Dietary phytic acid and its effects on *Macrobrachium rosenbergii* (De Man, 1879)

A thesis submitted for the Degree of Doctor of Philosophy

by

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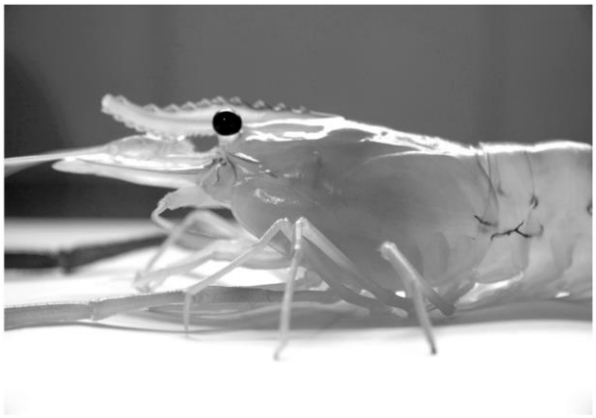

Malaysian Freshwater Prawn

To the other person who shares 99.89% of my DNA, and,

my loving family

Declaration

This thesis has been composed entirely by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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Abstract

The giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879), is gaining popularity as a key aquaculture species; global production currently exceed 220,000 tonnes, however, industry expansion is limited by high operational costs, with the feed accounting for between 40 to 60% of these. Attention, therefore, has been devoted to increasing the inclusion of plant proteins into the formulation of aqua feeds as a consequence of the limited, unpredictable supply and increasing price of fishmeal. The concomitant introductions of anti-nutritional factors (ANFs), such as phytic acid (PA) with the plant protein fraction, however, are major impediments in the efforts toward the increased use of plant protein ingredients in aqua feeds. Phytic acid is an anti-nutrient that can curtail the development of this as PA has been reported to suppress growth impairing proper nutrient intake, diminishing the availability of minerals, and causing damage to the body tissues and organs which can result in mortality. Although the anti-nutritive effects of PA have been studied extensively in terrestrial agriculture farm species, as well as in a variety of fish species, there is almost no information regarding the effects of PA in crustaceans, including the freshwater prawn, *M. rosenbergii*.

The aims of this present thesis were, therefore, to gain a greater understanding of dietary PA and microbial phytase and their effect on growth performance, feed utilisation, nutrient utilisation and digestibility, mineral availability and whole body proximate composition in juvenile *M. rosenbergii*. Specifically, the first major experiment set out to investigate the effect of including increasing amounts of PA in the diets presented to *M. rosenbergii* on growth. The dose-response relationships between PA and growth performance, feed utilisation, nutrient digestibility and utilisation and whole proximate composition were investigated. Four replicate groups of *M. rosenbergii* with a mean initial carapace length of 6.03 ± 0.30 mm and mean initial

weight of 0.29 ± 0.02 g were fed graded levels of PA for 140 days. The basal diet, to which different levels of PA were added to obtain 0.26 (control), 6.48, 11.28, 16.53, 21.45 and 26.16 g PA kg^{-1}, contained fishmeal, soy protein concentrate, wheat meal and corn starch. The results indicated that growth performance, feed utilisation and survival did not differ significantly between the groups receiving the different inclusions of PA within their diets. The apparent protein, lipid and energy utilisations responded negatively, decreasing significantly (p<0.05) with an increasing inclusion of PA, particularly within the groups of prawns fed the diet with the highest inclusions of PA, *i.e*. the 21.45–26.16 g PA kg^{-1} diets. The digestibility of protein and lipid were also reduced as the inclusion of PA increased. The whole body composition of protein (p<0.04), lipid (p<0.01) and gross energy (p<0.05) decreased significantly with an increasing supplementation of PA, while the ash content significantly increased (p<0.01), most notably in the groups of prawns receiving the highest levels of dietary PA.

