A Nitrogen budget for *Penaeus indicus* juveniles fed on diets of different protein levels

A thesis submitted to the University of Wales
in partial fulfilment of the requirements for the Degree of

Magister in Scientia.

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November 1997

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Menai Bridge,
Gwynedd, LL59 5EY.
DECLARATION

The work here submitted has not previously been accepted in substance for any degree
and is not being concurrently submitted in candidature for any other degree.

Candidate........................
Date 20 November 1994

This is to certify that the work submitted here was carried out by the candidate himself
with any help received being duly acknowledged.

Supervisor........................
Candidate........................
Date 20 November 1994
SUMMARY

Juvenile *Penaeus indicus* were fed isocaloric diets containing 10, 20 and 40% protein over a 6 week period. The diets containing 20 and 40% protein promoted the best growth in the present study.

The ingestion rate of shrimps fed on 40% protein diets was found to be significantly lower than for those fed on diets containing 10 and 20% protein. No significant differences existed between the latter two diets. Examination of the relationship between specific trypsin activity and percentage of protein present in the diets showed that these were correlated and a highly significant regression was obtained. No significant differences was found between digestibility coefficients of dry matter between the groups of shrimps fed on the 3 diets. Digestibility coefficients of protein in the diets was 89.57% for 20% and 94.23% for 40% protein diets. These were significantly higher than 71.83% for the 10% protein diets.

Shrimp fed at the highest protein concentration (40%), showed ammonia excretion levels 20 times higher than on the other 2 diets.

Of the three diets tested in this study, the 20% protein diet consistently provided the most cost effective benefits to the shrimp in terms of the high consumption, growth, digestibility and minimal ammonia excretion.
ACKNOWLEDGEMENTS

I am sincerely grateful to my supervisor, Dr. David A. Jones for his advice and valuable comments throughout all the practical work and writing of this dissertation. I am also indebted to Dr. A. Yule for his guidance during the practical work.

This study would not have been possible without the love and moral support of my beloved wife Lexy.

I wish to express my deepest thanks to my parents Carlos and Antonieta for putting up with me throughout my life. Their support, patience, understanding and incentive have made it all possible. I am also very grateful to my brother Renzo and my sister Lourdes.

The acknowledgements are extended to Mr. Enrique Olivares and Mr. Fernando Ribero for fruitful discussions during the course of this study.

Special appreciation to the School's technical staff Gwyn Hughes, Baerwyn Roberts, John Rowlands for their help, friendship and kind assistance during three months of practical work in the Tropical Laboratory.

My stay in Menai Bridge would not have been bright without the company of all the members of the MSc. Shellfish course.

Finally, I express my gratitude to the Foundation for Science and Technology (FUNDACYT) for the award that permitted me to undertake the MSc studies and the British Council for administration of this course.
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Chapter 1

INTRODUCTION

Decapods are the largest of the crustacean orders and contain widely diverse forms, including the many familiar shrimps, lobsters, crabs and crayfish.

There are over 140 species of penaeid shrimps in 11 genera but only 16 species in 6 genera are farmed (Pillay, 1993). Many species such as *P. schmitti*, have been cultivated experimentally often in laboratory settings; however, viable commercial aquaculture has developed for fewer than 10 species. The shrimps most widely cultivated are *P. monodon* (49.8%), *P. vannamei* (15.5%), *P. chinensis* (13.8%), *P. merguiensis* (6.7%), *P. indicus* (5.4%), *P. stylirostris* (1.8%) and *P. japonicus* (1.7%) (Csavas, 1994).

The global production of cultured shrimps has shown a dramatic increase in the last 10 years. World farmed shrimp production increased from over 300,000 metric tonnes (MT) during 1985 and reached 712,000 MT in 1995 (Rosenberry, 1995). The demand for shrimp is continuously growing which provides a strong stimulus for commercial shrimp culture throughout the world. The greatest commercial success, to date, in cultivating shrimps has been then establishment in China whose production rose 400,000 MT in 1995 (Rosenberry, 1995).

The aquaculture of these species requires the use of both nutritional and economical formulated diets. Such feeds typically represent the most significant operating costs (40-50%). In semi-intensive culture the amount of supplementary feeding increases to 40% of the total food requirement compared to about 60% in intensive culture (Csavas, 1994).

The potential value of shrimp as a food crop has resulted in many studies on their anatomy, physiology, biochemistry and nutrition (see reviews by Gibson and Barker, 1979; Dall and Moriarty, 1983; Brunet et al., 1994).
The occurrence and characterization of digestive enzymes in penaeids (Table 1) is of importance in order to determine the capacity of these species to utilize food sources.

The ability to digest and utilize these components depends on the presence of appropriate enzyme activities in the gut. Digestion in penaeids is dependant on the stage of development which involves anatomy, physiology and mechanical functions of the digestive system. The variation in gut structure and enzymic digestion across morphological changes reflects differences in dietary regimes.

Shrimp rely mainly on trypsin and to a lesser degree chymotrypsin for protein digestion (Lan and Pan, 1991). The primary catalytic sites of trypsin are the basic amino acids, lysine and arginine whilst chymotrypsin cleaves protein at the aromatic amino acids.

Several authors have reported the absence of chymotrypsin activity in penaeids (Gates and Travis, 1969; Galgani et al., 1984). This is in contrast to the contribution of chymotrypsin throughout development in P. monodon and P. vannamei which has been detected using an extended polypeptide substrate (Fang and Lee, 1992; Le Moullac et al., 1994). This enzyme has also been characterized by chromatography and immunoquantification in 4 penaeids and measured biochemically (Tsai et al., 1991; Le Moullac et al., 1997). Shrimp chymotrypsin present in crude extracts can be easily be overlooked by the use of unfavourable substrate. On the other hand, the presence of inhibitors may react with chymotrypsin and give false interpretations.
Table 1. Digestive enzymes present in penaeid shrimps at different stages of life. + = presence; ± = traces; - = absence

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>Trypsin</th>
<th>Chymotrypsin</th>
<th>Leucineaminopeptidase A</th>
<th>Leucineaminopeptidase B</th>
<th>Carboxypeptidase A</th>
<th>Carboxypeptidase B</th>
<th>Lipase</th>
<th>Esterase</th>
<th>Amylase</th>
<th>Macrocystisidas</th>
<th>Galactosidase</th>
<th>Lambdaconase</th>
<th>Chitinase</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penaeus monodon</em></td>
<td>Larva</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>MacDonald et al. (1989), Kumlu (1991),</td>
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<td>Glass et al. (1989), Chen &amp; Lin (1990),</td>
<td></td>
<td></td>
<td></td>
<td>Tsai et al. (1991), Lan and Pan (1991),</td>
</tr>
<tr>
<td></td>
<td>Juvenile/Adult</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Fang and Lee (1992)</td>
</tr>
<tr>
<td><em>Penaeus japonicus</em></td>
<td>Larva</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Laubier-Bonichon (1997), Galgani &amp; Benyamin</td>
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<td></td>
<td></td>
<td>(1985), Kamarudin (1992)</td>
<td></td>
<td></td>
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<td>Cuzon et al. (1980), Galgani et al. (1984,</td>
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<td></td>
<td></td>
<td>(1982ab, 1983ab), Chuang et al. (1985),</td>
<td></td>
<td></td>
<td></td>
<td>Tsai et al. (1986b), Tsai et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>Juvenile/Adult</td>
<td>+</td>
<td>±±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Gates &amp; Travis (1969, 1973), Lee &amp; Lawrence</td>
</tr>
<tr>
<td><em>Penaeus setiferus</em></td>
<td>Larva</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Lee &amp; Lawrence (1982), Galgani &amp; Benyamin</td>
</tr>
<tr>
<td></td>
<td>Juvenile/Adult</td>
<td>+</td>
<td>±±</td>
<td>±α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>(1983, 1988)</td>
</tr>
</tbody>
</table>
Table 1. Digestive enzymes present in penaeid shrimps at different stages of life. + = presence; ± = traces; - = absence

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>Protease</th>
<th>Trypsin</th>
<th>Chymotrypsin</th>
<th>Leucineaminopeptidase A</th>
<th>Leucineaminopeptidase B</th>
<th>Carboxypeptidase</th>
<th>Carboxypeptidase</th>
<th>Lipase</th>
<th>ESTerase</th>
<th>Amylase</th>
<th>Maltase</th>
<th>Sucrose Isomerase</th>
<th>Galactosaminidase</th>
<th>Cellulase</th>
<th>Chitinase</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penaeus occidentalis</em></td>
<td>Adult</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Lee &amp; Lawrence (1982).</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Penaeus aztecus</em></td>
<td>Adult</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Lee &amp; Lawrence (1982).</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. penicillatus</em></td>
<td>Juv/Adu</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Chuang et al. (1985), Tsai et al. (1986b, 1991).</td>
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</tr>
</tbody>
</table>
Enzymatic catabolism of ingested foods has been described for different species and is summarized in (Table 1). Investigations on several digestive enzymes have depended almost exclusively upon biochemical essays of tissue homogenates or luminar extracts. An important feature of shrimp is the absence of pepsin (Lee and Lawrence, 1982) and elastase (Tsai et al., 1991).

