein), but it declined gradually when TB-EAA levels increased up to 398 g kg^{-1} (635g kg^{-1} crude protein). Similar trend of PER values were also reported in other crustacean species (Koshio *et al.* 1993; Baillet *et al.* 1997; Jacinto *et al.* 2004).

When DE levels increased from 14.7 to 16.7 MJ kg^{-1}, the highest PER was observed in crabs fed diet consisting of 15.7 MJ kg^{-1} DE. This finding was similar to that of Jacinto *et al.* (2004) who reported that the PER in crayfish rose from 0.7 to 1.5 when DE of the diet increased from 13.4 MJ kg^{-1} to 14.0 MJ kg^{-1} but the PER value reduced to 0.6 as the DE of the diet increased to 14.9 MJ kg^{-1}.

5.4.4.2 Effects of the TB-EAA and DE levels on the apparent net protein utilisation

The apparent net protein utilisation (ANPU) of the mud crabs in the present study was affected by the increased levels of TB-EAA and an interaction between DE and TB-EAA levels (Figure 5.3). In the Chinese hairy crab, *Eriocheir sinensis,* when crude protein levels increased from 29.8 to 54.8 %, the highest ANPU was found in crabs fed diets containing 39.0 % crude protein (Mu *et al.* 1998). Similarly, the present study indicated that the highest ANPU value was observed in crabs fed diet containing 420 g kg^{-1} crude protein (or 258 g kg^{-1} TB-EAA) at the DE of 15.7 MJ kg^{-1}. However, there was little information of ANPU for crustaceans and fish.

5.4.4.3 Effects of TB-EAA and DE levels on the body chemical compositions

5.4.4.3.1 Dry matter

The present study shows that dry matter of the mud crab body was significantly affected by an interaction between DE and TB-EAA levels, whilst Catacutan (2002) indicated that there was no different in moisture content among crabs fed diets with gross energy values within 14.7 and 18.7 MJ kg^{-1}.

5.4.4.3.2 Crude protein

The present study shows that increasing TB-EAA levels from 195 to 398 g kg^{-1} (or as increased crude protein in diets from 310 to 635 g kg^{-1}) had a significant effect on the protein contents of the tissues of the crabs. In contrast, Catacutan (2002) found that there was no difference in the protein content of crabs when fed diets with increased protein levels from 32 to 48 %. Furthermore, Catacutan (2002) reported that the body protein content was influenced by increasing gross energy from 14.7 to 18.7 % at the same dietary protein levels. That finding contrasted to the present study showed that increased DE levels did not affect the protein content of the crabs. Nevertheless, the

different protein levels of mud crabs, as found in this study, were the result of an interaction between graded DE and TB-EAA (crude protein) levels. This is in agreement with the findings of Catacutan (2002), although the author used lipid and protein level instead of DE and TB-EAA.

5.4.4.3.3 Total lipid

In the present study, the lipid content of mud crab, *S. serrata*, was affected by DE levels, crude protein levels, and the interaction between DE and TB-EAA levels (or crude protein levels). The highest lipid body content was 1.9 % in crabs fed diet containing 195 g kg^{-1} TB-EAA (or 310 g kg^{-1} crude protein) and 15.7 MJ kg^{-1}DE. Catacutan (2002) reported that the lipid content of mud crabs was influenced by protein levels, gross energy levels, and interacted protein and lipid levels. The highest lipid content was found in a group fed a diet containing either 32 % or 48 % protein levels, at the gross energy level of 16.7 and 18.7 MJ kg^{-1}, respectively (Catacutan 2002).

The lipid content of the tissues of crabs in this study related to both TB-EAA and DE levels. At the low TB-EAA level, the higher DE in the diet the greater the lipid content in the tissues of mud crabs, suggesting that the excess DE over the TB-EAA level in balance, it was converted into tissue fat (Batterham 1992). By contrast, at higher crude protein levels (higher TB-EAA), for example TB-EAA of 327 g kg^{-1}, the lipid contents of crabs did not increase with the increase in DE. This suggested that the crabs can use the higher level of energy for protein synthesis (since the TB-EAA is higher) rather than fat synthesis.

5.4.4.3.4 Carbohydrate

In addition, the highest carbohydrate content was found in crabs fed diets containing the lowest TB-EAA of 195 g kg^{-1} (the lowest crude protein 310 g kg^{-1}). Similarly to this, the lower the protein content of diets, the higher the carbohydrate level found in the Chinese hairy crab, *Eriocheir sinensis*, (Mu *et al.* 1998).

5.5 Conclusion and applicability

In conclusion, the TB-EAA of 258 g kg^{-1} and DE of 15.7 MJ kg^{-1} were optimal dietary specification for juveniles *S. serrata*, in terms of growth, feed conversion ratio, protein efficiency ratio, body composition, and apparent net protein utilisation.

At the optimal level of 258 g kg^{-1} TB-EAA, the dietary content for individual essential amino acids were as follows (g kg^{-1}): 23.2 arginine, 16.3 histidine, 24.6 isoleucine, 31.5 leucine, 33 lysine, 23.3 methionine, 6 cystine, 22 phenylalanine, 23.7 tyrosine, 27.6 threonine, 22 valine, and 4.9 tryptophan.

To best meet EAA requirements, it would be better to combine different protein sources to obtain the right essential amino acid specification instead of using crystalline essential amino acids. Different protein sources have different EAA profiles, so it should be possible to match the EAA balance and requirements of mud crabs by a suitable mix of protein sources (Houser & Akiyama 1997).

Chapter 6 Quantitative dietary specification of linoleic acid and linolenic acid for juvenile mud crabs, *Scylla paramamosain* (Estampador, 1949)

6.1 INTRODUCTION

Not only farmed fish and crustaceans but also all animals are incapable of *de novo* synthesis of the n-3 and n-6 double bond in fatty acids such as linoleic acid (18:2n-6, LOA) and linolenic acid (18: 3n-3, LNA). These fatty acids must be obtained from the diet for *P. japonicus* (Kanazawa *et al.* 1977), for *P. monodon* (Glencross & Smith 1999), and for mud crab, *S. serrata,* (Sheen & Wu 2002). LOA and LNA are the key essential fatty acids of n-6 and n-3 families (Tacon 1990a; D'Abramo 1997). The LOA and LNA requirements have been determined in several crustacean species including *P. japonicus* (Kanazawa *et al.* 1977) and *P. monodon* (Merican & Shim 1997; Glencross & Smith 1999) but information related to mud crabs is not available.

This chapter describes an experiment carried out at the Bac Lieu Experimental Station for Aquaculture, Bac Lieu Province, Vietnam from 1st January to 30th February 2006 to determine the optimal level of both LOA and LNA required in the diet for crablets of *S. paramamosain* when EPA and DHA were present at 3 and 4 g kg^{-1} respectively, which were approximate optimal levels of these fatty acids found for black tiger shrimp (Glencross & Smith 2001).

The specific aims of this study were to find quantitative requirement of *S. paramamosain* crablets for LOA and LNA to: a) maximise their growth, b) minimise food conversion ratio, and c) to minimise inter-moult period.

6.2 MATERIALS AND METHODS

6.2.1 Juvenile mud crab preparation

Juvenile mud crabs of 0.1±0.01 g were used in this study. One thousand juvenile mud crabs (crablets) from one batch were obtained at stage C3 from the Bac Lieu Station for Aquaculture hatchery. They were acclimated to the trial conditions depending on the time of moulting, during which they were fed with prawn feed (Concord feed No.3 made by the Asian Aquaculture Co. Ltd., Thailand, for *P. monodon*) containing 41.0 % crude protein and 7.2 % lipid. This feed was previously used as a reference diet to determine digestibility values (Sections 3.2 of Chapter 3). When crabs moulted to crab 4 (four moults from the megalops stage), they were recorded as moult zero (M0). For the trial, 180 crablets at M0 with similar weights and carapace widths were selected from the population of acclimated crablets.

6.2.2 Culture system

For the trial, the animals were held in blue plastic tanks each with a volume of 60 L. There were sixty tanks, each containing 3 animals. Each tank contained two short pipes of 100 mm diameter and 300 mm length in which crabs were held to keep them separated. Sea water was recirculated at a rate of 16 L $hour^{-1}tank^{-1}$ through the experimental tanks from a 4 m^3 reservoir tank. During the course of experimentation, water temperature, salinity, pH, and dissolved oxygen (DO) were maintained within the ranges 26.3 ± 1.1 ^oC, 24.2 ± 0.7 g L^{-1}, 7.6 ± 0.1, and 4.8 ± 0.2 mg L^{-1} respectively. Details of system, water preparation, and water management are described in Sections 2.3-2.5 of Chapter 2.

6.2.3 Dietary ingredients

The dietary ingredients used in this study are listed in Table 6.1.

Table 6.1 Source of ingredients used in the formulation of experimental diets

Ingredients	Source
Vietnamese fish meal	Local product was supplied by RIA No 2, HCM City, Vietnam
Vietnamese acetes shrimp meal	As above
Bread wheat flour	As above
Korean fish oil	As above
Vietnamese beef tallow	Purchased locally in HCM City, Vietnam
Vietnamese anchovy fish sauce	Purchased locally in Bac Lieu, Vietnam
Vietnamese pure sunflower oil	Plant oil Co., Ha Long, Quang Ninh, Vietnam
Purified linoleic acid (>99.0 %)	Sigma-Aldrich, Inc., MO, USA
Purified linolenic acid (>99.0 %)	As above
Flaxseed oil	Melrose Health Supplies, Victoria, Australia
Astaxanthin 10 %	Imported product (Switzerland) was supplied from Huu Tin Company, HCM City, Vietnam
Cholesterol 80 %	Imported product (EU) was supplied from Tomboy company, HCM City, Vietnam
Soy lecithin (SLT)	TTET Union Corporation, Taiwan
Mono sodium glutamate	Ajinomoto Vietnam Co., Bien Hoa, Vietnam
Vitamin C-35 %	Hoffman-La Roche, Basel, Switzerland
Shrimp grow vitamin (VTM)*	Bayer Vietnam Ltd., HCM, Vietnam
Organic calcium and other essential minerals supplement (MNR)*	Asian Aquaculture Co. Ltd., Thailand
Vital wheat gluten	Roquette, France

*: Composition of shrimp grow vitamin-VTM (mg kg^{-1} VTM except where specified): Vitamin A & D, 3.45 MIU; vitamin E & K, 1,300; water soluble vitamin (B1, B2, B6, B12, H, Folic acid, Niacin, calpan), 42,000; vitamin C (coated), 42,000; amino acids, 36,000; arginine, 12,500; nucleotide, 4,000; batenoid pink, 1,500; inositol, 1,000; Fe, 107; Cu, 300; Mn, 500; phosphate, 15,000; organic selenium, 20; attractant, 4,000.

*: Composition of organic calcium and other essential minerals supplement - MNR (mg kg^{-1} MNR): calcium lactate, 100; sodium dihydrogenphosphate, 280; magnesium sulphate, 60.000; manganese sulphate, 40,000; ferrous sulphate, 68,000; zino oxide, 35,000.

All ingredients, except the Vietnamese fish meal (VINA fish meal), were used as supplied by the manufacturers without modification. VINA fish meal was used as a

protein source but its lipids were first extracted with a 2:1 mixture of hexane and ethanol (Smith *et al.* 2003).

Approximately 200 g fish meal was mixed with 800 mL of a solvent mixture of hexane: ethanol (2:1), stirred, and let to separate into two phases. After 20 minutes, the top phase was removed and the bottom phase was filtered with a hand vacuum pump to discard the solvent to form the first extracted fish meal. Next, another 800 mL solvent was mixed through the first extracted fish meal. The similar steps were repeated then the final defatted fish meal was spread on tray to dry with air fans. The dry defatted Vietnamese fish meal was blended into powder and stored in a -20 ^{o}C freezer until required.

6.2.4 Chemical analysis

Details of all analytical procedures are described in Section 2.8 of Chapter 2. Dry matter, lipid, protein, and ash were determined by the RIA2 (HCM City, Vietnam). The fatty acid composition of defatted fish meal, soy lecithin, pure sunflower oil, Vietnamese beef tallow, and fish oil were analysed by the Can Tho University (Can Tho City, Vietnam), and that of flaxseed oil was performed by the CSIRO Marine and Atmospheric Research (Hobart, Tasmania, Australia). The dry matters, crude protein, crude fat, ash, and nitrogen free extract (NFE) contents and the fatty acid profiles of major ingredients are presented in Table 6.2 and Table 6.3, respectively.

Table 6.2 Dry matter (DM), crude protein (CP), crude fat (CF), ash, and nitrogen free extract (NFE) of the dietary ingredients (on a dry weight basis)

Ingredients	DM (%)	CP (%)	CF (%)	Ash (%)	NFE (%)
Defatted Vietnamese fishmeal	88.9	70.2	1.5	18.8	9.6
Bread wheat flour	93.7	15.4	1.0	1.8	82.1
Vietnamese acetes shrimp meal	88.5	34.1	0.6	51.5	13.8
Taiwan soy lecithin	nd	nd	97.0	nd	nd
Vietnamese beef tallow	nd	nd	100.0	nd	nd
Korean fish oil	nd	nd	96.0	nd	nd
Vietnamese pure sunflower oil	nd	nd	100.0	nd	nd

Australian flaxseed oil	nd	nd	100.0	nd	nd

nd =not determined

Table 6.3 Fatty acid composition (g kg^{-1}) of defatted Vietnamese fish meal (DFFM), wheat flour (WF), fish oil (FO), Vietnamese beef tallow (tallow), Australian flax seed oil (FSO), sunflower oil (SFO), soy lecithin (SLT), linoleic acid (LOA), and linolenic acid (LNA) on a dry weight basis

FAs	DFFM	WF	FO	Tallow	FSO	SFO	SLT	LOA	LNA
14:00	0.4	0.0	49.9	0.0	0.0	0.0	0.0	0.0	0.0
14:1n-5	0.0	0.0	1.7	9.2	0.0	0.0	0.0	0.0	0.0
16:00	2.5	2.1	149.5	228.9	51.4	96.2	212.9	0.0	0.0
16:1n-7	0.5	0.0	73.4	13.0	0.0	0.0	0.0	0.0	0.0
18:00	1.2	0.3	36.5	296.8	41.3	13.2	0.0	0.0	0.0
18:1n-9	0.7	2.7	140.6	245.1	193.6	95.6	191.3	0.0	0.0
18:2n-6	0.2	1.9	37.6	13.3	148.2	510.9	440.2	1000	0.0
18:3n-3	0.1	0.2	8.7	4.7	538.4	9.6	36.1	0.0	1000
20:00	0.1	0.0	1.5	0.0	0.0	8.8	4.2	0.0	0.0
20:1n-9	0.3	0.0	28.2	0.0	0.0	0.5	12.3	0.0	0.0
20:2n-6	0.0	0.0	3.2	0.0	0.0	0.0	1.5	0.0	0.0
20:3n-6	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0
20:4n-6	0.4	0.0	5.9	0.0	0.0	0.0	0.0	0.0	0.0
22:00	0.1	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0
22:1n-9	0.0	0.0	0.0	0.0	0.0	0.0	7.3	0.0	0.0
20:5n-3	1.0	0.0	75.2	0.0	0.0	0.0	7.0	0.0	0.0
24:1n-9	0.2	0.0	4.1	0.0	0.0	0.0	1.9	0.0	0.0
22:6n-3	3.7	0.0	98.0	0.0	0.0	0.4	0.0	0.0	0.0
SFA	4.4	2.6	237.4	525.6	92.8	119.9	217.1	0.0	0.0
MUFA	1.6	2.7	247.9	267.3	193.6	96.1	212.8	0.0	0.0
PUFA	0.3	2.1	46.3	18.0	686.6	520.5	476.2	1000	1000
HUFA	5.1	0.0	183.9	0.0	0.0	0.4	8.5	0.0	0.0
Total	11.4	7.3	715.5	810.9	972.9	736.9	914.6	1000	1000

Note: FAs: Fatty acids
SFA (saturated fatty acids): 14:0, 16:0, 18:0, 20:0 and 22:00
MUFA (monounsaturated fatty acids): 14:1, 16:1, 18:1, 20:1, 22:1 and 24:1
PUFA (Polyunsaturated fatty acids): 18:2 and 18:3
HUFA (highly unsaturated fatty acids): 20:2, 20:3, 20:4, 20:5 and 22:6

6.2.5 Experimental diets

Twenty isonitrogenous and isolipidic diets containing five levels of LOA and four levels of LNA (Tables 6.4-6.5) were formulated. All diets contained 510 g kg^{-1} crude protein (chapter 4) and 120 g kg^{-1} crude fat (Sheen & Wu 1999). The experimental diet were

designed using feed formulation software (Feed mania, Mania Software Pty. Ltd., New South Wales, Australia) as detailed by Glencross and Smith (1999).