The second major experimental trial investigated the effect of microbial phytase on the growth of juvenile *M. rosenbergii*, when fed diets supplemented with various doses for a period of 80 days. The study set out to improve the growth performance, feed utilisation, nutrient digestibility and utilisation and body composition of *M. rosenbergii* when fed diets high in plant protein ingredients. To investigate this, four plant protein based diets, which included soybean meal, wheat gluten and wheat meal, were formulated and supplemented with microbial phytase at levels of 0, 500, 1000 and 2000 FTU kg^{-1} (one phytase unit per kg) and fed to sixty juvenile *M. rosenbergii* (mean initial carapace length of 8.51 ± 0.52 mm; mean initial weight of 0.40 ± 0.07 g) for 80 days. High levels of plant protein in the diets supplemented with 0–2000 FTU kg^{-1} did not result in any negative effect on growth performance, feed utilisation nor on the survival of *M. rosenbergii*. Noticeable moderate growth improvements in line with increasing microbial

phytase supplement levels were observed and the highest growth performance was seen in the group fed 2000 FTU kg^{-1}. Supplementation of the diets with 500–2000 FTU kg^{-1} were found to affect the nutrient utilisation, resulting in a significant (p<0.05) increase in the protein and lipid utilisation when compared to the prawns analysed from the control group. In addition to this latter finding, an increasing supplement of phytase in the diet also resulted in an increase in the dry matter fraction as well as improvements in the digestibility of protein and lipid. There were, however, no significant differences in moisture, protein, lipid, gross energy and the ash content of the whole body of the *M. rosenbergii* among the groups.

This thesis, in a third major trial, explored the impact of other potential ANFs associated to PA, the binding effect of PA with mineral. The effects of graded levels of PA (*i.e*. 0.26–control, 6.48, 11.28, 16.53, 21.45 and 26.16 g PA kg^{-1}) on the moult frequency and mineral availability in juvenile *M. rosenbergii* fed over a period of 140 days were determined. The levels of PA assessed in this feed trial had no major detrimental effects on moult frequency. Negative effects (p<0.05) of high PA levels (*i.e.* 21.45–26.16 g PA kg^{-1}), however, were found on the whole body P concentration. An inverse trend was recorded for the Ca content in the whole body (p<0.005) and carapace (p<0.004) with increasing PA inclusion. These results are consistent with findings for marine shrimp species such as *Marsupenaeus japonicus* and *Litopenaeus vannamei*. The graded inclusion of PA in the experimental diets also resulted in a significant reduced (p<0.03) P content in the carapace. Significant changes (p<0.05) were observed in the carapace Zn, Cu, K and Na compositions, particularly in the prawns fed the diet containing 11.28 g PA kg^{-1}, which suggests that the specific minerals were either selectively utilised or retained in the carapace.

Accordingly, this thesis investigates the potential of adding dietary supplements of microbial phytase in order to improve mineral availability as proven in several fish species. To explore this, four experimental diets were formulated - three incorporating different levels of microbial phytase (*i.e.* 0 FTU kg^{-1}, 1000 FTU kg^{-1} and 2000 FTU kg^{-1}) and a fourth, a control. An aliquot of 15 g PA kg^{-1} was also added to each treatment. The mineral premix was omitted from the test group diets except the control diet was identical to the 0 FTU kg^{-1} diet but included a mineral premix and an aliquot of 8 g kg^{-1} monosodium phosphate which replaced an equal amount of wheat meal fraction. The trial found no significant differences in growth, feed utilisation and moult frequency with the microbial phytase level within the diet, however, survival was compromised. The proximate composition of the prawn whole body was in most cases unaffected by the level of phytase. Supplementation of the diets with microbial phytase did, however, result in significant higher (p<0.05) concentrations of minerals including: 1) Ca, Mg, K and Na in the whole body; 2) Ca and Zn in muscle tissue; and, 3) Ca and Mg in the carapace. The Zn content of the carapace, however, was negatively affected by the inclusion of microbial phytase suggesting the necessity of this mineral within the diet of *M. rosenbergii*.

This thesis contributes to current understanding surrounding the inclusion of dietary PA and the benefits of microbial phytase within the experimental diets consumed by juvenile *M. rosenbergii*. The knowledge gained from this work provides a means to optimise the use of plant protein ingredients and with the potential to decrease the dependability of fishmeal without compromising *M. rosenbergii* production and profitability, thus ultimately promoting the sustainable expansion of *M. rosenbergii* aquaculture.