Among some 50 species of marine invertebrates studied, decapods showed the highest proteolytic activities (Kozlovskaya and Vaskovsky, 1970). This coincides with the fact that shrimps require between 40-60% protein in the diets (Rodriguez et al., 1994).

Trypsin is a member of the family of proteinases and the most abundant proteolytic enzyme in Crustacea. It represents 40-60% of the proteolysis activity in the hepatopancreas of penaeids (Galgani et al., 1984; Le Moullac et al., 1997). This enzyme has been carefully studied in a number of shrimps, but always with an active form of the enzyme; a zymogen, or inactive form, has not been identified (Tsai et al., 1991). Probably, based on the fact that a decreased number of basic amino acid residues reduces the possibility of autocatalysis, zymogen may not be as important in shrimps as in animals with more basic amino acid residues.

Adaptation of digestive enzyme level to quantity and quality of protein and carbohydrates in the diet and variation with animal size have been demonstrated in P. vannamei (Lee et al., 1984; Le Moullac et al., 1994; Le Moullac et al. 1997), P. setiferus (Lee and Lawrence, 1985) and Homarus gammarus (Kurmaly, 1989; Glass and Stark, 1995).

The physiological responses of an organism as a function of dietary changes can be assessed in terms of simple, integrated physiological parameters such as metabolic, excretory and growth rates. A combination of these provides an insight into physiological mechanisms involved in the nutrient processing, absorption and
utilization and thereby allow the optimization of food according to nutritional requirement of the studied species.

Studies on shrimp nutrition and digestive enzymes have led to the formulation of improved diets for the numerous developmental stages of widely cultured P. monodon, P. japonicus, P. vannamei, P. stylirostris, P. indicus and Macrobrachium rosenbergii (Jones et al., 1993). Many of the dietary requirements for adult penaeids are known (Cuzon et al., 1994) but few studies have been performed by determining specific metabolic requirements for penaeid larval growth.

However changes in nutritional requirements during development may modulate the enzyme levels of shrimp when food conditions are kept constant (Rodriguez et al., 1994). The relation between food quality and activity of digestive enzymes has been examined by modifying the biochemical composition of artificial feed (Jones et al., 1993; Le Moullac et al., 1994; Rodriguez et al., 1994). Le Moullac et al. (1997) showed a dose response effect between the amount of trypsin detected and the level of casein given to P. vannamei juveniles. In contrast amylase activity decreased when the casein was increased. Highest activities of chymotrypsin and amylase were measured for squid meal and casein.

The effect of protein level on trypsin, amylase adaptation which can vary with developmental stages, sex, season, temperature, water quality, and other environmental factors, may give clearer indications about how digestive enzymes are stimulated by nutritional components. For this reason it is necessary to understand how this adaptation is limited by the quality and quantity of the available food and shrimp requirements. Studies attempting to understand the adaptive significance of preferences in omnivorous species should focus on how trypsin synthesis occurs. Different experimental conditions should be provided to assess whether variations in protein and energy levels induce trypsin synthesis.
Chapter 1

There are both benefits and problems associated with feeding live animals. One of the most common problems is that artificial food has limited stability. In order to effectively and properly feed shrimp, it must be considered their natural feeding habits in terms of frequency and quantity. Many experiments are carried on different feeding regime and ration regardless of the species of the shrimp or properties of the diets.

Optimization of feed quality for shrimp culture is important for biological, environmental, health and economical reasons. The impact of feed on growth rate and feed conversion will depend on the ingredients used and on their quality. Digestibility of the diet or of any single nutrient of the diet is the proportion which is not excreted in the feces and hence apparently digested and absorbed (Schneider and Flatt, 1975; McDonald et al., 1988). Protein digestibility has been extensively used as a good measure to evaluate overall digestibility.

The estimation of nutrients digestibility directly in aquatic animals based on conventional method of the total collection of fecal material produced after consumption of food offered is difficult due to the problems of quantifying ingestion and egestion and time required to collect enough feces. In shrimp, the use of chromic oxide has been the most common marker although questions about different passing rates of food and marker through the digestive tract have been raised (Forster and Gabbot, 1971) further studies comparing the others markers to chromic oxide have found no statistical differences regarding digestibility coefficients between markers used. In addition chromic oxide is less expense, safer and easier to measure digestibility (Deering et al., 1996; Amouroux et al., 1997). For these reasons, chromic oxide is applied as a reference substance for determining the effects of protein concentration on absorption efficiency for the shrimp *P. indicus*.
Crustacea in general are ammoniotelic, meaning that the main nitrogenous waste is excreted as ammonia. Amino acids, urea and uric acid are also excreted but in lower concentrations (Claybrook, 1983). Ammonia is most efficient and least expensive way of excreting nitrogen. The metabolization of protein by aminooxidases, trans- and de-aminases results in carbon, ammonia, water and heat production (Claybrook, 1983).

Protein requirements tend to be species specific for penaeid shrimp ranging from 28% in *P. setiferus* (Andrews et al. 1972) to more than 60% in *P. japonicus* (Deshimaru and Shigueno, 1972). Protein is not only the most expensive ingredient in artificial diets but also a limiting nutrient for growth in shrimp which is why so much emphasis been put on it when formulating artificial diets.

When there is a lack of energy sources such as lipids or carbohydrates, dietary proteins can be easily deaminated and oxidized to provide energy rather than growth. On the other hand, excess energy is though to reduce ingestion rate and thereby lowering the total protein intake (McDonald, 1988). Several studies have shown that an adequate protein/energy balance could minimize the use of protein and reduce the amount of ammonia excreted by shrimp (Hajra et al., 1988; Shiau and Chou, 1991; Koshio et al., 1993a).

The aim of this work is to construct a complete nitrogen budget for *Penaeus indicus* juveniles fed on freshly prepared artificial diets containing 10, 20 and 40% protein contents. It is designed to give information regarding not only growth and ammonia excretion rates, but also the digestibility of dry matter and trypsin activity in the hepatopancreas for each diet. In addition, the effect of shrimp feeding activities on nutrient leaching rates, which may in turn affect the ingested and assimilated protein contents, are examined.
Chapter 2

MATERIALS AND METHODS

2.1 Growth trial

Penaeus indicus juveniles, from stock originating from the Red Sea, grown in the experimental facilities of School of Ocean Sciences, University College of North Wales, Bangor, were placed in a 2500 l raceway supplied by seawater aerated and recirculated through a biological filter. The shrimps were fed fresh mussel, Mytilus edulis L., to satiation once daily for a month and reared under laboratory conditions until they reached a size of more than 2g.

The feeding experiment was performed on juvenile P. indicus, which had attained a mean body weight of 2.53 ± 0.04g, stocked at a density of 5 shrimp for each of 12 plastic wash baskets (23x56x40cm, HxLxW) which were submerged in running seawater at 28.6 ± 0.6 °C in the 2500 l raceway. The basket windows were lined with 2mm mesh netting using aquarium silicon for attachment and the top was covered with 1cm mesh netting to prevent the shrimps from escaping. Shrimps were fed 15 times daily at 90 min intervals on diets containing 10%, 20% and 40% protein. The feed rations were equal to 8% of the body weight to promote growth and stimulate trypsin activity in the P. indicus (Srihar et al., 1995). Shrimps were fed daily from 08h00 to 00h30, where the last 3 of the 15 feeds were delivered together. The first 7 days were considered as a conditioning period to the artificial diets. Treatments were replicated 4 times in a randomized design.

Non-consumed food and faeces were siphoned out every morning before commencing with the first feeding while moults were removed throughout the day. Shrimp were weighed at the start of the experiment, after 25 days (to adjust feeding rations) and at the end of the trial. The shrimp were gently dried on soft paper and individually weighed to the nearest 0.1g using a Sartorius balance. Salinity was 32ppt and the photoperiod was set at 15h of light and 9h of darkness.
The shrimps were maintained on their respective diets for 43 days, in order to establish whether there were differences in growth, survival and trypsin activity, ammonia excretion, ingestion rate and digestibility with were also determined respect to each of the 3 different diets.

2.2 Diet formulation

2.2.1 Diet composition

Three isocaloric diets providing 10, 20 and 40% protein were prepared. These were based on the same formulation that was prepared and tested for growth experiments with *P. vannamei* by Velasco *et al.* (1996) and are summarised in Table 2.