Table 6.4 Composition of experimental diets (g kg^{-1}) expressed on a dry matter basis (data expressed in calculated value)

Diet code	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16	T17	T18	T19	T20
DFFM	602.6	602.4	602.2	601.9	602.5	602.2	601.9	601.7	602.3	602.0	601.7	601.5	602.1	601.8	601.5	601.2	601.9	601.6	601.3	601.0
WF	161.2	162.3	163.3	164.3	162.0	163.1	164.2	165.3	162.8	164.0	165.1	166.3	163.6	164.8	166.0	167.3	164.4	165.6	166.9	168.2
Tallow	67.5	59.7	51.8	43.9	61.7	53.0	44.3	35.7	55.8	46.3	36.9	27.4	49.9	39.7	29.4	19.2	44.0	33.0	22.0	11.0
FSO	0.0	1.2	2.4	3.7	0.0	4.0	8.1	12.1	0.0	6.9	13.7	20.6	0.0	9.7	19.4	29.1	0.0	12.5	25.0	37.5
SFO	0.6	0.4	0.3	0.1	3.5	2.9	2.4	1.9	6.4	5.5	4.6	3.7	9.2	8.0	6.7	5.5	12.1	10.5	8.9	7.2
LOA	0.0	0.0	0.0	0.0	2.4	2.2	2.0	1.8	4.8	4.4	3.9	3.5	7.2	6.6	5.9	5.3	9.6	8.7	7.9	7.0
LNA	0.0	6.0	12.1	18.1	0.0	4.5	9.0	13.6	0.0	3.0	6.0	9.0	0.0	1.5	3.0	4.5	0.0	0.0	0.0	0.0
Fish oil	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
SLT	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Chol	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
ACM	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
AFS	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
MSG	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
VWG	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
MNR	22.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
VTM	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
VTMC	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
ATXT	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Notes: DFFM: defatted Vietnamese fish; WF: bread wheat flour; FSO: Australian flax seed oil; SFO: pure sun flower oil; LOA: purified linoleic acid; LNA: purified linolenic acid; SLT: Soy lecithin; Chol: Cholesterol 80 %; ACM: VINA acetes shrimp meal, MNR: Organic calcium and other essential minerals supplement; AFS: anchovy fish sauce; MSG: Mono sodium glutamate; VWG: Vital wheat gluten; VTM: Shrimp grow vitamin; VTMC: Vitamin C -35%; ATXT: Astaxanthin

Table 6.5 Calculated composition of crude protein (CP), crude fat (CF), ash, and fatty acids of experimental diets on a dry matter basis (g kg^{-1})*

Diet code	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16	T17	T18	T19	T20
CP	509.5	509.6	509.6	509.6	509.6	509.6	509.5	509.5	509.6	509.5	509.5	509.5	509.6	509.5	509.5	509.4	509.6	509.5	509.4	509.3
CF	119.9	119.9	119.9	119.9	119.9	119.9	119.9	120.0	119.9	119.9	119.9	120.0	119.9	119.9	120.0	120.0	120.0	120.0	120.0	120.0
Ash	165.3	165.3	165.3	165.2	165.3	165.3	165.3	165.2	165.3	165.3	165.3	165.2	165.3	165.3	165.3	165.2	165.3	165.3	165.3	165.2
SFA	50.4	46.1	41.9	37.6	47.5	43.0	38.5	34.0	44.6	39.9	35.2	30.5	41.7	36.8	31.9	27.0	38.8	33.7	28.6	23.5
MUFA	32.0	30.0	28.0	26.0	30.6	29.0	27.3	25.6	29.2	27.9	26.6	25.3	27.9	26.9	25.9	24.9	26.5	25.8	25.2	24.5
LOA	7.5	7.5	7.5	7.5	11.5	11.5	11.5	11.5	15.5	15.5	15.5	15.5	19.5	19.5	19.5	19.5	23.5	23.5	23.5	23.5
LNA	1.1	8.1	15.0	22.0	1.1	8.1	15.0	22.0	1.1	8.1	15.1	22.1	1.1	8.1	15.1	22.1	1.1	8.1	15.1	22.1
ARA	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
EPA	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
DHA	4.1	4.1	4.2	4.2	4.1	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2
TFA	*98.6*	*99.3*	*100.0*	*100.7*	*98.3*	*99.2*	*100.0*	*100.8*	*98.1*	*99.0*	*100.0*	*100.9*	*97.8*	*98.9*	*100.0*	*101.0*	*97.5*	*98.7*	*99.9*	*101.2*

Notes:

SFA: Saturated fatty acids
MUFA: Mono-unsaturated fatty acids
LOA: Linoleic acid
LNA: Linolenic acid
ARA: Arachidonic acid
EPA: Eicosapentaenoic acid
DHA: Docosahexaenoic acid
TFA: Total fatty acids

* The data were calculated based on the chemical composition and quantity of the ingredients used in the formulation of the diets

6.2.6 Diet preparation

In general, the main steps are described in Section 2.6 of Chapter 2. However, since this study used some purified linoleic and linolenic acids then there were some specific steps undertaken. Firstly, dry ingredients were passed through a screen with mesh size of 450μm before being mixed together using a blender. Secondly, the lipid ingredients were mixed together in several steps. In step 1, the purified essential fatty acids (LOA and LNA) were mixed with sunflower oil and flaxseed oil, into which the fish oil was then added. In step 2, soy lecithin and Vietnamese beef tallow were warmed gently to liquefy them before being mixed with the lipid mixture in step 1, forming a lipid pre-mixture. Finally, the lipid pre-mixture was mixed with the dry ingredient pre-mixture. After that, hot water (60-70 ^{o}C) was added at rate of 0.5 L kg^{-1}. The resulting moist mixture was then pelletised using an electronic mincer to produce approximately 1.0 mm diameter and 100 mm length pellets. The pellets were then dried at 35 ^{o}C until the moisture contents were less than 10 %, then the long pellets were broken down into smaller pellets of 5-10 mm in length. The prepared diets were stored at -20 ^{o}C until required.

6.2.7 Feeding and collection of uneaten feed

The feed used for each replicate was weighed and kept separately in a small plastic jar to ensure consistency for the duration of the trial and for working out feed intake. Animals were fed twice daily at 9:00 and 16:00 hours. Three hours after feeding, uneaten feed was collected using a siphon which delivered the material to a mesh (30 μm mesh size) and the material collected on the mesh was gently rinsed with distilled water, then stored in a -20 ^{o}C freezer until the experiment was completed.

At the end of the experiment, the remainder of the unused feed in the plastic jar of each replicate was weighed. The weight difference between the beginning and end of the feeding trial gave the amount of feed fed in each treatment. All uneaten feed and fed feed were dried until constant. Amount of uneaten feed and feed intake were calculated on dry matter basis.

6.2.8 Measurements

The experiment was carried out over three moults intervals (from M0 to M3) - 60 days. To avoid the possibility of death after moulting (soft shell), crabs were weighed 2 days after moulting. Weight gain, inter-moult weight gain, specific growth rate, and feed conversion ratio were calculated according to Tacon (1990a). Inter-moult period (IMP) was also measured. Equations for these measurements are given in Section 2.10 of Chapter 2.

6.2.9 Experimental design

The experiment had 20 treatments (5 levels of LOA x 4 levels of LNA) with three replicates of each treatment. Thus, sixty tanks were used in this study. Each tank held 3 crablets. To prevent cannibalism, two crabs were separated individually in the two short pipes while the third one was kept out of the pipes. All treatments were set up using a randomised complete design (RCD).

6.2.10 Statistical analysis

Data as percentage were arcsine transformed prior to performing factorial ANOVA. Factor 1 was different LOA levels and factor 2 was different LNA levels. The Tukey HSD test was used to determine significant difference between treatment means. A distance weighted least squares model was used to explore the effect of interaction

between LOA and LNA on weight gain. The statistical analysis was carried out using *STATISTICA 7* (StatSoft, Inc., OK, USA, 2004).

6.3 RESULTS

6.3.1 Weight gain, specific growth rate, inter-moult period and feed conversion ratio

LOA/LNA had a significant effect on weight gain (WG) but not on specific growth rate (SGR), inter-moult period (IMP), or feed conversion ratio (FCR) (Table 6.6).

Table 6.6 Weight gain, specific growth rate, inter-moult period and feed conversion ratio of crabs fed diets containing different linoleic acid (LOA) and linolenic acid (LNA) levels (data were expressed as mean ± SE, n=3).

LOA (g kg^{-1})	LNA (g kg^{-1})	LOA /LNA	WG* (%)	SGR (% day^{-1})	MP (days)	FCR (kg kg^{-1})
7.5	1.1	6.8	717.9 ± 23.3^{abc}	5.8 ± 0.2	35.9 ± 0.9	1.9 ± 0.1
	8.1	0.9	779.8 ± 61.8^{abc}	6.3 ± 0.8	35.6 ± 3.6	2.0 ± 0.2
	15.1	0.5	667.8 ± 75.8^{abc}	5.8 ± 0.7	35.2 ± 3.8	2.1 ± 0.1
	22.1	0.3	588.8 ± 47.1^{a}	5.7 ± 0.7	34.2 ± 2.2	2.4 ± 0.2
11.5	1.1	10.5	739.7 ± 78.4^{abc}	6.9 ± 0.6	31.2 ± 2.6	2.3 ± 0.1
	8.1	1.4	965.8 ± 39.6^{c}	7.0 ± 0.5	33.9 ± 2.0	2.0 ± 0.1
	15.1	0.8	603.1 ± 97.2^{ab}	5.8 ± 0.5	33.6 ± 1.7	2.1 ± 0.1
	22.1	0.5	595.9 ± 58.8^{ab}	5.4 ± 0.4	36.8 ± 2.5	2.4 ± 0.2
15.5	1.1	14.1	691.7 ± 48.0^{abc}	5.7 ± 0.4	36.1 ± 3.3	2.1 ± 0.1
	8.1	1.9	898.1 ± 39.2^{bc}	6.3 ± 0.2	36.2 ± 1.6	1.8 ± 0.1
	15.1	1.0	715.7 ± 87.4^{abc}	5.9 ± 0.2	35.8 ± 1.6	2.0 ± 0.1
	22.1	0.7	668.9 ± 89.8^{abc}	5.3 ± 0.1	38.2 ± 1.9	2.1 ± 0.1
19.5	1.1	17.7	697.2 ± 34.8^{abc}	6.0 ± 0.5	35.0 ± 3.1	2.4 ± 0.2
	8.1	2.4	802.9 ± 62.3^{abc}	6.1 ± 0.4	36.3 ± 2.7	2.2 ± 0.1
	15.1	1.3	780.9 ± 27.6^{abc}	6.0 ± 0.2	36.9 ± 1.3	2.3 ± 0.1
	22.1	0.9	630.8 ± 9.6^{ab}	5.6 ± 0.5	36.7 ± 3.2	2.1 ± 0.1
23.5	1.1	21.4	756.9 ± 49.3^{abc}	6.8 ± 0.4	31.8 ± 1.7	2.3 ± 0.1
	8.1	2.9	649.8 ± 26.6^{ab}	5.2 ± 0.2	39.0 ± 1.2	2.3 ± 0.2
	15.1	1.6	644.4 ± 31.7^{ab}	5.6 ± 0.3	35.9 ± 1.1	2.4 ± 0.1
	22.1	1.1	658.6 ± 32.8^{ab}	6.0 ± 0.1	34.1 ± 1.6	2.2 ± 0.1

* Means within the same column having a similar superscript letter are not significantly different at 5 % of significance ($P > 0.05$)

Over the whole experiment there was a significant effect of LNA level on weight gain ($P < 0.05$), but no significant effect of LOA level on weight gain (Table 6.6) The highest weight gain was obtained in crabs fed diet containing 8.1 g kg^{-1} LNA and 11.5 g kg^{-1} LOA. Factorial ANOVA analysis (Appendix 6.1) showed that weight gain was not affected significantly by interaction between LOA and LNA (P=0.065).

A distance weighted least squares model showed that optimal values of LOA and LNA ranged from 10.5 -15 g kg^{-1} and 6-9 g kg^{-1} respectively (Figure 6.1). These results confirm the ANOVA results, although there was more plasticity in the optimal LOA: LNA ratio from the distance weighted least squares model.

By contrast to weight gains, specific growth rate (SGR), feed conversion ratio (FCR), and inter-moult period (IMP) were not affected significantly by the level of either LOA or LNA (P>0.05). Specific growth rates were slightly higher (not significantly) in crabs fed diets containing 8.1 g kg^{-1} LNA and either 7.5 or 11.5 g kg^{-1} LOA, and lower (not significantly) FCR values were observed for crabs fed a diet containing 8.1 g kg^{-1}LNA and 15.5 g kg^{-1} LOA (Table 6.6).