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Abbreviations and Acronyms

ACPD	Apparent Crude Protein Digestibility
ADC	Apparent Digestibility Coefficient
ADMD	Apparent Dry Matter Digestibility
ANEU	Apparent Net Energy Utilisation
ANF	Anti-nutritional Factor
ANLU	Apparent Net Lipid Utilisation
ANOVA	Analysis of Variance
ANPU	Apparent Net Protein Utilisation
Ca	Calcium
Cu	Copper
FAO	Food and Agriculture Organisation
FCR	Food Conversion Ratio
FFRC	Freshwater Fisheries Research Centre
Fe	Iron
FI	Feed intake
FM	Fishmeal
FTU	Phytase enzyme activity unit
HSI	Hepatosomatic index
IFFO	International Fishmeal and Fish Oil Organisation
K	Potassium
kg	Kilogram
L	Litre
mg	Milligram
ml	Mililitre
MDOF	Malaysian Department of Fisheries
Mg	Magnesium
Mn	Manganese
mt	Million tonnes
Na	Sodium
NFE	Nitrogen Free Extract
P	Phosphorus
PA	Phytic acid
PER	Protein Efficiency Ratio
PL	Post larvae
ppm	Parts per million
RCF	Relative Centrifugal Force
SBM	Soybean meal
SGR	Specific Growth Rate
SPC	Soy Protein Concentrate
t	Tonne
× g	RCF unit (where g represents the force of gravity)
Zn	Zinc

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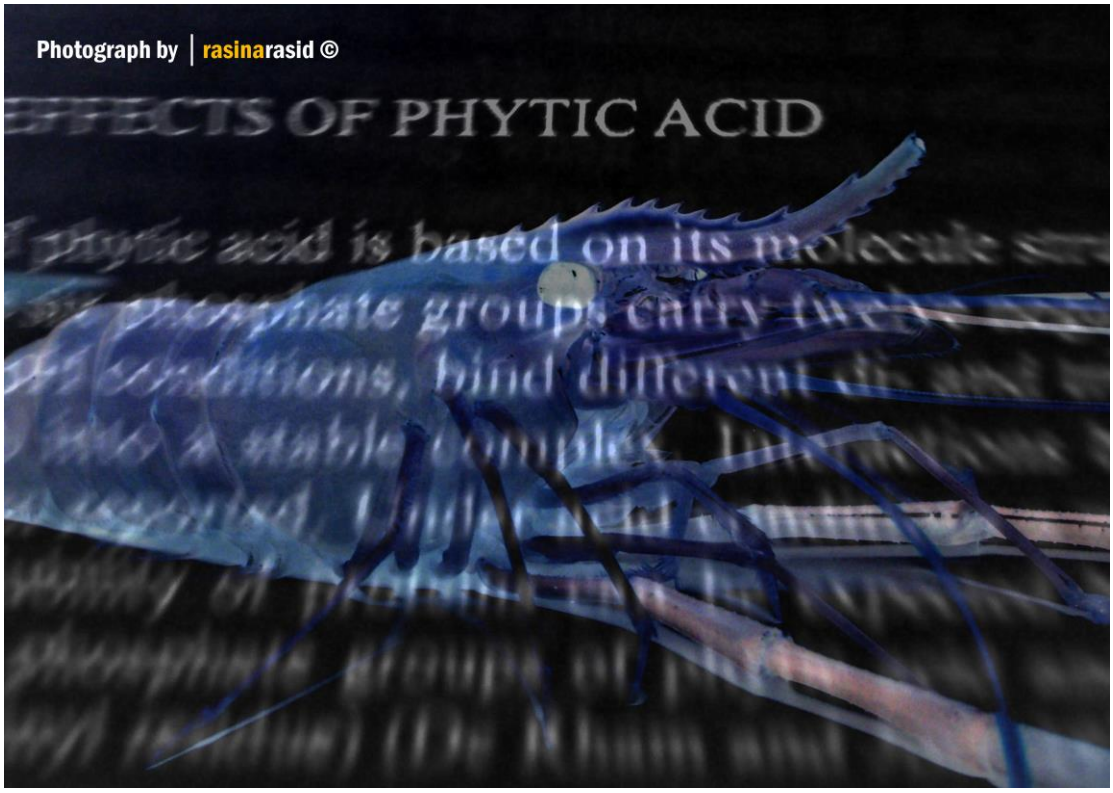
Chapter 1 - General introduction