Table 2. Composition of experimental diets 10, 20, %40 protein (% dry matter basis)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>10%</th>
<th>20%</th>
<th>40%</th>
</tr>
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<tbody>
<tr>
<td>Cod fish meal (2)</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Krill meal</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Lecithin (4)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Carboxymethylcellulose(1)</td>
<td>4</td>
<td>3.69</td>
<td>2.82</td>
</tr>
<tr>
<td>Na2HPO4 reagent (1)</td>
<td>1.9</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Mineral mixture (5)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Vitamin mixture (5)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Chromic oxide (3)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Wheat starch (1)</td>
<td>63.40</td>
<td>48.82</td>
<td>17.21</td>
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<tr>
<td>Soybean meal</td>
<td>0.00</td>
<td>7.79</td>
<td>29.41</td>
</tr>
<tr>
<td>Wheat gluten (1)</td>
<td>0.35</td>
<td>6.23</td>
<td>12.85</td>
</tr>
<tr>
<td>Cod fish oil</td>
<td>6.42</td>
<td>5.93</td>
<td>5.25</td>
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<tr>
<td>Kaolin (3)</td>
<td>1.93</td>
<td>3.65</td>
<td>8.56</td>
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</table>

<table>
<thead>
<tr>
<th>Calculated composition</th>
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<tbody>
<tr>
<td>Protein</td>
<td>10</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Lipid</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>63.4</td>
<td>50.3</td>
<td>22.8</td>
</tr>
<tr>
<td>Fibre</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Ash</td>
<td>4.93</td>
<td>7.11</td>
<td>13.32</td>
</tr>
<tr>
<td>Gross energy (Kcal,100g⁻¹)</td>
<td>382.59</td>
<td>385.38</td>
<td>385.63</td>
</tr>
</tbody>
</table>

1 SIGMA chemicals laboratory, USA
2 Richer & Son A/S, Bergen, NORWAY
3 BDH chemicals laboratory, UK.
4 Nutripur
5 INVE, Belgium
Soybean meal and wheat gluten were used as protein sources to adjust protein levels in the diets because of their high digestibility (89.9 and 98.0% respectively) (Akiyama et al., 1992).

The energy level was adjusted by varying the wheat starch and cod oil in the diets. The estimated energy level (382-385 kcal.100g⁻¹) was close to the values obtained in others studies (Bautista, 1986; Shiau and Chou, 1991) in which it has been reported that levels of 305-330 kcal.100g⁻¹ in diets with 36 to 40% protein are sufficient to promote good utilization of protein. The physiological values utilized for calculating the energy level, 5.65 kcal.g⁻¹ for protein; 9.45 kcal.g⁻¹ for lipid and 4.10 kcal.g⁻¹ for carbohydrate, were based on those used by Koshio et al. (1993a). Diet formulae were balanced with the aluminium-silicate based filler, kaolin.

2.2.2 Preparation

Experimental diets were made by weighing ingredients on a Metler balance (0.01g) and mixing them by hand. The vitamin and mineral mixes were each finely ground and blended in a mortar. As the mineral mix had hygroscopic salts, it was oven dried at 60 °C overnight before and after maceration. Before the water and oil were added for processing in a Kenwood meat mincer to produce 3 mm diameter strands, the vitamins and minerals were blended into the rest of the dry ingredients. Water, which was added last, was incorporated gradually until the resulting dough could be easily extruded. The pellet type diets of 5-10 cm were then dried in a fan-ventilated oven (Gallenkamp model OV-160) at 50 °C for 4h. After preparation, the diets were stored at -10 °C in a sealed plastic bags until use.

2.3 Biochemical analysis

All samples were weighed using an OHAUS analytical balance with a precision of 0.01mg. Each sample was ground to a fine powder using a mortar and pestle and then stored in sealed and labelled plastic bags.
2.3.1 Water content
Dried and pre-weighed aluminium trays with 300mg of each diet, were weighed and then oven dried at 60 °C for 24h. Final weights of the trays containing the dried diets were re-weighed and the initial weights of the trays were subtracted. Moisture contents were expressed as a percentage of the total amount of diet initially added.

2.3.2 Nitrogen content
Nitrogen content of the diets and feces was determined in a Carlo Erba 1106 elemental analyser using acetanilide as a standard. Dried and homogeneous samples (2-10 mg) were weighed using an analytical OHAUS balance (accuracy to 0.01mg), in tin capsules and dropped into a combustion furnace at 1000 °C from an autosampler. The samples were burned at 1800 °C and then, using helium as carrier, the combustion product was passed through a layer of chromic oxide at 1000 °C for oxidation. Complete oxidation was attained as the partially oxidised material was put in contact with a bed lined with a layer of silver wool covering a layer of copper oxide. The product of nitrogen oxide was then reduced to elemental nitrogen when passed through a second furnace containing copper at 600 °C. The amount of nitrogen present in the gases was measured in a gas chromatograph equipped with a thermal conductivity detector.
Acetanilide, weighed to the nearest 1 μg using a CAHN C-31 microbalance, was used to plot a calibration curve ranging from 15 μg to 1400 μg.
Values of protein content present in the samples were obtained by multiplying the nitrogen content by a conversion factor of 6.25, thereby assuming that all nitrogen in the samples was bound as protein.

2.3.3 Chromic oxide content
Chromic oxide present in diets and feces were measured by acid digestion of the oven-dried material on a Gallenkamp regulator hot plate for 30 min at 220 °C. The resulting solution was diluted to 50ml in a volumetric flask with distilled water. Ten millilitres of this solution were combined with 1 ml of
diphenylcarbazide reagent and after 15 min measured with a spectrophotometer at 540nm as described by McGinnis and Kasting (1964).

**2.3.4 Trypsin analysis**

A wheaton ground-glass tissue grinder was utilized to macerate the hepatopancreas in Tris-hydroxymethyl aminomethane hydrochlorite (Tris) buffer 46 mM at a pH of 8.1 at 4 °C. The supernatant obtained after centrifuging for 5 min at 13000 rpm at 4 °C was transferred to a new 1.5ml-microtest tube (Eppendorf) in order to keep the extract as clear as possible. The substrate N α-p-Toluenesulfonyl-L-arginine Methyl Ester (TAME) was used in an attempt to determine the activity of the trypsin at 25 °C according to Rick (1974). Dilutions 1:10 of supernatant with Tris buffer were made to measure trypsin activity, however when the TAME concentrations were insufficient to keep the absorbance from rising, dilutions of 1:20 and 1:40 were used instead. Microtest tubes of 1.5 ml with 800 μl of buffer were equilibrated at 25 °C in a Grant heater block prior to addition of 100 μl of substrate and 50 μl of the diluted supernatant. Assay mixtures were immediately transferred to a 1-cm path length quartz cuvette and measured with a Hewlett Packard 8452A Diode Array spectrophotometer equipped with a temperature cuvette holder suitable for measurements at 25 °C. The absorbance changes at 247 nm which occurred every 6 sec for 180 sec were logged on a Hewlett Packard Vectra E5/12 microcomputer. The assay was repeated in triplicate for each sample. The value for total trypsin activity was then multiplied by 10 if a 1:10 dilution had been used for the assay. Since enzyme activities are reported in international units, the total trypsin activity was expressed as total amount of toluenesulphonyl-L-arginine produced per gram of tissue per minute.

Trypsin specific activity which was obtained by dividing the total activity by the protein content.
Total activity = ((SV * AV) / VSA * ε * d) * Abs/ t

SV = buffer volume used to homogenize sample (1.5 ml)
AV = assay volume (buffer+TAME+aliquote of sample)
VSA = Aliquote of sample
ε = extinction coefficient for TAME (0.54 cm² µmole⁻¹)
Abs = Absorbance
t = time in min
d = pathlength (1 cm)

2.3.5 Soluble protein

Proteins were measured using a BCA protein assay reagent kit (Pierce™) with Human Albumin (HA) as standard. Concentrations of soluble protein in the whole hepatopancreas were determined from the supernatant used for trypsin analysis. One hundred µl of supernatant were diluted with 900 µl of Tris buffer to make a 1:10 dilution. In 5 ml glass test tube, 1 ml of reagent solution were added to 50 µl diluted supernatant and incubated for 30 min at 60 °C in water bath. After the assay mixture reached room temperature, it was immediately transferred to a 5-mm path length quartz cuvette and absorbance measured at 562 nm with a spectrophotometer. The soluble protein concentrations for each sample was established using the standard curve ranging from 0.2 mg HA.ml⁻¹ to 1.2 HA.ml⁻¹.

2.4 Determination of dry matter and protein digestibility

Chromic oxide present at a level of 1% per experimental diet was used as an inert marker to determine the digestibility of protein and dry matter (Deering et al., 1996).