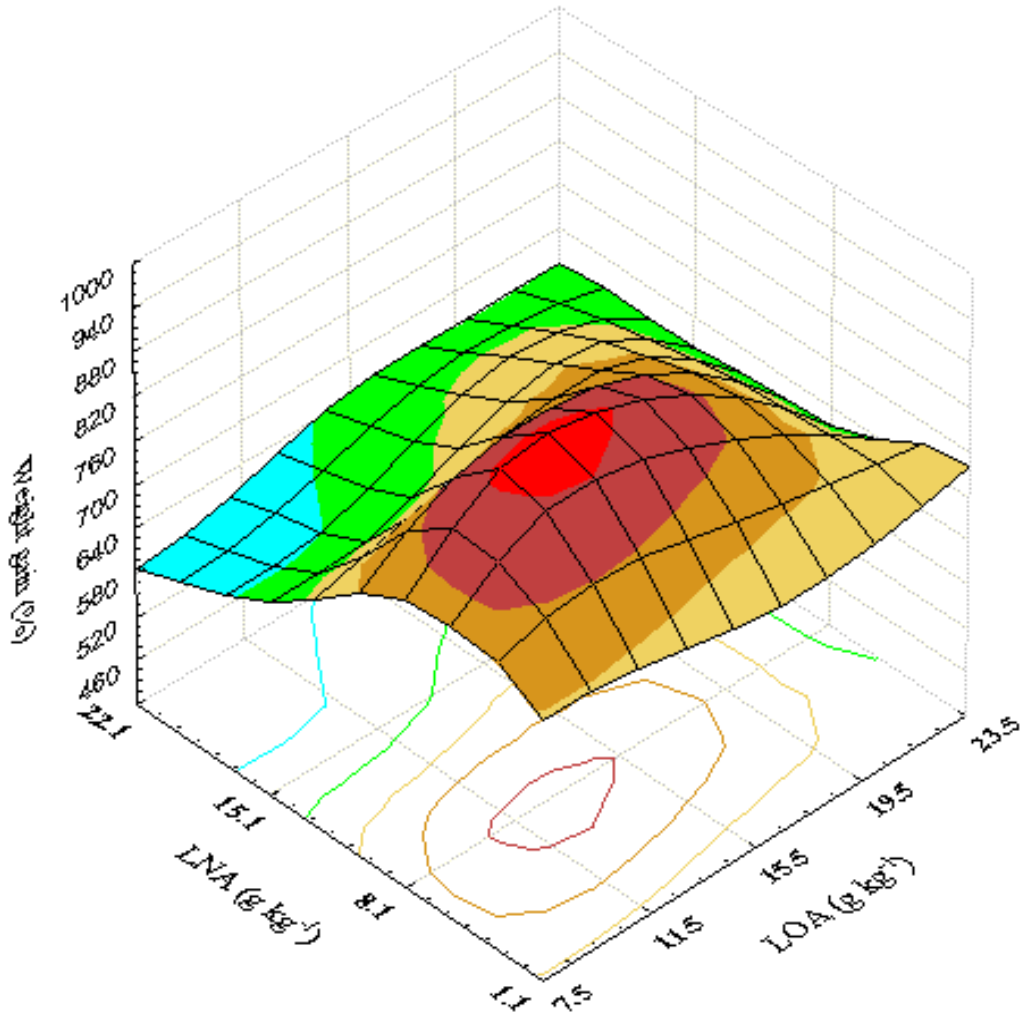

Figure 6.1 The distance weighted least squares model presents the weight gain response of crabs fed diets consisting of 4 levels of linolenic acid (LNA) and 5 levels of linolenic acid (LOA).

6.3.2 Inter-moult weight gain (IWG)

The IWG values are described in Table 6.7.

Table 6.7 The inter-moult weight gain (%) of juvenile mud crab fed diets containing different LOA and LNA ratios (data were expressed as mean ± SE, n = 3).

LOA (g kg^{-1})	LNA (g kg^{-1})	LOA/LNA	IWG 1 (%)*	IWG 2 (%)	IWG 3 (%)*
7.5	1.1	6.8	113.7 ± 6.9^{ab}	102.5 ± 7.6	95.3 ± 7.3^{a}
	8.1	0.9	135.6 ± 7.1^{ab}	102.5 ± 5.3	82.5 ± 8.0^{a}
	15.1	0.5	96.5 ± 11.7^{ab}	103.1 ± 15.0	91.1 ± 6.4^{a}
	22.1	0.3	91.1 ± 4.2^{a}	91.3 ± 7.9	84.9 ± 8.9^{a}
11.5	1.1	10.5	105.6 ± 19.3^{ab}	116.7 ± 2.2	103.7 ± 4.5^{ab}
	8.1	1.4	158.8 ± 17.7^{b}	106.1 ± 4.2	101.3 ± 6.7^{ab}
	15.1	0.8	103.5 ± 21.8^{ab}	79.4 ± 14.2	94.4 ± 7.9^{a}
	22.1	0.5	120.8 ± 10.8^{ab}	90.7 ± 26.2	84.4 ± 7.6^{a}
15.5	1.1	14.1	100.7 ± 9.4^{ab}	106.2 ± 14.3	84.0 ± 3.0^{a}
	8.1	1.9	122.7 ± 11.8^{ab}	94.7 ± 7.5	131.1 ± 11.3^{b}
	15.1	1.0	137.8 ± 18.9^{ab}	76.0 ± 16.8	99.2 ± 5.2^{ab}
	22.1	0.7	116.1 ± 6.5^{ab}	78.4 ± 16.8	97.9 ± 7.1^{ab}
19.5	1.1	17.7	94.7 ± 10.0^{ab}	106.4 ± 6.7	101.2 ± 1.9^{ab}
	8.1	2.4	130.1 ± 16.5^{ab}	103.8 ± 13.7	99.4 ± 4.7^{ab}
	15.1	1.3	118.9 ± 0.9^{ab}	120.3 ± 8.3	87.4 ± 7.4^{a}
	22.1	0.9	92.0 ± 6.8^{a}	105.4 ± 6.0	85.6 ± 3.1^{a}
23.5	1.1	21.4	110.9 ± 15.4^{ab}	103.9 ± 2.3	96.8 ± 4.7^{ab}
	8.1	2.9	107.9 ± 5.1^{ab}	91.0 ± 6.1	91.2 ± 4.2^{a}
	15.1	1.6	100.9 ± 4.8^{ab}	86.6 ± 4.5	97.2 ± 0.8^{ab}
	22.1	1.1	108.5 ± 4.2^{ab}	88.1 ± 7.6	95.5 ± 8.5^{a}

* Means within the same column having a similar superscript letter are not significantly different at 5 % of significance ($P > 0.05$)

In this study, M0, M1, M2, and M3 denote initial time, the first moult, the second moult, and the third moult, respectively. The inter-moult weight gain (IWG) between M0 and M1, M1 and M2, and M2 and M3 were designated IWG 1, IWG 2, and IWG 3 respectively.

Factorial ANOVA (Appendix 6.2) showed that inter-moult weight gains between M0 and M1 (IWG 1) were influenced significantly by LNA levels in the diet (Table 6.7).

Similarly, IWG 3 were influenced significantly ($P < 0.05$) by LOA levels and by interactions between LOA and LNA (Table 6.7).

Similarly to weight gains (Table 6.6), the highest IWG 1 value of 159 % were obtained from crabs fed LOA of 11.5 g kg^{-1} and LNA of 8.1 g kg^{-1} diet. The lowest IWG 1 was observed in crabs fed diet containing 7.5 g kg^{-1} LOA and 22.1 g kg^{-1} LNA.

The highest IWG 3 value was found in crabs fed a diet consisting LOA of 15.5 and LNA of 8.1 g kg^{-1}. The interaction between LOA and LNA is shown clearly by the distance weighted least squares model shown in Figure 6.2, where the optimal levels of LOA and LNA are around 15 g kg^{-1} LOA and 8.0 g kg^{-1} LNA. This is consistent with results from both the ANOVA and the Tukey test (Table 6.7).

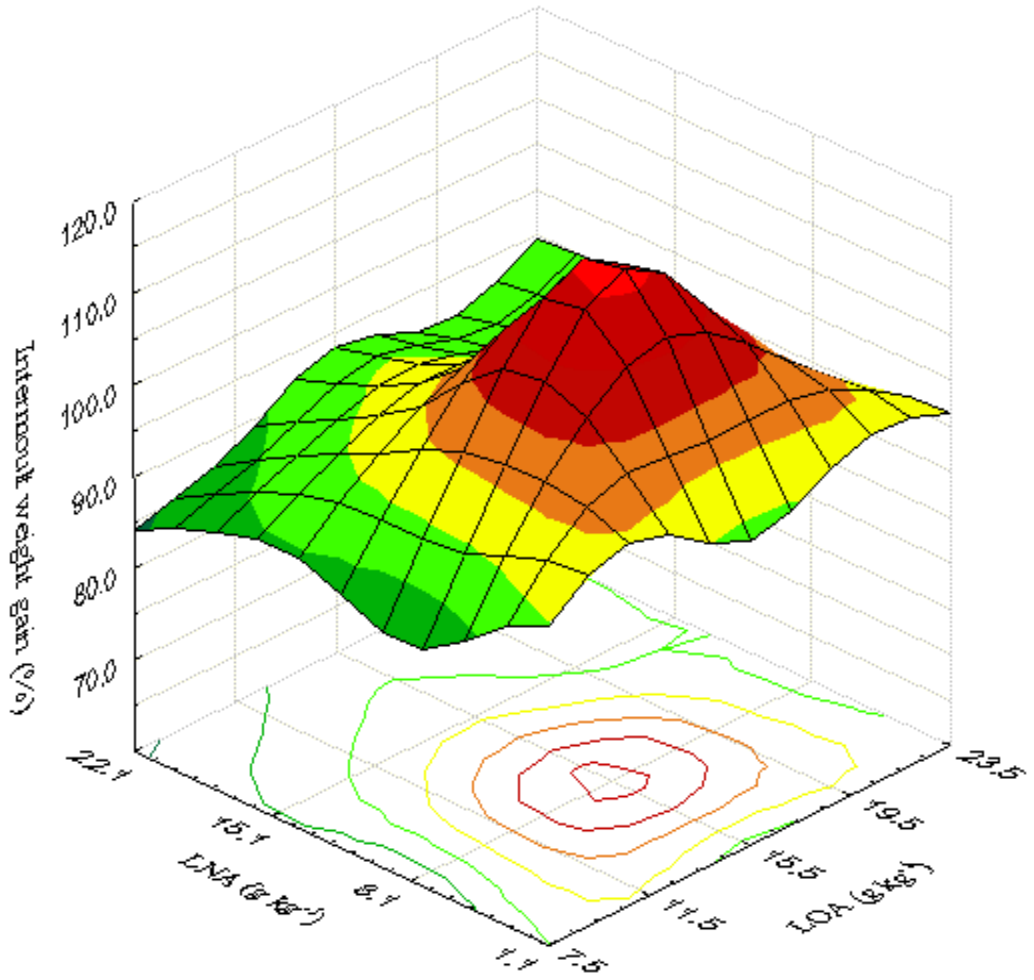

Figure 6.2 The distance weighted least squares model describes interactions between linoleic acid (LOA) and linolenic acid (LNA) levels on the inter-moult weight gain at third moult (IWG3).

6.4 DISCUSSION

6.4.1 The optimal specifications of LOA and LNA for maximum weight gain

The present study used constant levels of EPA (3.2 g kg^{-1}) and DHA (4.2 g kg^{-1}) in all diets. These values were based on the optimal requirements of *P. monodon* (Glencross & Smith 2001). There is evidence that the mud crab, *S. serrata,* has the metabolic ability to elongate and de-saturate LOA and LNA to HUFAs. Sheen and Wu (2002) indicated that the levels of arachidonic acid (20:4n-6, ARA), EPA (20:5n-3), and DHA (22:6n-3) were conserved in the polar lipid of the whole body tissue of mud crabs when these fatty acids were not provided in the diets. Similarly, studying the Chinese prawn, *P. chinensis,* showed that there was evidence of elongation of LOA and LNA to 20:2n-6 and 20:3n-3 (Xu *et al.* 1994). As a result, Glencross *et al.* (2002a) recently revealed that there was a significant interaction of the dietary n-3 and n-6 fatty acid classes affecting growth of the prawn, *P. monodon*. In other words, the requirement of LOA and LNA is dependent upon levels of other n-3 and n-6 fatty acids such as EPA, DHA, and ARA. Therefore, the present study used constant ratio for EPA and DHA to ensure that the LOA and LNA levels in diet were mostly used for growth.

In general, the weight gain of the crabs in the present study was highest when crabs were fed a diet consisting of 11.5 g kg^{-1} LOA and 8.1 g kg^{-1} LNA (Table 6.6 and Figure 6.2) at crude fat content of 120 g kg^{-1}. This LNA specification was similar to the finding of Xu *et al.* (1994) who suggested that *P. chinensis* required 10 g kg^{-1} LNA in their diet to maximise weight gain. By contrast, the value was lower than that of *P. monodon* which was reported to be 25 g kg^{-1} by Merican and Shim (1997) and 15 g kg^{-1} by Glencross and Smith (1999). With regard to the optimal dietary specification of LOA, the present study shows a similar result to that found by Glencross and Smith (1999) for juvenile *P .monodon*. However, it is noted that the findings of Glencross

and Smith (1999) were in diets that contained no long chain highly unsaturated fatty acids (EPA, DHA, and ARA). Further, the present finding could explain why the supplementation of only 2 g kg^{-1} LOA from Sheen and Wu (2002) did not significantly affect the growth of mud crab, *S. serrata*, as the presentation trial results suggests that linoleic acid in their trial was not high enough to affect the growth of mud crabs.

The effect of LNA on growth of mud crabs was greater than that of LOA in this study. The highest weight gain was found in crabs fed diet containing LNA of 8.1 g kg^{-1} and LOA of 11.5 g kg^{-1} for the total duration of the trial or 15.5 g kg^{-1} for the third moult period (see Table 6.6, Figure 6.1, Table 6.7 and Figure 6.2). Similar effects of both LOA and LNA requirements were found for *P. japonicus* (Kanazawa *et al.* 1977). The authors concluded that LNA enhanced the growth of prawns greater than LOA. Furthermore, LNA promoted faster growth than LOA in *P. vannamei* (Lim *et al.* 1997). In contrast, Glencross and Smith (1999) found that the reverse was true for *P. monodon*. The discrepancy in the results may reflect the natural feeding habits of the species examined. The less carnivorous a species (such as *P. indicus*) the more dependent its growth response is to LOA rather than LNA (Read 1981).

When the effects of the fatty acids were analysed on weight gains at different moult periods, the results were not consistent with time. In other words, the effects of LOA and LNA levels on inter-moult weight gains were different among moults (Table 6.7 and Figure 6.2). The LNA significantly influenced the maximum weight gain at the first moult (IWG 1), whilst the effect of LOA was most pronounced at the third moult (IWG 3). In general, the requirement for LNA was lower than that of LOA for mud crabs in the present study, but the LNA affected to the weight gain more sensitively than LOA.

There was an interaction of LOA and LNA levels on weight gains of crabs in the present study. The highest weight gain at the third moult was obtained at a ratio of LOA and LNA of approximate 2:1 (15.5:8.1). By contrast, Xu *et al.* (1994) indicated that the best ratio of LOA and LNA was 2:3 in *P. chinensis*. Furthermore, the interaction of LOA and LNA was reported in *P. monodon* by Glencross and Smith (1999) who found that when dietary LNA was absent from the diet, the requirement of LOA was 16 % of total fatty acid, whilst it was 14 % of total fatty acid when LNA was present and the best ratio of LOA and LNA found for this species was 1.0:1.5.

6.4.2 The importance of optimal LOA and LNA specification and applicability

Although the LOA and LNA levels were present in small amounts, their effects were significant on the growth of juvenile mud crabs. It has been known that the functions of LOA and LNA in body tissue are important because they are components of phospholipids and their precursors (Akiyama *et al.* 1992; De Silva & Anderson 1995). As the essential fatty acid requirement of fish, Sargent *et al.* (1999) revealed that determination of LOA and LNA specifications are important steps to further study of EPA and DHA requirements. Moreover, the deficiency of LOA and LNA in diets has been shown to be the cause of reduced growth *in P. japonicus* (Kanazawa *et al.* 1977) and *P. monodon* (Merican & Shim 1997; Glencross & Smith 1999).

The usefulness of this study is that it provides data on the optimum dietary content of LOA and LNA when the diet contains reasonable levels of the long chain highly unsaturated fatty acids. With this data, feed formulators who are providing EPA and DHA in the diets can ensure that the LOA and LNA balance is kept close to be optimal level through relatively easy manipulation of which vegetable oils they include in the formulation.