1.1 *Macrobrachium rosenbergii*

The giant river prawn, *Macrobrachium rosenbergii* (De Man, 1879), is indigenous to the tropical Indo-Pacific region (New, 2002). Freshwater prawns belong to the family Palaemonidae and *M. rosenbergii* is the largest known species with a total body length of up to 320 mm (New *et al.*, 2009). *Macrobrachium rosenbergii* (**Figure 1.1**) can be distinguished from other freshwater prawn species and from marine shrimp by: their very long rostrum; adult males have very long second chelipeds; the first and second legs (pereiopods) have chelae (pincers); the second pleuron of the abdomen overlaps both the first and the third pleuron; and, the tip of the telson reaches distinctly beyond the posterior spines of the telson (Holthuis, 2000).

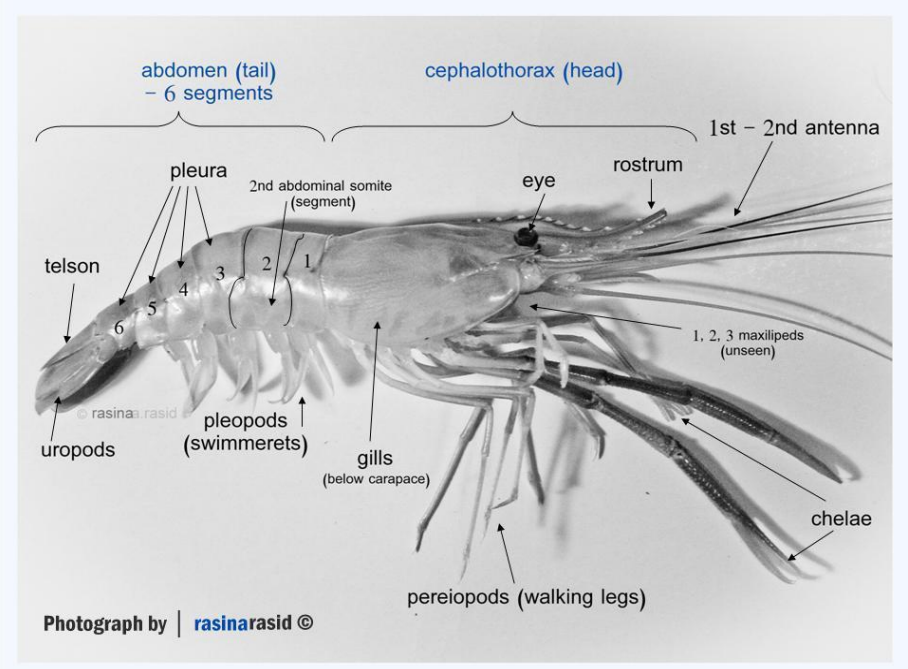


Figure 1.1. The principal anatomical features of the giant river prawn, *Macrobrachium rosenbergii* (De Man, 1879).

Macrobrachium rosenbergii, also known as the giant freshwater prawn or the Malaysian prawn, inhabits inland freshwater areas but the larval stages require brackish water. There are four distinct phases in the life-cycle of *M. rosenbergii*: eggs, larvae, post larvae (PL) and adults

(New, 2002). When larvae are released after hatching, they swim upside down and tail first. The larvae undergo 11 moults before transforming into post larvae (Uno & Kwon, 1969). Post larvae resemble miniature adults, changing from free-swimming and pelagic to principally benthic crawling, becoming positively rheotactic so that they can migrate upstream (New, 2002; Brown *et al.*, 2009). Although no standard definition for "juvenile" exists (Lee & Watson, 2003), it is generally used to describe individuals that are several weeks beyond the post larval stage in age but have yet to reach the adult stage.