During the second week of the growth trial, the feces produced in the 12 individual baskets were siphoned out at 2h intervals in a 12h period, and pooled in 12 individual Eppendorf tubes over 4 days of collection. Feces were separated from uneaten feed by sieving onto a fine mesh screen and hand sorting. Feces were rinsed 3 times in distilled water to remove any salt and kept in an ice bath.
until the end of the day. They were then centrifuged for 5 min at 13000 rpm in a refrigerated Heraus centrifuge at 4°C to decant and discard excess water. Two pools of 4 days worth of feces were frozen in Eppendorf tubes at -70 °C. Feces were dried for 48h in a freeze drier. Dried feces were ground to a fine and homogeneous powder in Eppendorf tubes with a metallic piston. Afterwards the feces powder was oven dried for 24h at 60°C and kept in an anaerobic atmosphere using silica gel to ensure complete dryness at the time of weighing. Chromic oxide and nitrogen contents were analysed in 5 replicas for fecal material from each pool as described previously. Because juvenile shrimps may ingest their own exuvia, baskets were checked for moults every time they were fed. Exuvia were removed immediately as soon as they were discovered, but when these were ingested by shrimp no feces were taken during the next 4 hours. Apparent digestibility of dry matter (ADM) and protein (ADP) were calculated by the indirect method based on the concentration of protein and chromic oxide in diets and feces:

\[
\% \text{ ADM} = \{1-(\% \text{Cr}_2\text{O}_3/\% \text{Cr}_2\text{O}_3 \text{ in feces})\} \times 100
\]

\[
\% \text{ ADP} = \{1-(\% \text{protein}/\% \text{Cr}_2\text{O}_3 \text{ in feces})/(\% \text{protein}/\% \text{Cr}_2\text{O}_3 \text{ in diets})\} \times 100
\]

### 2.5 Ammonia excretion

For analysis of ammonia production through excretion. Six plastic buckets (approximately 20 litres capacity) were filled with UV-irradiated and 0.2 mm-filtered seawater, before the shrimps (one per bucket) were transferred and covered with a perspex plate. Every 2 days, 5 shrimps were chosen per diet (10, 20 and 40% protein) and selected from the growth trial at the intermoult stage to avoid fluctuations in the ammonia excretion (Gerhardt, 1980; Regnault, 1981). The sixth bucket (without a shrimp) acted as a control to determine the loss of ammonia due to bacterial assimilation (Gerhardt, 1980). Each bucket was maintained without aeration at 28 °C. The shrimp were allowed to feed, after 1 h
they were placed in the buckets, on known quantities of diet which were supplied following the regular 15 times-daily feeding regime for a period of 24h. At the beginning and the end of this period 3 samples of water were sampled from each bucket after stirring the water. The samples were immediately filtered through filter paper (Whatman GF/C) to remove any particles of feces or uneaten feed and tested for ammonia. The samples were analysed using the oxidation method described by Parsons et al. (1989).

Ammonia excretion was measured at time 0 and 24h for each bucket. Standard solutions of ammonium sulphate in distilled water were used to plot a calibration curve ranging from 3 μg-atom NH₄-N to 15 μg- NH₄-N. One calibration point was run every sampling day to check for a drift in the curve.

Only in the 24 h samples from shrimps fed on diet 40% of protein, was it found necessary to make a 1:2 dilution of the sample with distilled water, in order to carry out the assay.

The resulting ammonia data were converted to μg-atom NH₄-N.day⁻¹.shrimp g⁻¹ and the sets of data were processed and analysed using Quattro-pro spreadsheets.

2.6 Ingestion rate

To determine the amount of food ingested in 24 h periods, the remains of uneaten feed from the ammonia excretion experiment were collected from each bucket, filtered through pre-weighed filter paper (Whatman GF/C) and dried at 60 °C for 24 h; then weighed again. The difference between final and initial weights was taken as amount that had not been eaten. The amount was subsequently subtracted from the amount of feed supplied and expressed as a percentage of ingestion rate.

2.7 Protein leaching from the diets into the sea water

One gram of feed was placed in 200ml of aerated seawater and left for 24 hours at 28 °C to determine the amount of protein leaching from the diets into the water. Five replicates were analysed per diet. Aliquots of 1ml were taken at 0, 1, 2, 3,
6, 9, 12, 24h intervals. Then total soluble proteins were analyzed using the bicinchoninic acid reaction method as described in soluble protein analysis. Absorbance was measured at 562nm by using a spectrophotometer.

2.8 Trypsin activity

Trypsin analysis was performed on premoult shrimps at the beginning and at the end of the growth trial. To avoid any circadian effects on trypsin activity, animals were sampled 15 h after the last feed - i.e. between 09:00 and 11:00 in the morning (Cuzon et al., 1982; Le Moullac et al., 1997). The hepatopancreas as dissected from the shrimps and frozen immediately to -70 °C until further analysis described previously in section 2.3.4. The samples from each treatment were analyzed in one day, to guarantee a good stability of samples.

2.9 Statistical analysis

All data are presented as means values ±1 standard error of the mean (SE). The Anderson-Darling test was used to check for normality in the data. Prior to performing a analysis of variance (ANOVA) and covariance, Bartlett's test for homogeneity of variance was employed with α=0.05 (Fry, 1993). Tukey's and Scheffé's pairwise comparison were used. Percent composition data was transformed by angular transformation (arc sin √percentage). Results were considered significant at the 0.05 probability level. Statistical analysis was performed using Minitab software.
3.1 Biochemical composition

The results of the various analyses performed on the diets are presented in Table 3. From the biochemical analysis it is apparent that the protein content is very close to the original formulation of each test diet. The chromic oxide content for each diet was slightly higher than the established 1% per diet. The results of the digestibility trial is not expected to be affected by this variation. The moisture levels in the 10% protein diet were 3 times higher than in the 40%, but again this was not expected to have a negative effect. Moisture levels for commercial diets range up to 10% moisture content.

**Table 3.** Results (%) of biochemical analysis of the test diets fed to *P. indicus* over a 6 week period.

<table>
<thead>
<tr>
<th>Dietary protein</th>
<th>Protein</th>
<th>Chromic Oxide</th>
<th>Moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10.35</td>
<td>1.37</td>
<td>3.61</td>
</tr>
<tr>
<td>20</td>
<td>22.26</td>
<td>1.48</td>
<td>2.69</td>
</tr>
<tr>
<td>40</td>
<td>41.52</td>
<td>1.41</td>
<td>1.19</td>
</tr>
</tbody>
</table>

Protein value for fresh mussel were not determined but Kuganathan (1994) showed that the content is normally 55.2%. This value was used in the trial for trypsin activity.

3.2 Leaching protein

The rate of soluble protein loss by pellets placed in 0.2 mm filtered seawater is presented in Fig 1. Although there is a trend for increase in soluble protein loss with increased time, the amount of soluble protein lost remained low (2.15, 13.33 and 6.50%) for diets of 10, 20 and 40% protein respectively after 90 min of incubation.
Figure 1. Regression lines for protein leaching of 10, 20 and 40% protein diets exposed to filtered seawater at 28 °C over 24 h. Diet 10%: \( y=0.107955+0.149424x \); Diet 20%: \( y=5.36543+6.31824x-0.673333x^2 \); Diet 40%: \( y=3.01257+2.80136x-0.316643x^2 \).

The regression lines for protein leaching of the 3 diets measured at different intervals of time are shown in figure 1. The 20% protein diet had the highest rate leaching of soluble protein compared with other diets in terms of percentage but in terms of the amount of soluble protein loss was nearly equal to the other 2 diets (Table 4). It was considered that the protein present in the diets was stable enough to be ingested by shrimp.
Table 4. Protein remaining in the diet after exposure to water in given amount of time for each of the three diets. Expressed in percentage of protein remaining compared to the original amount present in each diet (time 0).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Diet 10%</th>
<th>Diet 20%</th>
<th>Diet 40%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.35</td>
<td>22.26</td>
<td>41.52</td>
</tr>
<tr>
<td>90</td>
<td>10.13</td>
<td>19.29</td>
<td>38.83</td>
</tr>
<tr>
<td>180</td>
<td>9.98</td>
<td>18.19</td>
<td>37.96</td>
</tr>
</tbody>
</table>

3.3 Growth trial

All groups of shrimps had an equally high survival rate (95%) throughout the study. The final wet weight of shrimps fed on diets containing 20% and 40% of protein exhibited significantly ($p<0.005$) higher weight gain after 43 days than those fed on 10% of protein diets (Table 5). A general linear model (GLM) analysis also showed that there were significant differences ($p<0.001$) in growth between shrimp which were fed on 20% and 40% protein diets and those fed on a diet of 10% protein.