Although this study used purified LOA and LNA in the formulation of the diets, these fatty acids can be supplied through natural sources. There are many plant oils which are rich in LOA and LNA. For example, high LOA levels can be found in sunflower oil, corn oil, cottonseed oil, peanut oil, and soybean oil and LNA can be found in soy bean oil (8 %), rape oil (7 %), and linseed oil (56 %) (Tacon 1990a). Application of these optimal dietary specifications for LOA and LNA using natural lipid sources is described in Chapter 7.

Chapter 7 An evaluation of laboratory pellet feed, trash fish and prawn feed on survival rate, growth, and feed conversion ratio on juvenile mud crabs, *Scylla paramamosain* (Estampador 1949)

7.1 Introduction

Previous chapters of this thesis investigated the nutrition aspects of mud crabs, *Scylla* spp., in terms of apparent digestibility, digestible protein, total balanced essential amino acid, linoleic acid, and linolenic acid. Using this knowledge, several practical diets were formulated for use in the growth trial that is reported here. Specifically, these diets were tested against existing diets of trash fish and prawn diets.

It has been suggested that a low protein diet with properly-balanced essential amino acids is better than one with a higher protein diet but poorly-balanced essential amino acids (Houser & Akiyama 1997). Furthermore, replacing fisheries ingredients with inexpensive ingredients such as soybean meal has been used in a number of crustacean feeds (Floreto *et al.* 2000; Smith *et al.* 2000; Paripatananont *et al.* 2001; Du & Niu 2003; Ulloa *et al.* 2003). However, it is emphasised that practical diets in which fish meal and fish oil are replaced with plant ingredients should be balanced with essential amino acids as well as essential fatty acids (Watanabe 2002). This study evaluated three laboratory pelleted feeds on mud crablets, including one diet containing a high protein level without balanced EAA, one diet formulated with a low protein level with balanced EAA, and another having a low protein level with balanced EAA, which also had fish meal replaced with defatted soybean meal.

These three diets were formulated using ingredients reported in Section 3.2 of Chapter 3 and optimal dietary specifications for linoleic acid and linolenic acid as described in Chapter 6.

The specific aims of this experiment was to evaluate three experimentally formulated diets against the existing diets of trash fish, Vital® feed for the prawn *P. japonicus* and Concord® feed for the prawn *P. monodon,* in terms of survival rate, growth, and feed conversion ratio for crablets of *S. paramamosain*.

7.2 MATERIALS AND METHODS

The experiment was carried out at the Bac Lieu Experimental Station for Aquaculture, Bac Lieu province, Vietnam (see Section 2.1.2 of Chapter 2) for 6 weeks from 18th April to 30th May 2006 with juvenile mud crabs of 0.07 ± 0.01g (crab 3) from one batch of five hundred crabs at the crab 2 stage, which had been provided from the mud crab hatchery of the Fisheries Extension Service Centre of Tra Vinh province (Vietnam). Before experimentation, they were acclimatised to the conditions of the trial. During the acclimation period, the crablets were fed Concord® feed (a feed for *P. monodon*). This feed was previously used as a reference diet for mud crabs in Section 3.2 of Chapter 3. When crab 2 moulted to crab 3 (three moults from megalops), they were recorded as moult zero (M0) to signify the start of the trial. A total of seventy two M0 crablets having similar carapace width and weight were chosen for the trial.

7.2.1 Culture system and water supply

General culture system, water preparation and water measurement are given in Sections 2.3-2.5 of Chapter 2. Thirty six 60 L-tanks were used. Each tank held one short pipe of 100 mm diameter and 300 mm length to hold an individual crablet. For each tank, the water level was set at 200 mm in depth which was maintained with a seawater recirculation system at a flow rate of 16 L hour^{-1}tank^{-1}. During the

experimental period, the water temperature, salinity, pH, and dissolved oxygen (DO) were 29.2 ± 1.3 ^{o}C, 26.9 ± 0.6 g L^{-1}, 7.9± 0.1, and 5.2 ± 0.3 mg L^{-1} respectively.

7.2.2 Dietary ingredients

The ingredients included VINA fish meal, bread wheat flour, defatted soybean meal, VINA acetes shrimp meal, Korean fish oil, Australian flaxseed oil, Taiwan soy lecithin, cholesterol, local anchovy fish source, vital wheat gluten, vitamin mixture, mineral mixture, and astaxanthin, were the same as those described in Chapter 6. The crystalline essential amino acids were the same as those described in Chapter 5.

The essential amino acid (EAA) composition and fatty acid composition of the dietary ingredients are presented in Table 7.1 and Table 7.2, respectively.

Table 7.1 Essential amino acid (EAA) composition of the ingredients used in feed formulation, on dry matter basis (g kg^{-1})

EAAs	VINA fish meal	VINA acetes shrimp meal	Defatted SBM	Bread wheat flour
Arginine	32.5	27.5	34.8	4.3
Histidine	21.2	12.9	11.2	2.5
Isoleucine	23.2	19.6	21.4	4.7
Leucine	30.3	25.2	31.2	8.7
Lysine	35.6	28.1	27.6	2.5
Methionine	22.1	14.4	5.9	1.8
Phenylalanine	29.3	24.0	20.0	6.0
Threonine	28.8	23.6	16.2	3.3
Valine	23.1	19.1	22.7	5.0
Tryptophan	6.1	6.9	6.1	1.2
Tyrosine	26.0	20.6	13.3	3.4
Cystine	6.6	2.9	7.1	3.0

In Table 7.1, the essential amino acid (EAA) composition of defatted soybean meal (DFSBM) and bread wheat flour were taken from Tacon (1990b). The EAA composition of VINA fish meal and VINA acetes shrimp meal were determined by analysis at Can Tho University. The tryptophan values of VINA fish meal and VINA

acetes shrimp meal were taken from those of fish meal and shrimp meal in Mu *et al.* (2000), respectively (see Section 5.2.5, Chapter 5).

Table 7.2 Fatty acid composition of the ingredients on dry matter basis (g kg^{-1})

Fatty acids	VINA Fish meal	Wheat flour	Defatted soybean	Australian flax seed oil	Korean fish oil	Soy lecithin
14:00	1.62	0.04	0.13	0.00	49.95	0.0
14:1n-5	0.09	0.00	0.00	0.00	1.71	0.0
16:00	9.90	2.15	6.80	51.42	149.48	212.9
16:1n-7	1.79	0.00	0.00	0.00	73.41	0.0
18:00	4.80	0.34	1.08	41.34	36.46	0.0
18:1n-9	2.70	2.69	8.52	193.58	140.55	191.3
18:2n-6	0.88	1.91	6.05	148.21	37.57	440.2
18:3n-3	0.36	0.15	0.48	538.40	8.70	36.1
20:00	0.37	0.03	0.08	0.00	1.49	4.2
20:1n-9	1.05	0.00	0.00	0.00	28.16	12.3
20:2n-6	0.19	0.00	0.00	0.00	3.16	1.5
20:3n-6	0.10	0.00	0.00	0.00	1.62	0.0
20:4n-6	1.42	0.00	0.00	0.00	5.92	0.0
22:00	0.29	0.02	0.05	0.00	0.00	0.0
22:1n-9	0.06	0.00	0.00	0.00	0.00	7.3
20:5n-3	4.04	0.00	0.00	0.00	75.20	7.0
24:00	0.00	0.01	0.02	0.00	0.00	0.0
24:1n-9	0.66	0.00	0.00	0.00	4.12	1.9
22:6n-2	14.30	0.00	0.00	0.00	98.01	0.0
TFAs	44.59	7.34	23.21	972.94	715.48	582.00

Notes: TFAs = Total fatty acids

In Table 7.2, the fatty acid composition of fish meal and defatted soybean were analysed. The fatty acid profiles of flaxseed oil, Korean fish oil, soy lecithin and wheat flour are taken from Chapter 6.

7.2.3 Experimental diets

The experiment has 6 treatments, three laboratory pellet feeds developed specifically for mud crabs (P1, P2, and P3) and three control diets (P4, P5, and P6), the compositions of which are shown in Tables 7.3-7.5.

Table 7.3 The composition (dry matter basis) of experimental diets (g kg^{-1})

Ingredients	P1	P2	P3	P4	P5	P6
VINA Fish meal	650.0	470.0	300.0	-	-	-
Indian defatted soybean	0.0	0.0	300.0	-	-	-
Bread wheat flour	174.0	330.4	172.6	-	-	-
Australian flax seed oil	15.0	15.0	15.0	-	-	-
Korean fish oil	0.0	12.0	32.0	-	-	-
Taiwan soy lecithin	18.0	17.0	12.0	-	-	-
Cholesterol (80%)	2.0	2.0	2.0	-	-	-
VINA acetes shrimp meal	60.0	60.0	60.0	-	-	-
Anchovy fish sauce	5.0	5.0	5.0	-	-	-
Vital wheat gluten	40.0	40.0	40.0	-	-	-
Shrimp grow vitamin	25.0	25.0	25.0	-	-	-
MNR	10.0	10.0	10.0	-	-	-
Astaxanthin 10%	1.0	1.0	1.0	-	-	-
EAA pre-mixture	0.0	12.6	25.4	-	-	-
Vital feed	-	-	-	1000	-	-
Concord feed	-	-	-	-	1000	-
Small tilapia (trash fish)	-	-	-	-	-	1000
Analysed nutrition values						
Moisture	43.5	50.6	51.5	79.7	75.0	-
Crude protein	507.5	426.0	432.2	570.2	545.9	-
Ash	260.7	207.5	161.9	171.5	203.0	-
Crude fat	63.7	69.7	82.6	116.5	81.7	-
Nitrogen free extract	168.1	296.8	323.4	141.8	169.3	-
Calculated nutrition values						
Crude protein	450.8	363.2	357.5	-	-	-
Crude fat	72.1	73.9	76.8	-	-	-
Nitrogen free extract	218.8	328.4	327.2	-	-	-
LOA	9.1	9.0	9.7	-	-	-
LNA	9.1	9.1	9.2	-	-	-
EPA	2.6	2.8	3.6	-	-	-
DHA	9.3	7.9	7.4	-	-	-
GE (MJ kg^{-1})	16.21	16.30	16.28	-	-	-
CP:GE	27.8	22.3	22.0	-	-	-

Notes:

Dash (-) : not determined

LOA: Linoleic acid

LNA: Linolenic acid

EPA: Eicosapentaenoic acid

DHA: Docosahexaenoic acid

GE: Gross energy

MNR: Organic calcium and other essential minerals

GE (MJ kg^{-1})= (17.2*Nitrogen free extract + 39.5*Crude fat + 21.3*Protein) / 1000

Table 7.4 Calculated EAA composition of laboratory pellet feeds on a dry matter basis (g kg^{-1})*

Essential amino acids	Diets		
	P1	P2	P3
Arginine	23.5	18.4	22.6
Histidine	15.0	11.6	11.9
Isoleucine	17.0	15.9	18.0
Leucine	22.7	20.3	23.0
Lysine	25.3	21.3	24.2
Methionine	15.5	15.0	17.1
Phenylalanine	21.5	17.2	17.3
Threonine	20.7	17.8	20.2
Valine	17.0	14.1	16.1
Tyrosine	18.8	15.3	17.3
Cystine	5.0	4.3	4.8
Tryptophan	4.5	3.6	4.3
Total	**206.7**	**174.6**	**196.7**

* Data was calculated from EAA profile of each ingredient in diet

Table 7.5 Calculated fatty acid composition of laboratory pellet feeds on a dry matter basis (g kg^{-1})

Fatty acids	Diets		
	P1	P2	P3
14:00	1.1	1.4	2.1
14:1n-5	0.1	0.1	0.1
16:00	9.3	9.2	11.9
16:1n-7	1.2	1.7	2.9
18:00	4.2	3.8	3.9
18:1n-9	5.5	6.7	11.3
18:2n-6	9.1	9.0	9.7
18:3n-3	9.1	9.1	9.2
20:00	0.2	0.2	0.2
20:1n-9	0.7	0.8	1.2
20:2n-6	0.1	0.1	0.2
20:3n-6	0.1	0.1	0.1
20:4n-6	0.9	0.7	0.6
22:00	0.2	0.1	0.1
22:1n-9	0.0	0.0	0.0
20:5n-3	2.6	2.8	3.6
24:00	0.0	0.0	0.0
24:1n-9	0.4	0.4	0.3
22:6n-2	9.3	7.9	7.4
Total	**54.1**	**54.0**	**64.8**

The P1 diet was formulated relatively with high protein content (calculated value) of 45% and no supplementary essential amino acids. The diets P2 and P3 had lower protein levels (approximately 36 % - calculated values). The P3 diet had approximately 36 % of its fish meal replaced with defatted soybean meal. Both P2 and P3 diets were supplemented with exogenous EAAs to match the essential amino acid profile of mud crab tissues.

The P4 (Vital® feed) and P5 (Concord® feed) were the same sources from Section 3.1 and Section 3.2, respectively, of Chapter 3. The P6 diet was small wild tilapia (*Oreochromis mossambicus*) as trash fish. The small wild tilapia was available at the drainage canal system of the Bac Lieu Experimental Station for Aquaculture Research. This is very low value at the local market (USD 0.10-0.20 per kg).

All three laboratory pellet feeds (P1, P2 and P3) contained LOA levels of 9.0-9.7 g kg^{-1}, LNA levels of 9.1-9.2 g kg^{1}, EPA levels of 2.6-3.6 g kg^{-1}, DHA levels of 7.4-9.3 g kg^{-1} and lipid levels of 7.2-7.7 g kg^{-1}. The LOA and LNA contents were close to the optimal levels for mud crabs as described in Chapter 6. The EPA was similar to the optimal requirement for *P. monodon* (Glencross & Smith 2001), whilst the DHA level was similar to the supplemented level in the diet for *P. japonicus* (Kanazawa *et al.* 1979). The lipid contents were within the requirements of mud crab, *S. serrata*, as reported by Sheen and Wu (1999).

7.2.4 Calculation of essential amino acid balance

In this study, the essential amino acid profiles in diets P2 and P3 were supplemented with crystalline essential amino acid (C-EAA) based on the balance of EAA in diet and mud crab tissue. The A/E ratio in tissue of mud crab, *S. serrata*, was taken from Mukundan *et al.* (1981). The main steps to obtain essential amino acid balance were similar to those described in Chapter 5. However, in this study only some (but not

all) the main essential amino acids were balanced including isoleucine, leucine, lysine, methionine, threonine, valine, and tyrosine (and also histidine in diet P3). The levels of EAAs of arginine, phenylalanine, cystine, and tryptophan were higher than those in balance and therefore were not supplemented (Table 7.6). The main steps used to obtain balanced EAAs in a diet are presented in Appendix 7.1.