Growth in *M. rosenbergii* occurs through moulting or ecdysis where individuals periodically shed their old exoskeleton, resulting in the growth of somatic tissue (Ismael & New, 2000; Wickins & Lee, 2002; Brown *et al.,* 2009). The frequency of moulting varies naturally depending on the size, age, temperature, salinity and on the availability of food (Chang, 1995). Freshwater prawns increase their size by means of water absorption during the moult cycle, however, somatic growth (*i.e*. a slight increase in external dimensions) occurs during intermoult (Mauchline, 1977). Mykles & Ahearn (1978) demonstrated changes in net water transport in *M. rosenbergii* perfused midguts during the moulting cycle where water flux rates were elevated in animals approaching ecdysis and significantly reduced in those in the post-moult stage (Ismael & New, 2000). Taking these observations into account, the rate of growth is a function of the frequency of moulting and the increase in size at each moult (Wickins & Lee, 2002).

Following a moult, the cast exuviae are normally eaten as a convenient source of minerals (Wickins & Lee, 2002). Newly moulted prawns are particularly susceptible to cannibalism, particularly those held in high densities (Adisukresno, 1980; Wickins & Lee, 2002; Brown *et al.*, 2009). In addition, it has been suggested that an insufficient food supply may also increase cannibalistic behaviour (Ismael & New, 2000; Brown *et al.,* 2009).

This species has emerged as having high potential for aquaculture based on a number of advantages over many other crustaceans, most notably its large size (Tidwell *et al*., 2002); its ability to adapt to a relatively wide range of temperatures from a minimum of 15°C to a maximum of 35°C; its tolerance of a range of salinities (Soesanto, 1980); and, its apparently lower susceptibility to many of the viral diseases that have devastated marine shrimp production, although this suggested "resistance" may be linked to its culture at lower densities (Tidwell *et al.,* 2002). Fast growing individuals reach market size in about 6–9 months, and the meat is of high quality in terms of tastes and texture (Mitra *et al*., 2005).

Macrobrachium rosenbergii is also adaptable to a variety of different culture systems, both monoculture and polyculture (New, 2000; Mitra *et al*., 2005; Hossain & Islam, 2006). There are, for example, accounts of the polyculture of various *Macrobrachium* species in combination with single or multiple species of fish, including tilapias, common carp, Chinese carps, Indian carps, golden shiners, mullets, pacu, ornamental fish, and red swamp crayfish (New, 2002; Hossain & Islam, 2006). The cultivation of *M. rosenbergii* can also be integrated with rice farming (New, 2000; Phuong *et al*., 2001).

1.2 Overview of aquaculture

1.2.1 Global overview

The internationally accepted definition of aquaculture by the Food and Agriculture Organisation (FAO) of the United Nations Organisation (UNO) (FAO, 1990) is "*the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators etc. Farming also implies individual or corporate ownership of the stock being cultivated*".

Aquaculture remains a growing, vibrant and important sector for the production of food items that contain a high level of protein (FAO, 2010). In the early 1950s, aquaculture production (excluding aquatic plants) was less than 1 mt per year, but by 2013, for which the latest FAO figures are available, global production had increased to 97.2 mt, worth an estimated value of US$ 157.3 billion (FAO FishStatJ, 2013). With an annual growth rate of 6.2% in the period 2000–2012, supply from aquaculture has more than double from 32.4 mt in 2000 to 66.6 mt in 2012; aquaculture continues to be the fastest growing animal food producing sector (FAO, 2014).

1.2.2 Current and future status of crustacean production

FAO (2014) reported that freshwater farmed finfish continued to dominate aquaculture with a production of 40.5 mt valued at US$ 66.53 billion, while crustacean production was 6.84 mt valued at US$ 35.05 billion. Over the period 2000–2008, the production of crustaceans grew at an average annual rate of almost 15%, faster than the rates observed over the previous decade (FishStatJ, 2014). Culture production of crustacean in the Asean community is 8.04%, increasing at 8.31% and fish is 37.41%, increasing at 12.27%. Although cultured crustaceans still account for less than half of total global crustacean production, the culture production of penaeids (shrimps and prawns) in 2008 was 73.3% of the total production but unfortunately, production was affected by an economic crisis which resulted in the first decline since production entered international trade in the 1980s (FAO, 2010). Nonetheless, the United States of America continues to be the main shrimp importer followed by Japan and most major European countries, except Spain, all of whom have had a stable or a steadily increasing rise in shrimp imports (FAO, 2010).