Table 5. Survival and growth of juvenile shrimps *P. indicus* fed artificial diets for 43 days$^1$

<table>
<thead>
<tr>
<th>Dietary protein (%)</th>
<th>Survival (%)$^3$</th>
<th>Final weight (g)$^{1,2,4}$</th>
<th>Growth rate (mg.day$^{-1}$)$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>95.0 ± 2.3</td>
<td>2.8 ± 0.09$^a$</td>
<td>6.98 ± 1.97$^a$</td>
</tr>
<tr>
<td>20</td>
<td>95.0 ± 2.3</td>
<td>3.2 ± 0.10$^b$</td>
<td>16.28 ± 2.55$^b$</td>
</tr>
<tr>
<td>40</td>
<td>95.0 ± 2.3</td>
<td>3.3 ± 0.10$^b$</td>
<td>18.61 ± 2.58$^b$</td>
</tr>
</tbody>
</table>

$^1$ Mean ± SE  
$^2$ Values with different superscript are significantly different at 5% level  
$^3$ Mean initial wet weight 2.5 ± 0.04g (n=60)

Growth rate is reported in mg.day$^{-1}$ to allow for comparison with previous studies on *P. indicus*. 

20
3.4 Ingestion rate of *P. indicus*

Feeding trials showed that artificial diets were accepted, when offered to shrimps during the experiment.

The ingestion rate, expressed as a percentage of food eaten is derived from the amount of food offered as well its relation to body weight, and is analysed using analysis of variance (Table 6). The analysis of variances indicated that the mean ingestion rate of at least one diet was significantly different from one other when comparing body weight and percentage of food eaten (*p*<0.003). Using Tukey’s method for pairwise comparison the ingestion rate of shrimps fed on 40% protein diets was found to be significantly lower than for those fed on diets containing 10% and 20% protein (shrimps fed on 10% and 20% protein diets were not significant from each other).

Although there was no significant difference between the absolute amount of food ingested in terms of body weight between 10% and 20% protein diets, shrimp fed on 20% protein diets ate 2% more than shrimps fed on 40% protein diets and these shrimps ingested 1% less than shrimp fed on 10% protein diets (Table 6).

**Table 6. Ingestion rate of juvenile *P.indicus* at various concentrations of dietary protein.**

<table>
<thead>
<tr>
<th>Dietary protein</th>
<th>Ingestion rate in terms of:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% body weight</td>
<td>% food eaten</td>
</tr>
<tr>
<td>10 %</td>
<td>5.42 ± 0.17*a</td>
<td>75.26 ± 2.19*a</td>
</tr>
<tr>
<td>20 %</td>
<td>6.25 ± 0.30*a</td>
<td>76.49 ± 2.99*a</td>
</tr>
<tr>
<td>40 %</td>
<td>4.28 ± 0.32*b</td>
<td>57.91 ± 4.35*b</td>
</tr>
</tbody>
</table>

1 Data are the average values and SE of the 5 replicates.
2 Values with different superscript are significantly different at 5% level.

The effect of ingestion rate on the amount of ingested nitrogen for *P. indicus* juveniles fed on three different protein concentrations was also analysed by analysis of variance.
Results indicate that the average daily nitrogen ingestion of *P. indicus* increased with increasing protein concentrations in the diets (Table 6). The shrimps fed on 40% protein diets consumed 1.6 and 3.7 times more than those fed on 20% and 10% protein diets respectively.

### 3.5 Digestibility

The results presented in this study indicate that dietary protein content in the diets had an effect on the digestibility of protein. A comparison of diet performance using a balanced ANOVA analysis revealed that a significant difference exists between the arcsin transformed percentages of protein digestibility (*p*<0.001). Tukey's pairwise comparison showed that protein digestibility of 20% and 40% protein diets was significantly higher than 10% protein diets, and digestibility of protein for 20% and 40% protein diets was not significantly different (Table 7).

**Table 7.** The percentage dry matter and protein digestibility by *P. indicus* at different protein levels.

<table>
<thead>
<tr>
<th>Dietary protein</th>
<th>Digestibility (%)</th>
<th>Dry matter</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 %</td>
<td>82.04 ± 1.95 a</td>
<td>71.83 ± 4.76 a</td>
<td></td>
</tr>
<tr>
<td>20 %</td>
<td>81.15 ± 1.75 a</td>
<td>89.57 ± 1.53 b</td>
<td></td>
</tr>
<tr>
<td>40 %</td>
<td>77.26 ± 1.34 a</td>
<td>94.23 ± 0.29 b</td>
<td></td>
</tr>
</tbody>
</table>

1 Data are the average values and SE of the 8 replicates.
2 Different superscripts in the same column indicate significant difference *p*<0.05

Interestingly, no significant difference (*p*>0.091) was found between the arcsin transformed percentages of dry matter digestibility between the groups of shrimps fed on the 3 diets.
3.6 Ammonia excretion

Diets containing 10%, 20% and 40% protein were assessed for their effect on total ammonia production ($\mu$g-atom NH$_3$-N.day$^{-1}$.shrimp$^{-1}$) and ammonia excretion rate ($\mu$g-atom NH$_3$-N.day$^{-1}$.shrimp gram$^{-1}$) by shrimps fed regularly 15 times a day. The mean values of each are presented in Table 8.

The effect of dietary protein levels on the ammonia production and the ammonia excretion rate by P. indicus was that shrimp maintained on the 40% protein diet produced approximately 20 times more ammonia and demonstrated a rate of ammonia excretion 25 times higher than those maintained on the 10% and 20% protein diets. The mean values of total ammonia production analysed using a one-way analysis of variance showed significant differences ($p<0.001$) between diets. Tukey’s method for pairwise comparison (Table 8) confirmed that significant differences in ammonia production and ammonia excretion rate are between the 40% protein diet and the 10% and 20% diets. No differences existed between the latter two.

Table 8. Ammonia production ($\mu$g-atom NH$_3$-N.day$^{-1}$. shrimp$^{-1}$) and ammonia excretion ($\mu$g-atom NH$_3$-N.day$^{-1}$. shrimp gram$^{-1}$) rates for shrimp fed at various protein levels at 28 ºC.

<table>
<thead>
<tr>
<th>Dietary Protein</th>
<th>Total ammonia production$^2$</th>
<th>Ammonia excretion rate$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>10%</td>
<td>119.40±14.12$^a$</td>
<td>116.67±4.93$^a$</td>
</tr>
<tr>
<td>20%</td>
<td>122.41±15.87$^a$</td>
<td>131.43±5.32$^a$</td>
</tr>
<tr>
<td>40%</td>
<td>3214.14±38.79$^b$</td>
<td>2146.58±140.32$^b$</td>
</tr>
</tbody>
</table>

$^1$Data are the average values and SE of the 5 replicates.

$^2$Different superscripts in the same column indicate significant difference $p<0.05$

The ammonia values given are based on means of 5 individual shrimps per diet tested. Due to the large variation between 40% and the 20 an a 10% protein diets but only small differences between 10 and 20% protein diets, this experiment was
repeated. A similar result was obtained with a low ammonia excretion in shrimp fed on 10 and 20% protein diets and an obvious significantly higher value when 40% protein was present in the diet.

A second trial was performed to re-test the total ammonia production and ammonia excretion rates especially for shrimps fed on the 10 and 20% protein diets as no significant differences were detected. The second trial, however, yielded comparable results to the first trial, thereby confirming the first results. The only change made in the second trial was that filtered seawater was used instead of raceway seawater to exclude the possibility that nitrifying bacteria in the biological filter for the raceway were transforming the ammonia into nitrate.

3.7 Trypsin activity

The measurement of total trypsin activity and soluble protein made it possible to test for specific trypsin activity for shrimp fed on each diet. The effect of protein concentrations on enzyme adaptation of *P. indicus* was studied at the moult stage D2. Shrimp fed on mussels (55% protein) before starting the growth trial showed a significantly (*p*<0.001) higher trypsin activity (0.276 ± 0.011) than any of those fed on artificial diets, demonstrating the effect of dietary protein on digestive enzyme level in shrimp (Figure 2).

The result of the one-way ANOVA shows that dietary protein levels have a significant effect on trypsin activity. The specific activity of trypsin to increased with increasing dietary protein level. An increase of 10% to 20% in protein level of the diet elevated the specific trypsin activity substantially (20%) in *P. indicus* juveniles to levels similar to those of juveniles fed on a 40% protein diet.
Figure 2. Effect of dietary protein level on specific trypsin activity. Line represents regression analysis.

No significant differences ($p<0.001$) were found between the 20% protein diet and the 10% and 40% protein diets when the energy level in the diets was held constant. The lowest specific trypsin activity was observed in shrimps fed on the lowest protein level.