Table 7.6 Quantity of each supplemented EAA in diets (on dry matter basis)

EAAs	**Diets**	
	P2 (g kg^{-1})	**P3 (g kg^{-1})**
L-arginine	0.00	0.00
L-histidine	0.00	0.99
L-isoleucine	2.25	2.66
L-leucine	1.62	1.55
L-lysine HCl	2.57	3.93
L-methionine	3.20	7.50
L-phenylalanine	0.00	0.00
L-threonine	1.73	4.72
L-valine	0.51	0.33
L-tyrosine	0.67	3.72
L-cystine	0.00	0.00
L-tryptophan	0.00	0.00
Total	**12.55**	**25.39**

Note: EAAs: essential amino acids; L-lysine HCl contained 77.84 % pure L-lysine

7.2.5 Diet preparation

The steps in diet preparation were similar to those presented in Section 2.6 of Chapter 2. However, since diets P2 and P3 which contained supplementary EAAs, the EAAs were pre-coated before being mixing with dry ingredients. Therefore, these diets (P2 and P3) were prepared in a similar manner to the method described in Chapter 5. The small wild tilapia as trash fish, *Oreochromis mossambicus*, (Figure 7.1) were caught from the canals at the Bac Lieu Experimental Station for Aquaculture. The fish were cleaned with fresh water and filleted. The fillets were chopped into small pieces of approximately 3 mm^3, and then stored in a -20 ^{o}C freezer until required. One piece of fish was fed to one crab at one time.

Figure 7.1 Small tilapia (*Oreochromis mossambicus*) used to prepare the feed (diet P6) for juvenile mud crabs

7.2.6 Feeding and collection of uneaten feed

The feed for each replicate was weighed separately and kept in a small plastic jar to allow recording the feed used in each replicate. Animals were fed twice a day at 7:00 and 18:00 hours. Three hours after feeding, all uneaten feed was collected by siphoning through a filter mesh of 30 μm mesh size, gently rinsed with distilled water, pooled and stored at a -20 ^{o}C freezer until required for analysis.

At the end of the experiment, the remainder of the unused feed in the plastic jar of each replicate was weighed. All uneaten feed and unused feed were dried until constant. The weight difference between amount of fed feed at the beginning (the amount of feed in each jar) and amount of the uneaten feed and the unused feed at the end was the amount of feed intake in each treatment.

7.2.7 Chemical analyses

Details of the chemical analyses are described in Section 2.8 of Chapter 2. Dry matter, protein, crude fat, and ash were determined by the RIA2 (HCM City, Vietnam) using the methods described by AOAC International (1996). Composition of essential amino acids and composition of essential fatty acids were determined by the Can Tho University (Can Tho City, Vietnam) using the methods described AOAC International (2000). The amino acid composition of the fish and acetes shrimp meals were analysed with a Biochrom 20 Plus amino acid analyser (Biochrom Ltd., Cambridge, England). The fatty acid composition of fish meal and defatted soybean meal were determined using a gas chromatograph (model GC-14, Shimadzu Co., Kyoto, Japan). The fatty acid profiles of Australian flax seed oil, Korean fish oil, wheat flour, and soy lecithin were taken from Chapter 6.

7.2.8 Measurements and calculations

The experiment was carried out for three moult-periods of mud crab (M0 to M3). The parameters monitored included weight gain, specific growth rate, and feed conversion ratio, carapace width increase, and inter-moult period, which were calculated according to equations described in Section 2.10 of Chapter 2. The survival rate of mud crabs in each treatment was calculated according the following formula

$$\text{Survival rate (\%)} = \frac{\text{Final number}}{\text{Initial number}} \text{ x } 100$$

where: final number is the number of crabs from all replicates of one treatment at the end of the experiment. Initial number is the number of crabs from all replicates of one treatment at the beginning of the experiment.

7.2.9 Experimental design

Six treatments were distributed across 36 tanks using a randomised complete design (RCD). Each treatment had 6 replicates. Each replication had two crabs, one of which was held in the short pipe while another crab was kept free outside the pipe (the isolation of crabs was necessary to prevent cannibalism). During the course of the experiment, a few replicates were lost as both crabs in the replicates died. The lowest number of replications was 3 for treatment P6 (see Table 7.7).

7.2.10 Statistical analysis

The growth and feed data were processed using *STATISTICA 7* (StatSoft, Inc., OK, USA, 2004) with unequal replications. The percentage data were arcsine transformed prior to performing a one-way ANOVA (Appendix 7.2). Tukey HSD test was used to determine the significant differences between treatment means ($P < 0.05$).

The survival rates were simply described with means of total crablets for each treatment using a line graph in Microsoft Excel. The survival data could not be subjected to ANOVA because high variability between the lowest and the highest survival rate; there were only two crabs per replicate, so both died the survival rate was 0 % and if both survived the survival rate was 100 %).

7.3 RESULTS

7.3.1 Survival rate

In this study, M0, M1, M2, and M3 denote the initial time, the first moult, second moult, and third moult, respectively.

Overall, the survival rate of crabs fed six diets varied from 66 to 100 %, except for those crabs fed P6 (trash fish) (Figure 7.2). The survival rate for all treatments was similar at M1. At M2, diets P3 and P2 gave the best survival rates and diets P4, P5 and P6 the worst, with diet P1 somewhere in between (Figure 7.2). The survival rates at M3 followed the same trend as those of M2, except for diet P6 where the survival rate dropped to a very low level of 25 %. Most of the dead crabs exhibited the same symptoms, uncompleted moulting or having a soft shell for a relatively long period of time before dying.

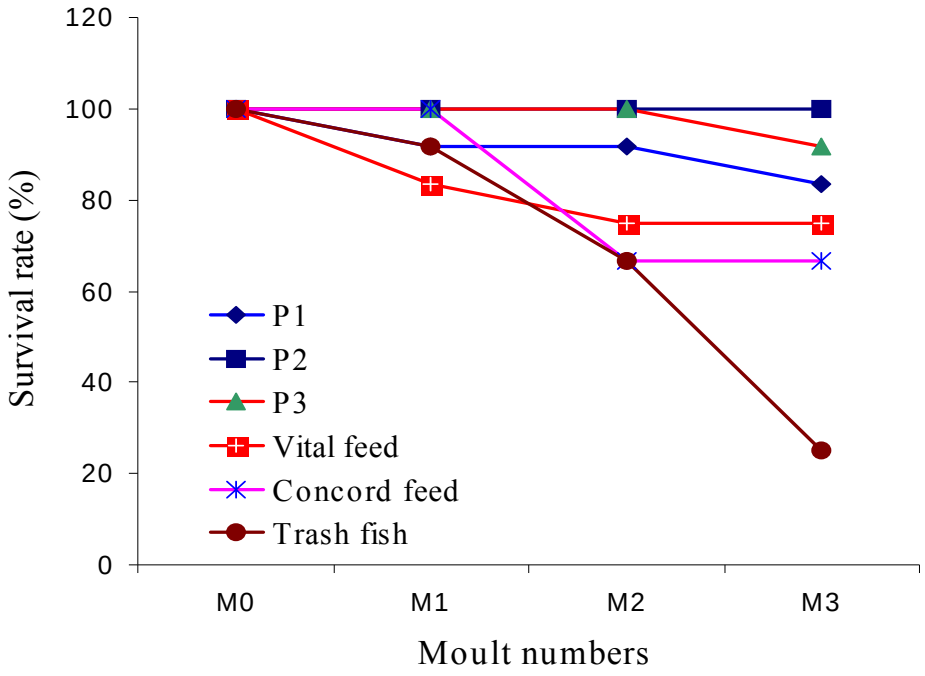

Figure 7.2 A trend of the survival rates of crabs fed different diets during three inter-moult-periods

7.3.2 Weight gain, carapace width increase, inter-moult period, specific growth rate, and feed conversion ratio

In general, the lowest weight gain was observed in crabs fed trash fish, whilst the highest value was obtained in the group fed the Vital® feed (Table 7.7).

Table 7.7 The weight gain (WG), carapace width increase (CWI), inter-moult period (IMP), specific growth rate (SGR), and feed conversion ratio (FCR) (data were mean ± SE with N = number of replicates)*

Diets	WG (%)	CWI (%)	IMP (days)	SGR (% day^{-1})	FCR	N
P1	644.2 ± 11.8^{ab}	157.7 ± 7.0^{a}	26.0 ± 1.7^{a}	8.1 ± 0.50^{a}	2.1 ± 0.1^{c}	5
P2	785.1 ± 44.2^{b}	178.1 ± 7.1^{ab}	24.8 ± 0.9^{a}	8.9 ± 0.26^{a}	1.6 ± 0.1^{bc}	6
P3	751.9 ± 32.7^{b}	173.6 ± 9.5^{a}	26.1 ± 1.1^{a}	8.5 ± 0.49^{a}	1.7 ± 0.1^{bc}	6
P4	1234.6 ± 68.1^{c}	219.8 ± 14.3^{b}	21.0 ± 1.5^{a}	13.1 ± 1.17^{b}	0.8 ± 0.0^{a}	5
P5	645.8 ± 38.1^{ab}	163.3 ± 10.4^{a}	24.1 ± 1.9^{a}	8.8 ± 0.91^{a}	1.8 ± 0.3^{bc}	4
P6	516.7 ± 44.1^{a}	151.1 ± 20.2^{a}	24.3 ± 0.3^{a}	7.5 ± 0.20^{a}	1.4 ± 0.1^{ab}	3

* Means within the same column having a similar superscript letter are not significantly different at 5 % of significance ($P > 0.05$)

7.3.2.1 Weight gain and specific growth rate

The best weight gain was obtained by crabs fed P4 (Table 7.7). The P4 diet produced a maximum weight gain of approximately 1235 %, whilst the lowest WG (517 %) was observed in crabs fed P6 (trash fish) ($P < 0.05$). The weight gain of the crabs fed the diet in which 50 % of the fish meal was replaced with defatted soybean meal (P3) was similar to those of crabs fed P1 and P2 ($P > 0.05$), both of which were 100 % fish- meal- based diets. However, the WG values of crabs fed P2 and P3 were significantly higher than that of crabs fed P6 (trash fish) ($P < 0.05$), whilst the WG of crabs fed P1 was similar to that of crabs fed diet P5 ($P > 0.05$).

Similarly, the highest SGR was recorded for the crabs fed P4 ($P<0.05$) whilst SGR values for the other treatments were similar ($P > 0.05$).

7.3.2.2 Carapace width increase

Unlike weight gain, the carapace width increase (CWI) of crab fed the P4 diet was similar to that of crab fed the P2 diet ($P > 0.05$) and it was significantly wider than those of crabs fed P1, P3, P5, and P6 diets ($P < 0.05$). Furthermore, the CWI values of crabs fed P1, P2, P3, P5, and P6 diets did not differ significantly ($P > 0.05$).

7.3.2.3 Inter-moult period

Although crabs fed P4 diet had the highest weight gain and widest carapace width, there was no significant difference in inter-moult period (IMP) for between diets ($P > 0.05$).

7.3.2.4 Feed conversion ratio

For feed usage efficiency, the lowest feed conversion ratio (FCR) of 0.8 was found in crabs fed Vital® prawn feed (P4), which was not significantly different from that of crabs fed trash fish (P6). It should be noted that all FCR values in this study were expressed as g dry matter of feed per g wet weight increase of crabs. Therefore, the FCR values of crabs fed trash fish was also calculated on a dry matter of feed basis. The highest FCR value (2.1) was observed in crabs fed with P1 diet but this value did not differ significantly from those of crabs fed P2, P3, P5, and P6 diets (Table 7.7).

7.4 DISCUSSION

7.4.1 Low and high protein diets

A low protein diet with balanced EAA was better than high protein diet without balanced EAA. This finding supports the previous result for the optimal requirements of total balance amino acid for *S. serrata* (Chapter 5). When the diet was formulated with balanced EAA, the optimum protein requirement was reduced from 530 g kg^{-1} crude protein (see Chapter 4) to 420 g kg^{-1} (Chapter 5). That finding represents a

significant saving of 110 g kg^{-1} in crude protein requirement. Similarly, the diet formulated with a partially balanced EAA profile (P2) could save 80 g kg^{-1} crude protein compared to diet P1 which had high protein content and no supplementary essential amino acids. These findings support the recommendation of Houser and Akiyama (1997) that a low protein diet with balanced essential amino acids is better than one with higher protein diet, but poorly-balanced essential amino acids.

7.4.2 Fish meal replacement diet

In the present study, replacement of about 36 % of the fish meal with soybean meal in the isoproteic diets (approximately 43 % crude protein - analysed values) produced the same survival rate, weight gain, specific growth rate, inter-moult period, and feed conversion ratio as the 100 % fish- meal- based diet. This is an exciting finding as reducing the fish meal content of aquatic feeds is encouraged worldwide (for details, see Section 1.2.1 of Chapter 1).

Some previous studies have found that somewhat less than 50 % of the fish meal in a diet can be replaced by soybean meal in diet for juvenile American lobster *Homarus americanus* (Floreto *et al.* 2000), in tiger prawn, *P. monodon* (Paripatananont *et al.* 2001), in crayfish *Cherax quadricarinatus* (Ulloa *et al.* 2003), and in the freshwater prawn, *Macrobrachium rosenbergii,* (Du & Niu 2003). However, this study found that substitution of about 36 % of the fish meal with soybean meal was possible without compromising the growth rate. This could be linked to the application of balanced EAAs in this feed formulation. This conclusion is supported by the work of Floreto et al (2000) who reported that when EAAs were supplemented in a 50 % soybean diet, the same weight gain (408 %) in *Homarus americanus* was achieved as that with a 100 % fish-meal-based diet with EAA supplementation. These authors also reported that when 50 % of the fish meal in the diet was replaced with soybean

meal without supplementary EAAs, the weight gain (148 %) was lower than that of lobster fed the 100 % fish-meal-diet without EAA supplementation (258 %), or far lower than the weight gain of lobsters fed the 100 % fish-meal-diet with EAA supplementation. In general, the present study was in agreement with the conclusion of Watanabe (2002) that it is critical to have a balanced EAA profile in diets where fish meal is replaced with plant ingredients to provide similar outcomes.

7.4.3 Small tilapia as trash fish

The effect of small tilapia (trash fish) on the mud crablets was poor in the present study as demonstrated by their low survival rate and low weight gain. This finding contrasted with the previous results using trash fish and mussel as feeds for mud crabs. For example, Cheong *et al.* (1991) showed that the biomass increase was better in crabs fed a mussel diet compared with two formulated feeds. More recently, trash fish fed to mud crabs, were kept with pens (in mangrove forest) in the Philippines showed good weight gain and survival rate (Trino & Rodriguez 2002). The contrasting results between the present work and others listed above could be due to the different culture systems used. In the present study, a lab-based, closed system was used where trash fish was the only available feed. Whilst the open systems of outdoor concrete tanks (Cheong *et al.* 1991) and the mangrove pens (Trino & Rodriguez 2002) may allow the crabs access to food in the environment, in addition to the mussel or the trash fish provided. It is possible that the trash fish alone may be insufficient in minerals or vitamins required for successful moulting and survival. Crabs that were grown in outdoor systems can have access to algae, worm, and prey organisms which are rich in minerals, vitamins, water soluble minerals and vitamins that are present in the water (Conklin 1997; Davis & Lawrence 1997).

Recently, lower survival rate of spiny lobsters was detected as they were fed mussel flesh compared with them fed with pelleted feeds (Smith *et al.* 2005b).

Recently, it has been demonstrated that supplementary trash fish for mud crabs are not always advantageous. Working in the Mekong Delta, Vietnam, Christensen, Macintosh and Phuong (2004) fed young *S. paramamosain* and *S. olivacea* in earthen ponds with trash fish (mainly Tilapia *Oreochromis mossambicus*) and mangrove sesarmid crabs (Ba khia in Vietnamese). They found no difference in growth between these two supplements and unfed controls after 115 days of feeding. This supports previous work which demonstrated that small crabs with a carapace width less than 70 mm feed mainly on detritus (Prasad & Neelakantan 1988). Similar feeding habits of other crabs species have been reported, for example the juvenile dungeness crabs, *Cancer magister*, were found to ingest filamentous diatoms (Jensen & Asplen 1998) and the stomach of the inter-tidal crab (*Pachygrapsus marmoratus*) was full of plant items such as filamentous algae and macro-algae (Cannicci *et al.* 2002).