Examination of the relationship between specific trypsin activity and percentage of protein present in the diets showed that these are well correlated ($r=0.585$) and a highly significant regression ($r^2 = 34.3\%, \ p<0.001$) was obtained.
3.8. Nitrogen Budget

Nitrogen available for growth (G) was calculated by subtracting the nitrogen ingested (I) from the nitrogen lost through excretion (Ex) and defecation (D) by applying the following equation:

\[ G = I - (Ex + D). \]

All elements of the budget, I, Ex, D and G, were calculated in terms of nitrogen (expressed in \( \mu g \text{ N.day}^{-1}.\text{shrimp.day}^{-1} \)). A nitrogen budget for the shrimps can be constructed from the results (Table 9). The shrimps fed on 20% and 40% protein diets demonstrate a remarkable digestion efficiency which is close to 90%.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10%</td>
</tr>
<tr>
<td>Nitrogen offered (C)</td>
<td>975.93</td>
</tr>
<tr>
<td>Nitrogen ingested (I)</td>
<td>701.01</td>
</tr>
<tr>
<td>Ammonia Excretion (Ex)</td>
<td>116.67 (16.6)</td>
</tr>
<tr>
<td>Defecation (D)</td>
<td>274.92 (39.2)</td>
</tr>
<tr>
<td>Growth (G)</td>
<td>309.42 (44.4)</td>
</tr>
<tr>
<td>% Assimilation (A)</td>
<td>44.14</td>
</tr>
<tr>
<td>% Efficiency (E)</td>
<td>60.78</td>
</tr>
</tbody>
</table>

Note: Values in brackets represent calculations allowing for feed lost into the water.
For 40% protein diets, ingestion of nitrogen is higher than that required for maintenance and growth (Table 9). When dietary protein concentration is decreased to 20%, growth remains similar (3.2 ± 0.10g) to 40% protein diets (3.3 ± 0.10g). Table 9 shows the nitrogen budget at each dietary protein concentration and demonstrated that the pattern of nitrogen allocation varies with protein concentration present in diets. The proportion of food nitrogen lost in excreta ranged from 5.3% in the 20% protein diet to 52.5 % in the 40% diet. The lowest mean loss through defecation was on the 40% protein diet (6.1 %) and there was a tendency for this proportion to increase with decreasing protein concentration.
Chapter 4

DISCUSSION

4.1 Growth

Weight gain of shrimp was significantly affected by different dietary protein concentrations. The diets containing 20 and 40% protein were found to be the best diets in the present study in terms of growth. Since all diets contained the same ingredients and all were isocaloric, the results indicate that protein concentration was the major factor influencing growth rates. The effects of protein on growth rates of shrimp may be caused by differences in the presence and availability of essential amino acids. The diets contained cod fish meal, krill meal, soybean meal and wheat gluten as protein sources all of which have been reported to have a high availability of essential amino acids (Akiyama et al., 1992) and as same ingredients were used in all diets should not effect results.

The growth rate of 6.98 to 18.61 mg.day\(^{-1}\) of *P. indicus* tested in this is lower than that obtained in previous studies. Colvin (1976) for example, reported growth rates of 84 and 105 mg.day\(^{-1}\) for juvenile *P. indicus* fed on 20 and 40% protein diets respectively, whereas Forster and Beard (1974) reported growth rates of 52 and 43 mg.day\(^{-1}\) for *P. indicus* cultured in low and high stocking densities during the first 6 weeks (25 and 116 shrimps.m\(^{-2}\), respectively). Different growth rates within the same species have also been found in *P. monodon* by Alava and Lim (1983), who reported a 23.8 mg.day\(^{-1}\) growth increase in 1.32g shrimps fed on a casein-dextrin-based diet (40% protein). Cruz-Suarez et al. (1992) on the other hand reported a 95.8 mg.day\(^{-1}\) increase in 2.2g *P. monodon* fed a squid meal-based diet (49% protein), while Shiau and Peng (1992) obtained a growth rate of 38.2 mg.day\(^{-1}\) in 0.57g *P. monodon* fed on 20% starch based diet (40% protein). In the present work, stress due to excessive handling is thought to be the most likely explanation for the low growth rate. The shrimps in this experiment displayed classic symptoms of stress due to handling (Foster and Beard, 1974). These authors described that some *P. indicus* showed opaque abdominal
musculature, while others remained passive for several minutes following a disturbance, tolerances to stress were found to vary and similar behaviour was observed in present work. Stress may well have been inflicted during the daily 90-min feedings throughout the entire experiment; together with feces collection at 2 h intervals over a period of 8 days, regular examinations under the microscope for the duration of one week (ammonia excretion experiment) and weight measurements made on 3 separate occasions.

The poor growth rate obtained in this study is not likely to be the result of the various protein concentrations in the diets. On the contrary, although the growth rate is much lower than observed in previous studies, significant differences exist between diets containing 10% protein and those containing 20 and 40% protein.

Protein requirement of shrimp may be associated with species specific feeding habits. For example, *P. japonicus* was observed to require a 60% protein diet to attain fastest growth rates (Deshimaru and Shigeno, 1972) while *P. indicus* is reported to only need a diet containing 43% protein (Colvin, 1976). A likely reason for these differences is that *P. japonicus* is mostly carnivorous while *P. indicus* is more herbivorous (Van Wormhoudt et al., 1995).

Colvin (1976) observed that live weight gain per gram of protein consumed (PER) is inversely proportional to successive increases in dietary protein level, indicating that excess protein may be utilized as an energy source rather than for growth. In the same study, shrimps fed a 60% fish meal and 40% prawn meal-based diet gave better growth rates than those fed diets containing either fish meal or prawn meal alone. This appears to suggest that a better combination of protein sources may reduce the total protein requirement for *P. indicus* to less than 43%.

Survival over the whole experiment was very high (95%) for the three groups of shrimp in which only 1 shrimp was lost per treatment. Two of these 3 shrimps are reported 'missing in action' while the third died an untimely death trapped between the net and the wall of the basket. Mortality was not related to the stocking density at which the shrimp were cultured.
4.2 Ingestion:

Shrimps fed on 20% protein diets were apparently eating as much as was necessary to cover their protein requirement for growth, while those fed on 40% protein reduced consumption when their metabolic demand for protein was covered.

Kurmalny (1989) noted that the appetite of postlarvae fed to satiation, returns after 1 to 2 h. Ingestion rates of penaeid juveniles are inversely proportional to immersion time of diets in the water (Sick et al., 1975). In this study, food left for more than 2-3h was observed not to be handled by P. indicus which may represent the intermeal period. In the same experiment, Sick et al. (1975) observed that immersion of artificial diets for more than 6h in water was shown to greatly reduce the feeding activity of shrimp as a result of decreased palatability (less attractive and poorer in quality) and physical characteristics.

Nitrogen ingested was proportional to the amount of protein present in each diet. The lowest nitrogen ingestion rate supplied by the 10% protein diet was enough (54 mg nitrogen.day\(^{-1}\).100g shrimp\(^{-1}\)) to cover minimum metabolic demand of between 30 and 50 mg nitrogen which is necessary to maintain the weight of a 100g shrimp per day according to Koshio et al. (1993b). In addition, Koshio et al. (1993b) using P. japonicus, which is a naturally more carnivorous species than P. indicus, concluded that 200 to 300 mg proteins are necessary for renewing body protein. A presumption is therefore made that the minimum nitrogen intake is even lower for P. indicus and has been met by all three diets which is confirmed by the increase from 2.5g initial weight to 2.8g final weight attained by shrimps fed on diet 10% protein.

4.3 Enzymes

Variability in trypsin activity of shrimps may be the result of many factors, including the moulting stage (Van Wormhoudt et al., 1995), size of the shrimp (Lee et al., 1984), quality and quantity of protein in the diet (Lee et al., 1984, Smith
et al., 1985, Le Moullac et al., 1994, Le Moullac et al., 1997), ration sizes (Sridhar et al. 1995) and cycadian rhythm (Van Wormhoudt, 1972; Cuzon et al., 1982; Maugle et al., 1982).

The relation between food quality and activity of digestive enzymes has been examined in previous studies by modifying the biochemical composition of artificial feed. Lee et al. (1984) who demonstrated that specific trypsin activity was affected by type and level of protein as well as the size of the shrimp. Although large (24.2g) and medium (17.6g) shrimp exhibited higher trypsin activity than small (9.9g) shrimp when fed on diets containing 1:1 animal to plant protein ratio, there were no significant differences amongst those fed diets containing 2:1 animal to plant protein ratio. Shrimp trypsin activity increased inversely proportional to rise in protein levels when fed on diets containing 2:1 animal to plant protein ratio. Van Wormhoudt et al. (1980), working with Palaemon serratus, reported that the highest specific protease activity was attained in prawns fed a 70% Atlanta meal based-diet containing 45% protein, whereas other shrimps fed on equal or greater amounts of protein composed of different protein sources, exhibited lower activities.