7.4.4 The Vital® feed and Concord® feeds

The crabs fed Vital® feed had the fastest growth in this study. The crude protein, crude fat and ash of Vital® feed were 570.2, 116.5, and 171.5 g kg^{-1}, respectively. The weight gain of crablets increased approximately 1235 % after being studied for 21 days (Table 7.7). The weight gain of crabs fed Concord fed® was considerably less (645 %), although this feed had high nutrition values such as 545.9, 81.7, and 203.0 g kg^{-1} for crude protein, crude fat, and ash, respectively. These results agree with those from Shelley (2001) who found that *S. serrata* fed Vital® feed and Ebistar® feed had a high weight gain of 1650 % after 46 days of growth while those fed *P. monodon* feed (Aquafeed®, CP® or Grobest®) had lower weight gains.

Although Vital® and Ebistar® feeds produce the best weight gain in mud crabs, they are very expensive at A$ 11 and A$ 6 per kg respectively (Shelley 2001).

7.5 CONCLUSION

Based on the results of survival rate and growth, it is concluded that the small Tilapia (*Oreochromis mossambicus*) - trash fish is a poor food source for young mud crabs. It is clear that although Vital® feed was the best performing feed in this study, the price of this feed is too high for large scale mud crab culture. The cheaper commercial feed, Concord® for *P. mondon,* was better than trash fish, but inferior to the two experimental feeds, P2 and P3. Although P2 and P3 were manually pelleted in the laboratory with a mincer, they still supported better growth and survival of mud crablets than those of trash fish and *P. monodon* (Concord) feed. As a result it is considered that with further refinement and development (e.g., better micronutrients and other feed additives, and pellet feed production technology); P2 and P3 have the potential to become cost-effective feeds to support the grow-out phase of mud crabs.

Chapter 8 General outcomes and recommendations

In general, the outcomes of the work presented in this thesis have satisfied the aims and specific objectives of this study in terms of determining the apparent digestibility of various ingredients, estimating the optimal specifications for the digestible protein, the total balanced essential amino acid, the digestible energy, the linoleic acid and the linolenic acid, and obtaining a satisfactory formula for a pellet feed. This final chapter summarises the outcomes of the work and suggests a strategy for future improvements of the formulation of feed for the grow-out of mud crabs.

8.1 THE OUTCOMES OF THE WORK

8.1.1 Apparent digestibility for dietary ingredients

The apparent digestibility (AD) of *S. serrata* for 7 ingredients (fish meal, shrimp meal, blood meal, defatted soybean meal, wheat flour, alpha cellulose, and cod liver oil) has been found. Furthermore, the AD of *S. paramamosain* were found for 13 ingredients fish meal, acetes shrimp meal, defatted soybean meal, full fat soybean meal, expelled coconut oil cake, expelled peanut oil cake, ground cassava meal, whole rice bran, bread wheat flour, Korean fish oil, Korean squid oil, local pig fat oil, and vegetable oil. These AD values are good tools to evaluate the quality of ingredients.

For example, the AD for dry matter and energy of *S. serrata* show that the high digestible ingredients were soybean meal, fish meal, and blood meal, followed by shrimp meal and cod liver oil with somewhat lower digestibility. The ingredients with the low apparent digestibility were wheat flour and alpha cellulose. In this study suggested that AD of *S. serrata* for the bread wheat flour needs to be re-examined.

Similarly, the AD for dry matter of *S. paramamosain* indicates that the ingredients with the highest digestibility were defatted soybean meal, cassava meal, wheat flour, fish meal, acetes shrimp meal, and rice bran. The intermediate AD values were full fat soybean meal and expelled peanut oil cake. The least digestible ingredient tested was expelled coconut oil cake. Based on AD for crude fat, all lipid ingredients were well digested by *S. paramamosain*.

Although, some of the plant ingredients such as the defatted soybean meal were well digested by both species, AD values for wheat flour are different. The AD values for wheat flour should be re-examined for *S. serrata* due these values were lower than those of the finding of Catacutan (2003) for this species.

In general, plant ingredients can be utilised in formulated crab feeds to minimise their cost. This result contributes to the knowledge of the global trend for aquafeed development, which encourages replacement of marine products with plant sources (Tacon 1999).

In summary, these AD values are fundamental to further development of formulated feeds for mud crabs with minimum wastage and cost (De Silva & Anderson 1995; Lee & Lawrence 1997; Cho & Bureau 2001). However, selection of ingredients should be considered relative to the target culture system. Ingredients with low AD should not be used for pellet formulation in an intensive culture system because the indigestible part of feed is a cause to damage water quality of there system.

8.1.2 Optimal specification for digestible protein by *S. serrata*

Protein is usually the most expensive component of aquaculture feeds. Therefore, it is important to find the optimum level of dietary protein for mud crabs to reduce the cost of feed. In addition, high protein feeds are likely to be wasted and this could

impact negatively on the environment (Cho & Bureau 2001; Watanabe 2002). In the present study, the optimal digestible protein requirements of *S. serrata* were estimated around 530 g kg^{-1} for maximising weight gain and 460 g kg^{-1} for minimising the feed conversion ratio (Chapter 4). These values are higher than those of crayfish, *Cherax quadricarinatus*, (Jacinto *et al.* 2003; Jacinto *et al.* 2004; Jacinto *et al.* 2005) and of the tiger prawn, *P. monodon* (Shiau & Chou 1991; Burford *et al.* 2004) but similar for lobsters (Smith *et al.* 2005b). The apparently high dietary protein specification for mud crabs may be a result of imbalanced essential amino acid profiles in their dietary protein sources. This hypothesis was tested in Chapter 5 and Chapter 7 where diets having balanced essential amino acids were evaluated.

8.1.3 Optimal levels of total balanced essential amino acid and digestible energy by *S. serrata*

In Chapter 5 it was demonstrated that the optimal requirements of essential amino acids to provide a balanced diet for *S. serrata* was obtained at 258 g kg^{-1}(or 420 g kg^{-1} crude protein) in terms of the maximum weight gain, maximum carapace width increase, minimal feed conversion ratio, highest body protein content, and highest apparent net protein utilization. This finding confirmed that the crude protein requirement can be reduced significantly if the diet contains an essential amino acid balance that matches the EAA profile in the body of the mud crab. That being the case, it follows that the essential amino acid profiles of ingredients should also be examined when ingredients for feed formulation are being assessed.

The importance of balanced essential amino acids in diets was further shown in Chapter 7. It was found that a high protein diet without balanced essential amino acids (diet P1) produced poorer growth than a low protein diet with properly-

balanced EAAs (diet P2), even when the low protein diet had 50 % of the fish meal replaced by defatted soybean meal.

At the optimal TB-EAA level of 258 g kg^{-1}, the optimal requirement of *S. serrata* for digestible energy was 15.7 MJ kg^{-1}. While proteins and amino acids are good sources of energy they are more expensive energy sources than carbohydrates and lipids. When a diet is not balanced in its EAA, some of the amino acids are converted to energy regardless of the level of the DE of the diet. Therefore, determining the optimal DE level from lipid and carbohydrate sources with optimal level of TB-EAA is important to ensure that essential amino acids (proteins) are used for tissue synthesis rather than as an energy source (Cuzon & Guillaume 1997).

8.1.4 Optimal levels of linoleic acid and linolenic acid by *S. paramamosain*

Linoleic acid (LOA) and linolenic acid (LNA) are essential for the synthesis of body tissue because they are components of phospholipids and their precursors (Akiyama *et al.* 1992; De Silva & Anderson 1995). Furthermore, obtaining the optimal requirements for these particular fatty acids is an important step to further determine optimal levels of highly unsaturated fatty acids (HUFAs) (Glencross & Smith 1999; Sargent *et al.* 1999) in diets. In this study, the optimal levels of linoleic acid and linolenic acid for *S. paramamosain* were 11.5 and 8.1 g kg^{-1}, respectively.

8.1.5 A recommended feed formula

Using the data available, the following formulated diet was found to be the best performer for the grow-out phase of mud crabs, *S. paramamosain*. The diet delivers 432 g kg^{-1} crude protein, 78 g kg^{-1} crude lipid, and 16.3 MJ kg^{-1} gross energy, 9.7 g kg^{-1} LOA, and 9.2 g kg^{-1} LNA (Tables 8.1-8.3). When juvenile mud crabs were fed

this diet, their weight increased 751 % after 26 days and the survival rate obtained was approximately 98 %.

Table 8.1 Composition of the recommended feed formula on a dry matter basis

Ingredients	**Amount (g kg^{-1})**
VINA fish meal	300.0
Indian defatted soybean meal	300.0
Bread wheat flour	172.6
Australian flax seed oil	15.0
Korean fish oil	32.0
Taiwan soy lecithin	12.0
Cholesterol 80 %	2.0
VINA acetes shrimp meal	60.0
VINA anchovy fish sauce	5.0
French vital wheat gluten	40.0
Shrimp grow vitamin	25.0
Organic calcium and other essential minerals	10.0
Astaxanthin 10 %	1.0
Essential amino acid pre-mixture	25.4
Analysed values	
Crude protein	432.2
Ash	161.9
Crude fat	82.6
Nitrogen free extract	323.4
Calculated values	
Crude protein	357.5
Crude lipid	76.8
Nitrogen free extract	327.2
Gross energy (MJ kg^{-1})	16.28
Crude protein: Gross energy (g MJ^{-1} kg^{-1})	22.0

Table 8.2 The essential amino acid composition of the recommended feed formula

Essential amino acids	**Amount (g kg^{-1})**
Arginine	22.6
Histidine	11.9
Isoleucine	18.0
Leucine	23.0
Lysine	24.2
Methionine	17.1
Cystine	4.8
Phenylalanine	17.3
Tyrosine	17.3
Threonine	20.2
Valine	16.1
Tryptophan	4.3
Total	**196.7**

Table 8.3 The calculated fatty acid composition of the recommended feed formula

Fatty acids	Amount (g kg^{-1})
14:00	2.1
14:1n-5	0.1
16:00	11.9
16:1n-7	2.9
18:00	3.9
18:1n-9	11.3
18:2n-6	9.7
18:3n-3	9.2
20:00	0.2
20:1n-9	1.2
20:2n-6	0.2
20:3n-6	0.1
20:4n-6	0.6
22:00	0.1
20:5n-3	3.6
24:1n-9	0.3
22:6n-2	7.4
Total	**64.8**

8.2 General recommendations

As stated previously, the recommended diet for the grow-out of mud crabs will require further refinement. The following further work is proposed:

1) Determine apparent digestibility of mud crabs for other available ingredients. Although AD for various ingredients have been known for both *S. serrata* and *S. paramamosain*, the AD for poultry meal, shrimp head meal, sunflower oil, flaxseed oil, and catfish fat oil should be examined, because these ingredients are currently available at reasonable prices and they are likely to have nutritional value. This information would expand the list of usable ingredients for the formulation of the pelleted feeds for the grow-out phase of mud crabs, particularly in Vietnam.

2) Obtain requirements for balanced essential amino acids and optimal digestible energy in *S. paramamosain*.

3) Estimate the optimal level of linoleic acid and linolenic acid in *S. serrata*. The determination of the optimal requirement of *S. serrata* for LOA and LNA should be done before determining optimal levels of arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic (DHA).

4) An evaluation of the optimal requirements of both species for ARA, EPA and DHA should be undertaken. According to Sheen and Wu (2002) effects of ARA and DHA were greater than LNA in the mud crab, *S. serrata* but their optimal requirements were not determined. Furthermore, EPA is an important essential fatty acid in crustacean species (Glencross & Smith 2001). Therefore, the optimal levels of these highly unsaturated fatty acids should be identified for *S. serrata* and *S. paramamosain.*

5) Studies will be required on the vitamin, mineral, binder and feed additives required in feeds for both species. As stated previously in Section 1.2.2 of Chapter 1, vitamins, minerals, attractants, and carotenoids serve an important role in the body structure of crustaceans. As in shrimp feed, feed additives (enzymes, antioxidants, antibiotics, zeolite, phytosterols, olaquindox, thyroprotein, and bile acid) should be tested in growth trials for mud crabs.

6) Examine the method of pelleting and the form of the pellets for large-scale industrial production. Since mud crabs used their claws to hold the feed, larger crabs may require pellets of a larger size than shrimps, or of a different form.

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Appendix 2.1 The method used to determine crude protein at the Charles Darwin University, NT, Australia

Method

The nitrogen content of the sample from the experiments conducted at the DAC was determined using the Kjeldahl method with a Lachat 8000 (Lachat Instruments Milwaukee, WI, USA) as described by Diamond (1992).

Procedures

Firstly, the powder of feed pellets and faeces was weighed, approximately 20-25 mg, into separate tubes. To each tube 1.625 g mixture of 1.5 g K_2SO_4, and 0.125 g $CuSO_4.5H_2O$ were added. Approximate 1 dozen anti-bumping granules (BDH) were added to each tube. After that, 3.5 mL H_2SO_4 was added slowly to each tube (2.5mL at first and 1 mL later). The tubes were left for 24 hours in the fume-hood before carrying out the next step.

Next, the samples were heated gradually to 390 ^oC for three hours with a temperature–programmed block digester. When samples turned from brown to green/blue the digestion was completed. The samples were then removed from the block and let cooled for 10 minutes. In the fume-hood, 20 mL high purity (HP) water was carefully added to each tube. The mixtures were then mixed with a vortex mixer and water was added to obtain the final volume of 50 mL, then the tubes were covered with thinned plastic and let stand overnight before reading.

A Lachat 8000 flow injection analyser (FIA) was used to determine nitrogen (total Kjeldahl nitrogen) content. Firstly, the digested solutions were injected into the chemistry FIA manifold, where the pH was maintained by a buffer prior to reacting with the reagents of sodium salicylate and sodium hypochlorite to produce blue product. Then, the colour was further intensified by reaction with sodium nitroprusside. Finally, the absorbance of a blue product was measured at 660 nm and reported as nitrogen digest (C_D), then the percent of nitrogen in the sample was calculated by the formula:

$$\%N = \left[\frac{C_D \text{ x } V_D}{W_S} \right] \text{x } 10^{-4}$$

where:

- V_D= Total volume (mL), default = 50 mL
- W_S= Weight of sample (g)
- C_D= Concentration in the nitrogen digest (mg L^{-1}) after adjusting for the blank value

Crude protein was determined as a following equation:

Crude protein (%) = % N x 6.25

Appendix 2.2 The method used to determine crude protein by the RIA No. 2, HCM City, Vietnam

Method

Nitrogen content of the samples from the experiments carried out at the BLESA was determined by the RIA 2 (HCM, Vietnam) using the Kjeldahl method described by AOAC International (1996)

Procedures

A known weight (approximately 500 mg) of dried sample was placed in a Kjeldahl digestion flask (Velp, Italy), into which 5 g K2SO4 (Merck, Pure), 1 g anhydrous CuSO4 (Merck, Pure), 0.5-1.0 g pumice stone (Fluka Co. Ltd., Tokyo, Japan), and 20 mL concentrate sulphuric acid (Merck, Pure) were added.

The sample mixture above was digested at 400 °C (Digestion system) for one hour until the solution was clear, and heating was then continued for another 30 minutes. Then the mixture was removed from the heat and left for cooling at room temperature, after which 100 mL distilled water was added. The sample was then gradually mixed with 25 ml of 40 % sodium hydroxide solution.