In the present study, the body weight of shrimps maintained on the 10% protein diet for 43 days were significantly lower compared to those fed on 20 and 40% protein diets (Table 5). However, at the end of this experiment the specific trypsin activity did not differ significantly from shrimp fed the 20% protein diet which attained the same final weight as those fed on 40% protein diet. This suggests that shrimps fed on 10% protein diets fed cannot raise enzyme activity to compensate for the lower level of protein in the diet. Rather, enzyme activity level is directly linked to the amount of substrate present (Fig. 2) so that increase in dietary protein stimulates increase in enzyme activity levels. As there were no significant differences in final weights despite a rise in specific trypsin activity in shrimp fed on 20% and 40% protein diets it appears that a limit in the use of protein for growth exists.
The results of the present study, indicating that increases in dietary protein content are correlated to a rise in specific trypsin activity, agree with previous observations made by Van Wormhoudt et al. (1980), Lucien-Brun et al. (1985) and Le Moullac et al. (1997). A dose response effect between protein level and specific protease activity has been observed in *Palaemon serratus* fed on diets containing between 6.5 and 45.2% protein (Van Wormhoudt et al. 1980), in juvenile *Homarus gammarus* fed on diets containing 26.1 to 36.6% protein (Lucien-Brun et al. 1985) and in juvenile *P. vannamei* fed on 25 to 40% protein diets (Le Moullac et al., 1997). In each case the trypsin activity increased with successive increases in protein levels.

The significantly higher trypsin activity in shrimp fed fresh mussel flesh at the beginning of the experiment compared to those fed artificial diets could be a combination of two factors. On one hand, the mussel flesh contained the highest protein concentration (55%) which may have boosted the trypsin secretion as described in earlier studies. On the other hand, the presence of a natural appetite stimulant in the fresh mussel flesh and possibly other fresh diets which stimulates trypsin activity, may also by a possible cause. While shrimp react with little interest when artificial diets are introduced to the water, high scurrying activity and 'excitement' is observed upon the introduction of fresh mussel diets. Interestingly, Le Moullac et al. (1994) reported a 60% increase of trypsin activity in larvae *Penaeus vannamei* fed algae (25 and 37% protein) and *Artemia* (55% protein) compared to those fed an artificial diet containing 50% protein. Another indication that fresh diets may have a stimulatory effect on digestive enzyme production is supported by Le Vay et al. (1993) who reported that algal secretions are responsible for enzyme stimulation.

Cuzon et al. (1982) observed 2 peaks of enzymatic activity in *P. japonicus*. While the largest peak occurred at 20h00, 3h after sunset, a second, smaller one occurred
at 07h00. The shrimp in the present study were sampled for trypsin activity 15 h after their last feed (18h00). The gut of each shrimp was empty when the hepatopancreas was dissected. Over the 15 hours the maximum amount of trypsin needed for digestion of the protein diet on which each shrimp had been acclimatised (10, 20 or 40%) over a period of 6 weeks, had been allowed to accumulate. The low variability both between replicates within samples and between individual shrimps of the same treatment (coefficient of variation less than 5%), demonstrates that the results obtained in this study reflect the real effect of protein concentration on trypsin activity.

4.4 Digestibility

There are some concerns about the use of chromic oxide to determine apparent digestibility of specific nutrients. These include variations of chromic oxide concentrations in feces over time, rate differentials in passage of food and chromic oxide through the alimentary tract (Forster and Gabbit, 1971) and leaching of water soluble nutrients from diets before ingestion and prior to feces collection (Fenucci et al., 1982; Cuzon et al., 1982).

Differential passage rates of food and digestibility marker used is a reason why some studies have tested other digestibility markers. Deering et al. (1996) evaluated the use of Ytterbium acetate, acid insoluble ash and chromic oxide in isocaloric diets for *P. monodon* where shrimps were fed 3 times a day and feces were collected within 4h of egestion. A comparison of digestibility of protein showed no significant differences between the three markers. In a preliminary work, carried out on Rainbow trout, Atkinson et al. (1984) demonstrated that digestibility of dry matter, protein and energy were similar regardless of indicator used (acid insoluble ash and chromic oxide).

Since digestibility of dry matter analysed in all three diets of the current study was not found to be significantly different, one can assume that the passage rates the chromic oxide marker was similar in all cases. The fact that the feces was
consistently green also implies that chromic oxide was passed through at the same rate as the food.

An overestimation of apparent digestibility due to leaching of nutrients into water can occur through prolonged immersion of feed and fecal material in water (Cousin et al. 1996, Sudaryono et al., 1996). Fenucci et al. (1982), studying *P. stylirostris*, estimated a minimal 4% loss of protein through leaching from feces after 6 h of exposure to water. In the present trial, the fecal material was collected over two 4-day periods and within 2h after egestion, to avoid any errors associated with differential passage rates of chromic oxide and nutrients and losses of protein and chromic oxide from feces into the water.

No significant effects on shrimp nutrition studies have been assumed when diets are immersed in water for less than 2h (Clark et al., 1993). Interestingly Clark et al. (1993) based their assumption of insignificant nutrient losses through leaching on Fenucci (1981) and Coelho (1984). While Goldblatt (1980), quoted in his introduction that "losses of total nitrogen and trace minerals were found to be negligible when examined on a per gram ration recovered basis". These kinds of assumptions based on previous studies may generate erroneous conclusions as the diets used vary enormously between studies in terms of moisture content, fibre, protein and carbohydrate sources, binders, process of manufacture, stability and therefore the leaching of nutrients. Not only have the differences in diets shown to have an effect on leaching, but also the length of time each diet has been exposed in the water before ingestion. Cuzon et al. (1982) evaluated leaching of nutrients by exposing known quantities of diets to seawater for 30 min, 1h and 3h. To reduce nutrient losses due to leaching the shrimp were fed when feeding activities were high. The loss of protein into the water reached 11 and ~14% after 1h and 3h, respectively. A strong argument is therefore made for testing each new diet for leaching rates before assuming it to be an insignificant variable affecting nutrient leaching.
The possibilities of leaching were greatly reduced throughout the present trials by feeding the shrimp at 90-min intervals throughout the current study. Nevertheless, protein leaching was measured and taken into account to ensure that no information was lost in calculating each component of the nitrogen budget.

Leaching of protein was tested by analysing the amount of soluble proteins in the water. While after 90 min 2.15 to 13.33% leaching of soluble protein occurred, 3.60 to 18.26% occurred after 3 h. The applied bicinchoninic acid reaction method for analysing protein leaching is still new and has both advantages and disadvantages. On one hand the method is easier and faster to apply and uses smaller sample sizes compared to the generally accepted Kjeldahl method, but on the other hand the method only tests for peptide bonds and 4 amino acids (cysteine, cystine, tryptophan and tryrosine). The Kjeldahl method, which tests for the amount of crude protein present in the diets, may have been more useful to compare results of the current study to previous studies. No publications could be found which tested for differences in accuracy between the 2 methods which test for amounts of protein leaching.

Apparent digestibility coefficients of dry matter above 77% obtained in all diets in the present work are in agreement with preliminary studies by Koshio et al. (1993a) who, using *P. japonicus*, reported 74.1% and 72.7% for isocaloric diets containing 21% and 41% protein, respectively. Shiau and Peng (1992), working with *P. monodon*, noted that digestibility coefficients of dry matter in isocaloric semipurified diets containing 40% protein with 20% dextrin or 20% starch varied from 68.0 - 72.7%.

The high digestibility coefficient for dry matter suggests that composition and palatability of diets were several of the variables which did not interfere in the ingestion and digestion processes.

Apparent digestibility coefficients of protein (ADP) of the diets 20 and 40% protein were 89.57 and 94.23%. Again, the results coincided with the previous
works (Colvin, 1976; Koshio et al., 1993; Sudaryono et al., 1996). Koshio et al. (1993a), working on *P. japonicus* fed on 21 and 41% protein diets gave an ADP of 92.5 and 94.4% respectively while the work by Sudaryono et al. (1996) on *P. monodon* gave an ADP range of 85.4 - 92.8% on practical diets containing 40% protein (based on fish processing waste products). Colvin (1976), using *P. indicus* fed on non-isocaloric diets containing 21.3 and 42.8% protein (based on prawn meal) obtained an ADP of 88.0 and 86.3%. Shrimp were fed a 38% protein diet containing either fish meal, prawn meal or a combination of the two, obtained the best result in terms of ADP when the fish and prawn meal were combined (Colvin, 1976). A higher digestibility based on the ADP results obtained for the 20 and 40% protein diets in the current study (89.57 and 94.23%, respectively) indicates that the combination of various protein sources allowed for an even higher digestibility than observed by Colvin (1976). The presence of highly digestible amino acids (Akiyama et al., 1992) in the different ingredients used to prepare test diets resulted in an increase in ADP proportional to supplement of protein level in the diets as consequence of increase of protein sources. This has been quoted by Schneider and Flatt (1975) and McDonald et al. (1988) who suggested that rises in ADP of the diets appear to be related the increase protein concentration in the test diets, which has been evidenced by Smith et al. (1985). The low protein digestibility (71.83%) obtained with the 10% protein diet was perhaps due to high amount of carbohydrate present (low digestibility) and the overall lower protein proportion in comparison to the 20 and 40% diets.

**4.5. Ammonia**

Of the nitrogen excreted, ammonia is the most important, constituting more than 60% of the final metabolic waste products in crustacea (Claybrook, 1983). The ammonia fraction contributes up to 72.6% of the total nitrogenous excretion in juvenile *P. indicus* kept at 28 °C (Gerhardt, 1980). Variability in ammonia
excretion of shrimps can be the result of many factors, including temperature, salinity, pH, oxygen, volume of the medium, type of food, size of shrimp, moulting stage and physiological condition of the animals (Talbot, 1960). In starvation conditions some species such as *Crangon franciscorum* and *Euphausia pacifica* for example, can excrete between 7 and 9 times more ammonia than those which have been fed (Takahashi and Ikeda, 1975; Nelson *et al.*, 1979).

The fraction of the nitrogen intake lost as ammonia in the experiments ranged from 5.31 to 52.5% and an indication that nitrogen losses increase with higher ration sizes exists, although the relationship is not significant. There was a distinct relationship between the dietary protein concentration and the subsequent excretion of ammonia by the shrimp in the current study. With the highest protein concentration (40%), ammonia excretion rose to levels 20 times higher than the other 2 diets. The ammonia excretion rate reached a peak-level (751.1 \( \mu \text{g.g}^{-1}.24\text{h}^{-1} \)) only in shrimp fed on the 40% protein diet, matching the results reported by Koshio *et al.* (1993a) for *P. japonicus* fed 41.6% protein diet (900 \( \mu \text{g.g}^{-1}.24\text{h}^{-1} \)).

The observed pattern has also been reported by Udayakumara and Ponniah (1987), who found that juvenile *P. indicus* fed on diets 22.45 and 40.87%, at 7% of body weight, excreted 288 and 816 \( \mu \text{g.g}^{-1}.24\text{h}^{-1} \). Therefore the maximum peak level obtained for *P. indicus* was exclusive to the diet containing 40% protein. The magnitude of ammonia production increase in the present study was somewhat higher than the 18.9 time increase reported for 40 and 65% protein diets fed to *P. setiferus*, or 3.3 times increase for juvenile shrimp *P. duorarum* (Rosas *et al.*, 1996). An unexpected result was that *P. schmitti* fed on 65% protein diet excreted 1.5 times less ammonia than shrimp fed on 60% protein diets, but similar amounts (ratio close to 1) to those fed on 50% protein diet (Rosas *et al.*, 1996). These large variations may have been caused by differences in dietary protein content and digestibility, temperature variations, body weight and even feeding regime.
Each of the three groups of shrimp showed an increase in ammonia excretion related to the level of protein present in the diet. In view of the levels of ammonia excretion shown in Table 8 two groups of shrimp response appear present: those with low ammonia excretion (shrimps fed on 10 and 20% protein diets) and those with high ammonia excretion (fed on 40% protein diets).

Shrimp fed the 20% protein diet (131.4 μg.g⁻¹.24h⁻¹) showed a much lower ammonia excretion compared to those fed the 40% protein diet (906.5 μg.g⁻¹.24h⁻¹). As the trials on ammonia excretion were performed twice at different stages in the study and on different shrimp each time with the same results, errors in the methodology or readings can be ruled out. The most likely explanation for the striking trend is that shrimp on the 20% protein diet demonstrate the optimum protein utilization and hence produce exceedingly low amounts of ammonia. Thus the lower (20%) percentage protein diet which results in equal or higher growth (efficiency) than the higher (40%) percentage protein diet also, minimises the amount of ammonia production through excretion. Should further studies reveal similar conclusions in other, commercially farmed species, the implications of present results are that cheaper diets may be produced, less food would be wasted and pollution through effluents may be greatly reduced.

Hewitt and Irving (1990), reported that a 40% protein diet fed to juvenile *P. esculentus* had no significant difference between pre- and post-feeding ammonia production and was overall the lowest compared to shrimps fed the 30 and 50% protein diets. The trend Hewitt and Irving (1990) observed was the same as that in the present experiment although the range of protein level is different. However in contrast, Zuniga et al. (1984) using juvenile shrimp *Rhynchocinetes typus*, found that ammonia production rate was not influenced by concentration of protein present in 44, 55 and 66% protein diets. The protein content in the aforementioned diets may therefore have been too high to detect differences in ammonia excretion.
Changes in the ammonia excretion appear to be relative to variations in dietary protein concentration which are influenced by the adaptive capacity of species to use protein as a metabolic substrate. Rosas et al. (1995, 1996) demonstrated that *P. setiferus*, a herbivorous species, excreted less ammonia than *P. duodarum*, a carnivorous species. A reduction in the use of protein as an energy source in more herbivorous species as compared to the preferential use of protein as an energy source in carnivorous species is thought to explain the differences in ammonia excretion.

Present results suggest that when *P. indicus* is fed on diets of 10 and 20% protein, amino acids are used more for growth than as a metabolic substrate, whereas in shrimp fed 40% protein the amino acids are utilized both for growth and as a metabolic substrate. This is in agreement with earlier work in which *P. orientalis* utilized primarily protein and then lipid as energy source to supply metabolic energy (Zhou, 1990).

### 4.6 Nitrogen Budget

A total nitrogen budget for *P. indicus* has been constructed by incorporating all components from the individual nitrogen analyses and is summarised in Table 10.

**Table 10.** Nitrogen budget for juveniles *P. indicus*. All values are given in percentage.

<table>
<thead>
<tr>
<th>Protein Content of Diet</th>
<th>Growth (G)</th>
<th>Ingestion (I)</th>
<th>Excretion (E)</th>
<th>Defecation (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>54.7(44.4)</td>
<td>100.0</td>
<td>6.0 (16.6)</td>
<td>39.2</td>
</tr>
<tr>
<td>20%</td>
<td>86.0(83.0)</td>
<td>100.0</td>
<td>2.3 (5.31)</td>
<td>11.6</td>
</tr>
<tr>
<td>40%</td>
<td>58.2(41.4)</td>
<td>100.0</td>
<td>35.7 (52.5)</td>
<td>6.1</td>
</tr>
</tbody>
</table>

*Values in brackets represent calculations when including results of feed lost into the water*
The nitrogen available for growth of *P. indicus* was 32 - 42% higher in the 20% protein diet than in the 10 and 40% protein diets. The lack of growth in the 10% diet can most likely be attributed to the lack of proteins and the high (63%) carbohydrate content. The low nitrogen available for growth and up to 52.5% nitrogen excreted as ammonia by shrimp fed on 40% diets on the other hand is more likely to be a result of an over-metabolisation of amino acids. When fed on diets which are too high in proteins, shrimp metabolise excess amino acids to ammonia after acquiring sufficient amino acids for repair and build-up of new tissue Velasco *et al.* (1996) reported a dose response effect between the amount of the dissolved total inorganic nitrogen (TIN) accumulated in the water and the level of protein given to *P. vannamei* postlarvae regardless of dietary lipid concentration. The same authors suggested that net nitrogen utilization was 85.5, 71.0 and 37.0% for 10, 18 and 33% protein diet, respectively based on the difference between nitrogen content of diet and accumulation of TIN. It was also assumed that a part of unaccounted nitrogen was present in the remaining sediments. These results differ from the current study, the best availability of nitrogen for growth (Assimilation) was given for 20% protein diet whereas the 10 and 40% protein diets gave the poorest result (Table 9).
CONCLUSIONS

Although feeding rates of 15 times per day resulted in desirable growth rates and possibly more efficient protein uptake, this type of feeding regime would be impossible to uphold in extensive culture. Nevertheless, the costs of feeding several times per day should to be weighed against increasing costs of fresh and artificial feed and impact of pollution through sediment and water loading.

In conducting similar experiments, nutrients lost into the water due to handling and leaching should be carefully monitored, as the exclusion of these factors may distort the actual in- and output of nutrients per diet into the shrimp.

Of the three diets tested in this study, the 20% protein diet consistently provided the most benefits to the shrimp in terms of the high consumption, growth, digestibility and minimal ammonia excretion. The implications of feeding artificial diets which contain less protein than the average commercial diet are threefold. If marketable, production costs of artificial diets for some or all shrimp species should be drastically cut, since protein is the most expensive ingredient. Also, if leaching of diets can be reduced by ensuring that the animals are fed at intervals when they are producing peak amounts of digestive enzymes (i.e. hungriest), food wastage and therefore pollution loading into the water and sediment might be significantly reduced. Finally, the greatest advantage in producing a properly protein-content adjusted diet is the improvement in water quality not only in the ponds but also the discharge waters when ammonia production is reduced to as little as 2.3%. Naturally further work is needed to confirm the findings of this study and to test whether these findings are relevant in commercial culture systems.
REFERENCES


References