Both distillation and titration were carried out using a semi-automatic steam distilling unit, Model UDK 132 (Velp, Italy). The distilled solution was titrated with 0.2 N hydrochloric acid. Crude protein was calculated as follows:

$$\text{Crude protein (\%)} = \frac{\text{(A – B) x C x 14.007 x 6.25}}{\text{D (mg)}} \text{ x 100}$$

Where

- A: a volume of hydrochloric acid used in sample titration (mL)
- B: a volume of hydrochloric acid used in blank titration (mL)
- C: Normality of hydrochloric acid (C=0.2)
- D: Sample weight (mg)
- 14.007: Molecular weight of nitrogen
- 6.25: Protein is assumed to be 16 % nitrogen

Appendix 2.3 The method used to determine total lipid at the Charles Darwin University, NT, Australia

Method

Total lipid content of the samples from the experiments conducted at the DAC was determined gravimetrically using the method described by Mason and Nell (1995).

Procedures

The samples were freeze-dried and stored in a -20 ^{o}C freezer. At appropriate time, a known weight (approximately 100 mg) of sample was placed in 100 mL conical flask, into which. 5 mL methanol, then 10 mL chloroform, and 4 mL HP water were added. The mixtures were warmed by the ultrasonification for 10 minutes before being filtered through a Whatman paper (No 40) into 50 mL beakers. Boiling chips were then added. Then beakers were heated at 60 ^{o}C with a hot plate (SEM) until dryness.

When the lipid extract was dried, 5 mL chloroform were added, the content rinsed, and then the extract and washing were transferred into 25 mL- pre-weighed beakers containing the boiling chips. The pre-weighed beakers and lipid solution were placed on the hot plate (60 ^{o}C) again for evaporation. The lipid content was weighed to constant weight and the total lipid was calculated as follows.

$$\text{Total lipid (\%)} = \frac{\text{Lipid mass (mg)}}{\text{Sample mass (mg)}} \text{ x } 100$$

Appendix 2.4 The method used to determine crude fat by the RIA No 2, HCM City, Vietnam

Method

Crude lipid content of the samples from the experiments conducted at the BLESA was analysed by the RIA2 using the method provided by AOAC International (1996).

Procedures

Clean and empty flasks from a 105 ^{o}C oven were cooled and weighed. Samples were weighed (500-1000 mg) and put into the thimbles. Both thimble and flask were attached to the Soxhlet extraction apparatus. The temperature was set at 40-60 °C (boiling point of petroleum ether). The samples were boiled for 30 minutes, and then rinsed for 10 hours. The solvent was evaporated from the flasks to concentrate the lipid. The flasks with concentrated lipid were then placed in the 105 °C oven for 1 hour for drying. The dried crude fat was then weighed. The crude fat was calculated as below:

$$\text{Crude lipid (\%)} = \frac{\text{Extraxted lipid (mg)}}{\text{Sample weight (mg)}} \text{ x } 100$$

Appendix 2.5 The method used to determine carbohydrate at the Charles Darwin University

Method

Soluble carbohydrates were measured using a method described by Kochert (1978).

Procedures

A known weight (approximately 15 mg) of feed or body crabs powder was placed into 15 mL tubes. To each tube 10 mL of 0.5 M H_2SO_4 was added and its level was marked. The tubes were then warmed with a steam bath at 90 ^oC for 60 minutes. At the end of 60 minutes, the tubes were removed from the water bath for cooling to room temperature. Evaporation was compensated using 0.5 M H_2SO_4 before the tubes were centrifuged at 2000 rpm for 10 minutes.

A glucose standard curve was prepared using a series of glucose standards (BDH, Analar). The glucose concentrations of the standard solution were 0, 50, 100, and 200 μg mL^{-1}.

One mL aliquots of each test sample, glucose standard solution or distilled water was pippeted into the test-tube, standard tube or blank tube, respectively. One mL 5% phenol (BDH, analar) reagent each was added to all test samples, standards, or blank tubes. The tubes were mixed with a vortex (Stansen Model VM1). In a fume hood, 5mL concentrated H_2SO_4 (Ajax, Univar) was rapidly measured with an acid dispenser and added to each tube (staggered at 1 minute per tube), then mixed using the vortex for mixing. After 45 minutes, orange colour was developed, then each test sample, standard or blank solutions were measured at 485 nm using a ultra-violet visible spectrophotometer, model U-1100 (Hitachi, Tokyo, Japan). Soluble carbohydrate was calculated according to formula:

$$\text{Total soluble carbohydrate (\%)} = \frac{\text{Solution concentration } (\mu\text{g L}^{-1}) \text{ x Volume (mL) x } 10^{-4}}{\text{Mass (g)}}$$

where: solution concentration was calculated from a glucose standard curve.

Appendix 2.6 The method used to determine gross energy by the South Australian Research and Development Institute, SA, Australia

Procedures

Firstly, all materials were ground and passed through a 0.5 mm screen, and then 0.2-0.5 gram of each sample was pelleted into a tared crucible. Next, the crucible was placed in the support assembly stand (crucible holder attached to bomb head), then cotton thread of 10 cm was attached to the wire (this is attached between the electrodes where the current comes through), and rested against the sample. Then, the assembled bomb head was then placed into the Parr 1281 bomb calorimeter. After that, lids were closed. After closing the lids, a start button was pressed and then bomb ID, sample ID and sample weight were entered. After this, an enter button was pressed. In principle, the calorimeter established a balanced temperature. Upon establishment of a stable temperature, the calorimeter gives a few fast beeps to alert the operator that ignition is taking place. When completed, the calorific value was displayed in MJ kg^{-1} on the screen. Same steps were used for all other samples.

Appendix 2.7 The method used to determine chromic oxide by the Queensland University of Technology, Brisbane, Queensland, Australia

Method

Chromic oxide (Cr_2O_3) content of the samples from the experiment carried out at the DAC was determined by the Queensland University of Technology (QUT) using the method described by Furukawa and Tsukahara (1966).

Procedures

At the beginning, the samples of feed pellets and faeces had been dried using a freeze-drier then ground into powder. Then, 40-70 mg of each samples were weighed into 100 mL Kjeldahl flasks into which 5 mL Nitric acid (HNO3) was added and let digested at 165 ^{o}C for 30 minutes. During this period, the samples were monitored to prevent evaporation of the acid. When the solutions were clear and greenish, they were removed from the heat. When the solutions were cool, 3 mL perchloric acid was added in each tube, in the fume hood. The flasks were placed in the digester again and boiled until the solution turned from green to lemon yellow. The content of the flasks were filtered into 100 mL volumetric flasks to remove undissolved materials. Distilled water was then added to make up to 100 mL. Finally, concentrations of chromium samples were read using a Liberty 200 ICP spectrometer (Varian, Inc., CA, USA) at 350 nm after adjusting to zero for the blank tubes. The blank tubes were also prepared with acids and distilled water at the same time. There are two steps to calculate the percentage of chromic oxide in the sample.

The amount of chromic oxide (mg) present in the sample:

$$X\,(\text{mg}) = \frac{Y - 0.0032}{0.2089}$$

where: X = the amount of chromic oxide in the sample, Y= the absorbance, 0.2089 and 0.0032 = constants

The percentage of chromic oxide in sample

$$Cr_2O_3\ (\%) = 100 \text{ x } \frac{\text{Amount of chromic oxide in sample (mg)}}{\text{Sample mass (mg)}}$$

Appendix 2.8 The method used to determine chromic oxide by the Can Tho University, Can Tho City, Vietnam

Method

Chromic oxide was analysed using the method described by Temminghoff (2000).

Procedures

The concentrations of chromium in the digested solution were measured with an atomic absorption spectrophotometer (model: Z5000, Hitachi, Tokyo, Japan) at 359.3 nm.

$$\text{Chromic oxide (\%)} = \frac{C * V * 152}{104 * M * 10000}$$

where C: Chromium concentration from the calibration graph; V: Volume of sample solution; M: Sample mass (mg); 104: molecular weight of chrome; 152: molecular weight of Cr_2O_3; 10000: a constant

Appendix 3.1 Chromic oxide (Cr_2O_3) contents in diets and faeces (g kg^{-1}) from experiment determined AD for *S. serrata*

Treatments	In diets	In faeces				
		Replicate1	Replicate2	Replicate3	Replicate 4	Replicate5
T1	3.1	22.4	20.7	25.2	31.9	27.9
T2	2.9	22.4	22.6	24.6	21.6	m
T3	3.3	21.4	22.2	21.4	25.1	m
T4	2.9	22.1	22.3	22.9	21.6	23.1
T5	2.5	25.6	25.8	23.0	23.7	33.1
T6	4.0	23.6	23.2	22.8	m	m
T7	3.4	22.6	23.7	21.7	23.7	20.4
T8	3.5	23.7	27.4	23.2	23.8	m

m=missing

Appendix 3.2 ADs of *S. serrata* for eight experimental diets

Sources	**ADD (%)**	**ADE (%)**	**ADP (%)**
T1	87.8 ± 0.9^{c} (5)	90.9 ± 0.7^{c}(5)	94.5 ± 0.5^{bc}(5)
T2	87.2 ± 0.4^{bc} (4)	91.1 ± 0.4^{cd}(4)	94.7 ± 0.3^{bc}(4)
T3	85.2 ± 0.5^{abc} (4)	88.9 ± 0.1^{abc}(4)	93.5 ± 0.2^{b}(4)
T4	86.9 ± 0.2^{bc} (5)	89.2 ± 0.4^{abc}(4)	94.1 ± 0.2^{bc}(5)
T5	90.3 ± 0.6^{d} (5)	93.1 ± 0.6^{d}(5)	95.5 ± 0.4^{c}(5)
T6	82.7 ± 0.2^{a} (3)	87.9 ± 0.3^{ab}(3)	91.4 ± 0.2^{a}(5)
T7	84.9 ± 0.4^{ab} (5)	87.5 ± 0.5^{a}(5)	nd
T8	85.7 ± 0.5^{bc} (4)	90.3 ± 0.4^{bc}(4)	nd

Appendix 3.3 Chromic oxide (Cr_2O_3) contents in diets and faeces on a dry matter basis from experiment determined AD for *S. paramamosain* (g kg^{-1})

Treatments	In diets	In faeces		
		Replicate 1	Replicate 2	Replicate 3
V1	3.57	13.80	15.70	14.36
V2	4.01	17.10	15.27	16.09
V3	3.73	14.13	15.66	13.89
V4	3.61	16.63	16.45	15.59
V5	4.14	14.97	16.10	16.02
V6	4.40	14.05	13.99	15.16
V7	3.70	14.44	14.48	14.26
V8	3.61	15.97	16.47	16.56
V9	3.52	14.66	15.10	14.83
V10	3.79	15.00	15.57	16.02
V11	3.25	16.37	15.82	16.28
V12	3.26	15.75	14.00	15.93
V13	3.62	16.54	15.68	14.40
V14	3.63	15.28	16.68	15.52

Appendix 3.4 Apparent digestibility for dry matter (ADD), crude fat (ADF) protein (ADP), ash (ADA), and nitrogen free extract (ADN) in juvenile mud crab, *S. paramamosain*, for fourteen diets (data was expressed in mean ± SE with n = 3)*

Experimental diets	ADD (%)	ADP (%)	ADA (%)	ADN (%)	ADF (%)
V1	75.5 ± 0.9^{bcd}	80.4 ± 0.7^{b}	48.6 ± 3.6^{a}	79.7 ± 0.5^{bc}	83.7 ± 2.8^{abcd}
V2	76.0 ± 0.8^{bcd}	82.2 ± 1.0^{bc}	54.1 ± 1.7^{ab}	77.5 ± 0.6^{b}	80.9 ± 1.1^{a}
V3	75.4 ± 0.9^{bcd}	81.5 ± 0.9^{b}	68.3 ± 1.2^{c}	72.0 ± 1.6^{a}	82.1 ± 0.3^{ab}
V4	78.1 ± 0.4^{d}	84.7 ± 0.4^{c}	51.2 ± 1.5^{ab}	78.9 ± 0.4^{bc}	83.2 ± 0.4^{abc}
V5	73.3 ± 0.6^{ab}	80.8 ± 0.4^{b}	47.8 ± 2.3^{a}	72.5 ± 1.2^{a}	85.8 ± 0.6^{abcde}
V6	70.6 ± 0.8^{a}	77.1 ± 0.2^{a}	51.6 ± 0.8^{ab}	69.7 ± 1.4^{a}	87.0 ± 0.3^{bcde}
V7	73.8 ± 0.1^{bc}	79.9 ± 0.2^{b}	58.4 ± 1.1^{b}	69.6 ± 0.3^{a}	90.5 ± 0.8^{e}
V8	78.0 ± 0.3^{d}	81.4 ± 0.2^{b}	49.5 ± 0.2^{a}	81.8 ± 0.4^{c}	82.4 ± 0.3^{ab}
V9	76.0 ± 0.2^{bcd}	81.1 ± 0.3^{b}	51.2 ± 0.9^{ab}	81.4 ± 0.2^{bc}	84.2 ± 0.4^{abcd}
V10	76.4 ± 0.5^{cd}	81.3 ± 0.5^{b}	48.9 ± 0.2^{a}	78.4 ± 0.6^{bc}	82.7 ± 0.2^{ab}
V11	nd	nd	nd	nd	88.4 ± 0.4^{de}
V12	nd	nd	nd	nd	89.3 ± 0.7^{e}
V13	nd	nd	nd	nd	90.1 ± 0.2^{e}
V14	nd	nd	nd	nd	87.8 ± 1.2^{cde}

*Means within the same column having a similar superscript letter are not significantly different at 5% of significance (P>0.05); nd = not determined.

Appendix 4.1 Statistical analysis of data of Chapter 4

ANOVA Table for weight gain

Effect	SS	Degree of freedom	MS	F	P
DP	627426	9	69714	7.857	0.000001
Tanks	4484	2	2242	0.253	0.777777
DP*Tanks	80856	18	4492	0.506	0.941473
Error	408171	46	8873		

ANOVA Table for carapace width increase

Effect	SS	Degree of freedom	MS	F	P
DP	15336.8	9	1704.1	11.382	0.000000
Tanks	565.5	2	282.7	1.888	0.162847
DP*Tanks	1287.2	18	71.5	0.478	0.955170
Error	6887.2	46	149.7		

ANOVA Table for specific growth rate

Effect	SS	Degree of freedom	MS	F	P
DP	19.5514	9	2.1724	7.998	0.000001
Tanks	1.0210	2	0.5105	1.879	0.164204
DP*Tanks	2.9096	18	0.1616	0.595	0.884623
Error	12.4939	46	0.2716		

ANOVA Table for moult period

Effect	SS	Degree of freedom	MS	F	P
DP	3050.5	9	338.9	2.993	0.006845
Tanks	702.6	2	351.3	3.102	0.054487
DP*Tanks	1084.6	18	60.3	0.532	0.927154
Error	5209.5	46	113.3		

ANOVA Table for feed conversion ratio

Effect	SS	Degree of freedom	MS	F	P
DP	14.5870	9	1.6208	9.371	0.000000
Tanks	0.3825	2	0.1912	1.106	0.339608
DP*Tanks	2.5675	18	0.1426	0.825	0.663209
Error	7.9557	46	0.1730		

ANOVA Table for protein efficiency ratio

Effect	SS	Degree of freedom	MS	F	P
DP	8.5969	9	0.9552	9.641	0.000000
Tanks	0.3341	2	0.1670	1.686	0.196508
DP*Tanks	2.2047	18	0.1225	1.236	0.274125
Error	4.5575	46	0.0991		

Appendix 5.1 The main steps to balance essential amino acid in diet (Chapter 5)

Step 1: Design a crude protein level for each diet by calculating the crude protein from the ingredients (see Table 3)

Step 2: Computing all EAA of each diet from the ingredients including: Arginine-Arg, histidine-hist, isoleucine-iso, leucine-leu, lysine-lys, methionine-met, phenylalanine-phen, threonine-thr, valine-val, tyrosine-tyr, cystine-cyt, and tryptophan-tryp.

EAAs	Diets											
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Arg	17.5	17.5	17.5	23.2	23.2	23.2	29.4	29.4	29.4	35.8	35.8	35.7
Hist	8.5	8.5	8.5	11.3	11.3	11.3	14.2	14.2	14.2	17.3	17.3	17.3
Iso	13.6	13.6	13.6	17.8	17.8	17.8	22.4	22.4	22.4	27.1	27.1	27.1
Leu	20.2	20.2	20.2	26.2	26.2	26.2	32.9	32.9	32.9	39.4	39.4	39.4
Lys	19.3	19.3	19.3	25.9	25.9	25.9	33.1	33.1	33.1	40.6	40.6	40.6
Met	6.9	6.9	6.9	9.2	9.2	9.2	11.6	11.6	11.6	14.1	14.1	14.1
Cyt	3.3	3.3	3.3	4.1	4.1	4.1	5.0	5.0	5.0	5.7	5.7	5.7
Phen	16.3	16.3	16.3	21.4	21.4	21.4	26.9	26.9	26.9	32.4	32.4	32.4
Tyr	9.6	9.6	9.6	12.5	12.5	12.5	15.8	15.8	15.8	19.0	19.0	19.0
Thr	12.0	12.0	12.0	15.9	15.9	15.9	20.1	20.1	20.1	24.4	24.4	24.4
Val	12.9	12.9	12.9	16.9	16.9	16.9	21.2	21.2	21.2	25.5	25.5	25.5
Tryp	2.7	2.7	2.7	3.5	3.5	3.5	4.3	4.3	4.3	5.2	5.2	5.2
Total	142.8	142.8	142.8	187.8	187.8	187.8	236.8	236.8	236.8	286.3	286.3	286.3

Step 3: Computing A/E of EAA in each diet and in mud crab tissue

A/E of EAA in diet

EAAS	A/E in EAAs of diets												A/E of EAAs in Mud crab
	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**T12**	
Arg	122.5	122.5	122.5	123.4	123.4	123.4	124.0	124.0	124.0	124.9	124.9	124.9	89.8
Hist	59.8	59.8	59.8	60.0	60.0	60.0	60.1	60.1	60.1	60.3	60.3	60.3	63.1
Iso	95.0	95.0	95.0	94.9	94.9	94.9	94.7	94.7	94.7	94.6	94.6	94.6	95.4
Leu	141.1	141.1	141.1	139.7	139.7	139.7	138.8	138.8	138.8	137.5	137.5	137.5	121.9
Lys	135.5	135.5	135.5	137.9	137.9	137.9	139.6	139.6	139.6	141.8	141.8	141.9	127.9
Met	48.5	48.5	48.5	48.8	48.8	48.8	49.0	49.0	49.0	49.2	49.2	49.2	90.4
Cyt	23.1	23.1	23.1	21.9	21.9	21.9	21.0	21.0	21.0	19.9	19.9	19.9	23.1
Phen	114.3	114.3	114.3	113.9	113.9	113.9	113.6	113.6	113.6	113.2	113.2	113.2	85.1
Tyr	66.9	66.9	66.9	66.7	66.7	66.7	66.6	66.6	66.6	66.4	66.4	66.4	91.9
Thr	84.2	84.2	84.2	84.6	84.6	84.6	84.9	84.9	84.9	85.2	85.2	85.2	107.1
Val	90.3	90.3	90.3	89.8	89.8	89.8	89.4	89.4	89.4	88.9	88.9	88.9	85.1
Tryp	18.7	18.7	18.7	18.4	18.4	18.4	18.3	18.3	18.3	18.1	18.1	18.1	19.2
Total	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0

Step 4: Comparison of A/E from diet with A/E crab tissue (Difference = A/E diet – A/E of mud crab tissue)

The difference between A/E in based ingredient diet and A/E in mud crab tissue

	Diets											
EAA	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**T12**
Arg	32.7	32.7	32.7	33.6	33.6	33.6	34.2	34.2	34.2	35.1	35.1	35.1
Hist	-3.3	-3.3	-3.3	-3.1	-3.1	-3.1	-3.0	-3.0	-3.0	-2.8	-2.8	-2.8
Iso	-0.4	-0.4	-0.4	-0.6	-0.6	-0.6	-0.7	-0.7	-0.7	-0.9	-0.9	-0.9
Leu	19.2	19.2	19.2	17.8	17.8	17.8	16.9	16.9	16.9	15.6	15.6	15.6
Lys	7.5	7.5	7.5	10.0	10.0	10.0	11.7	11.7	11.7	13.9	13.9	13.9
Met	-41.9	-41.9	-41.9	-41.6	-41.6	-41.6	-41.4	-41.4	-41.4	-41.1	-41.1	-41.1
Cyt	0.0	0.0	0.0	-1.2	-1.2	-1.2	-2.1	-2.1	-2.1	-3.2	-3.2	-3.2
Phen	29.2	29.2	29.2	28.8	28.8	28.8	28.5	28.5	28.5	28.1	28.1	28.1
Tyr	-25.0	-25.0	-25.0	-25.1	-25.1	-25.1	-25.3	-25.3	-25.3	-25.5	-25.5	-25.5
Thr	-22.9	-22.9	-22.9	-22.5	-22.5	-22.5	-22.2	-22.2	-22.2	-21.8	-21.8	-21.8
Val	5.2	5.2	5.2	4.7	4.7	4.7	4.3	4.3	4.3	3.8	3.8	3.8
Tryp	-0.5	-0.5	-0.5	-0.7	-0.7	-0.7	-0.9	-0.9	-0.9	-1.1	-1.1	-1.1

Appendix 5.2 Factorial ANOVA for growth and feed data of Chapter 5

ANOVA Table for Weight gain

Effects	SS	Degree of freedom	MS	F	P
TB-EAA	899116	3	299705	8.085	0.000087
DE	48926	2	24463	0.660	0.519586
TB-EAA*DE	447135	6	74522	2.010	0.073360
Error	3076822	83	37070		

ANOVA Table for Carapace width increase

Effects	SS	Degree of freedom	MS	F	P
TB-EAA	3597	3	1199	4.218	0.007922
DE	417	2	209	0.734	0.483080
TB-EAA*DE	1958	6	326	1.148	0.342308
Error	23594	83	284		

ANOVA Table for Specific growth rate

Effects	SS	Degree of freedom	MS	F	P
TB-EAA	9.849	3	3.283	6.794	0.000375
DE	1.621	2	0.810	1.677	0.193153
TB-EAA*DE	4.236	6	0.706	1.461	0.201664
Error	40.104	83	0.483		

ANOVA Table for Inter-moult period

Effects	SS	Degree of freedom	MS	F	P
TB-EAA	3899.6	3	1299.9	6.322	0.000649
DE	649.7	2	324.9	1.580	0.212134
TB-EAA*DE	264.1	6	44.0	0.214	0.971390
Error	17067.0	83	205.6		

ANOVA Table for Feed conversion ratio

Effect	SS	Degree of freedom	MS	F	P
TB-EAA	3.1998	3	1.0666	10.496	0.000006
DE	0.5669	2	0.2835	2.790	0.067220
TB-EAA*DE	0.3094	6	0.0516	0.508	0.801048
Error	8.4342	83	0.1016		

ANOVA Table for Protein efficiency ratio

Effect	SS	Degree of freedom	MS	F	P
TB-EAA	35.7947	3	11.9316	88.581	0.000000
DE	1.2211	2	0.6106	4.533	0.013541
TB-EAA*DE	0.7034	6	0.1172	0.870	0.520316
Error	11.1799	83	0.1347		

ANOVA Table for Apparent net protein utilisation

Effect	SS	Degree of freedom	MS	F	P
TB-EAA	3786.25	3	1262.08	72.658	0.000000
DE	62.76	2	31.38	1.807	0.170599
TB-EAA*DE	416.72	6	69.45	3.998	0.001451
Error	1441.73	83	17.37		

Appendix 5.3 Factorial ANOVA for body composition data of Chapter 5

ANOVA Table for Dry matters (DM)

Effect	SS	Degree of freedom	MS	F	P
TB-EAA	7.05	3	2.35	144	0.0000
DE	5.98	2	2.99	183	0.0000
TB-EAA*DE	54.20	6	9.03	554	0.0000
Error	0.39	24	0.02		

ANOVA Table for Crude proteins (CP)

Effect	SS	Degree of freedom	MS	F	P
TB-EAA	91.743	3	30.581	37.361	0.000000
DE	5.321	2	2.661	3.250	0.056338
TB-EAA*DE	13.745	6	2.291	2.799	0.032952
Error	19.645	24	0.819		

ANOVA Table for Total lipid (TL)

Effect	SS	Degree of freedom	MS	F	P
TB-EAA	2.44585	3	0.81528	25.879	0.000000
DE	0.43987	2	0.21993	6.981	0.004076
TB-EAA*DE	0.90405	6	0.15067	4.783	0.002442
Error	0.75608	24	0.03150		

ANOVA Table for Carbohydrates (CHO)

Effect	SS	Degree of freedom	MS	F	P
TB-EAA	0.69291	3	0.23097	16.110	0.000006
DE	0.29544	2	0.14772	10.303	0.000589
TB-EAA*DE	0.39324	6	0.06554	4.571	0.003157
Error	0.34410	24	0.01434		

Appendix 6.1 Factorial ANOVA for weight gain of Chapter 6

ANOVA Table of weight gain for whole period (three moults)

Effect	SS	Degree of freedom	MS	F	P
LOA	38295	4	9574	1.002	0.417682
LNA	291264	3	97088	10.165	0.000042
LOA*LNA	217450	12	18121	1.897	0.064635
Error	382046	40	9551		

ANOVA Table for weight gain at the first moult

Effect	SS	Degree of freedom	MS	F	P
LOA	2283.2	4	570.8	1.320	0.279176
LNA	6617.3	3	2205.8	5.100	0.004400
LOA*LNA	8304.9	12	692.1	1.600	0.130752
Error	17299.2	40	432.5		

ANOVA Table for weight gain at the second moult

Effect	SS	Degree of freedom	MS	F	P
LOA	2859.1	4	714.8	1.856	0.137111
LNA	2432.9	3	811.0	2.106	0.114657
LOA*LNA	3212.7	12	267.7	0.695	0.746076
Error	15401.5	40	385.0		

ANOVA Table for weight gain at the third moult

Effect	SS	Degree of freedom	MS	F	P
LOA	1333.0	4	333.3	2.641	0.047713
LNA	1026.6	3	342.2	2.712	0.057661
LOA*LNA	4170.9	12	347.6	2.754	0.008055
Error	5047.6	40	126.2		

Appendix 7.1 Main steps to balance of essential amino acids in Chapter 7

Step 1: As described in main Text.

Step 2: Calculate A/E of EAA in diet and A/E of EAA in mud crab tissue

The quantity of EAAs and A/E of EAA in diet and A/E of EAA in mud crab

EAAs	Diets				A/E in mud crab tissue
	P2		P3		
	g kg^{-1}	A/E	g kg^{-1}	A/E	
Arginine	18.4	112.8	22.6	131.2	89.80
Histidine	11.6	71.1	10.9	63.4	63.12
Isoleucine	13.6	83.7	15.4	89.2	95.43
Leucine	18.6	114.6	21.5	124.7	121.92
Lysine	19.3	118.4	21.1	122.5	127.94
Methionine	11.8	72.7	9.6	55.5	90.36
Phenylalanine	17.2	105.7	17.3	100.3	85.10
Threonine	16.1	98.8	15.5	90.0	107.08
Valine	13.6	83.8	15.7	91.4	85.10
Tyrosine	14.6	89.7	13.6	79.1	91.87
Cystine	4.3	26.3	4.8	27.9	23.11
Tryptophan	3.6	22.4	4.3	24.7	19.16
Total	162.7	1000	172.2	1000	1000

Step 3: Compute a difference = A/E in diet minus A/E in mud crab tissue

Differences between A/E in diet and A/E in mud crab tissue

EAAs	P2	P3
Arginine	23.1	41.4
Histidine	8.0	0.3
Isoleucine	-11.7	-6.3
Leucine	-7.3	2.8
Lysine	-9.5	-5.4
Methionine	-17.7	-34.9
Phenylalanine	20.6	15.2
Threonine	-8.3	-17.1
Valine	-1.3	6.3
Tyrosine	-2.1	-12.7
Cystine	3.2	4.8
Tryptophan	3.2	5.6

Step 4: Determine which EAA need to supply, lysine of 2 g kg^{-1} was added in diet P2 and methionine of 7.5 g kg^{-1} was added in diet P3

Step 5: Set up a balance EAA in diet P2 and P3

The quantity of each EAA in balance

Essential amino acids	P2	P3
Arginine	14.9	17.0
Histidine	10.5	11.9
Isoleucine	15.9	18.0
Leucine	20.3	23.0
Lysine	21.3	24.2
Methionine	15.0	17.1
Phenylalanine	14.1	16.1
Threonine	17.8	20.2
Valine	14.1	16.1
Tyrosine	15.3	17.3
Cystine	3.8	4.4
Tryptophan	3.2	3.6
Total	166.2	188.8

Step 6: Determine amount of each EAA need to supplement for each diet

The amount of each EAA need to supplement for each diet

Essential amino acids	P2	P3
Arginine	-3.4	-5.6
Histidine	-1.1	1.0
Isoleucine	2.2	2.7
Leucine	1.6	1.5
Lysine	2.0	3.1
Methionine	3.2	7.5
Phenylalanine	-3.1	-1.2
Threonine	1.7	4.7
Valine	0.5	0.3
Tyrosine	0.7	3.7
Cystine	-0.4	-0.4
Tryptophan	-0.5	-0.6
Total	12.0	24.1

Note: Negative values indicate that the level of this EAA was in excess.

Appendix 7.2 ANOVA for growth and feed utilization data of Chapter 7

ANOVA Table for weight gain

Effect	SS	Degree of freedom	MS	F	P
Treatments	1410111	5	282022	30.104	0.000000
Error	215473	23	9368		

ANOVA Table for carapace width increase

Effect	SS	Degree of freedom	MS	F	P
Treatments	13822.8	5	2764.6	4.875	0.003473
Error	13043.5	23	567.1		

ANOVA Table for inter- moult period

Effect	SS	Degree of freedom	MS	F	P
Treatments	88.85	5	17.77	1.995	0.117492
Error	204.90	23	8.91		

ANOVA Table for specific growth rate

Effect	SS	Degree of freedom	MS	F	P
Treatments	95.964	5	19.193	8.568	0.000107
Error	51.518	23	2.240		

ANOVA Table for weight gain

Effect	SS	Degree of freedom	MS	F	P
Treatments	4.64722	5	0.92944	12.8046	0.000005
Error	1.66950	23	0.07259		