1.2.3 Current and future status of *Macrobrachium rosenbergii*

The first real breakthrough in production came about in 1961 when Dr Ling successfully developed larvae rearing (New & Valenti, 2000). This was followed by successful methods in the mass production of post larvae (PL) by Fujimura & Okamoto (1972). Since then, this freshwater prawn has been introduced throughout the tropical and sub-tropical zones for farming purposes. *Macrobrachium rosenbergii* is now farmed in many countries including Bangladesh, Brazil, China, the Dominican Republic, Fiji, Ecuador, Guatemala, Mexico, Senegal, Thailand and the USA (New, 2002, 2005; New *et al*., 2009).

Prawn farming is regarded as one of the more sustainable forms of crustacean aquaculture as freshwater prawns cannot be reared at densities as high as those commonly used in the farming of marine shrimp. Consequentially, the potential abuse or waste of resources is minimal and expensive coastal sites are not required for production (Wickins & Lee, 2002; FAO, 2005-2012).

New (2005) predicted a rapid rise in production and this has proven to be the case. The most recent available production figures from FAO (2012), put the global production of *M. rosenbergii* as 220,254 tonnes worth an estimated US$ 1.27 billion (**Figure 1.2**). China is currently the top producer followed by Bangladesh and Thailand. Other major producers are Vietnam, Taiwan, Indonesia and Myanmar, whilst Malaysia is the ninth largest producer (FAO FishStatJ, 2013). It is not surprising, therefore, that Asia dominates and accounts for more than 90% of production (FAO FishStatJ, 2013). This species is highly valued in the market, and demand is increasing. Hence, necessary efforts to increase production and maintain low production cost are essential (Hanson & Sempier, 2005). *Macrobrachium rosenbergii* are commonly sold live, whole, headed and peeled (Galitzine & Morgan, 2009).

The development of commercial farming of *Macrobrachium*, however, has been limited due to a several factors which include the comparatively long larval stage which is twice as long as that typically seen for farmed shrimp species, cannibalism, the cost of seed which is expensive, marketing difficulties, and, the lack of nutritionally efficient and economical diets (New, 2000, 2009).

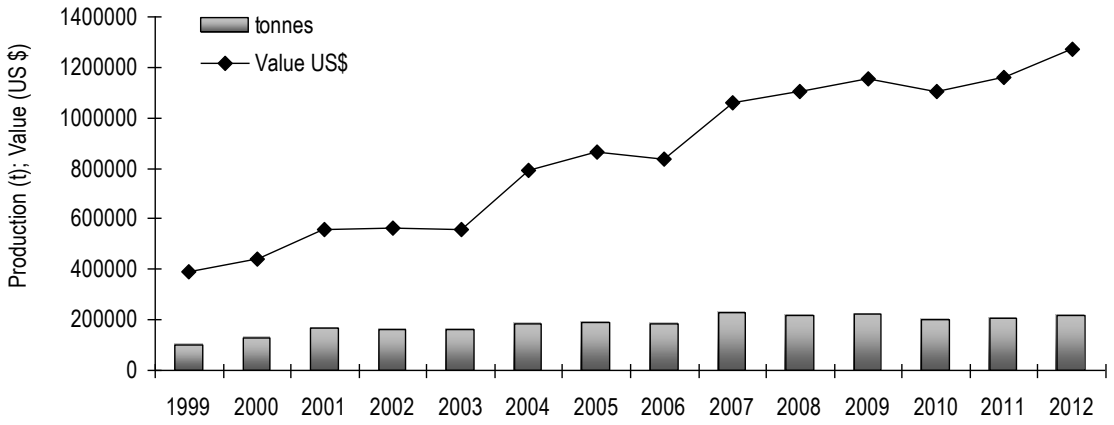


Figure 1.2. Global production and value of *Macrobrachium rosenbergii* over the period of 1999 to 2012 (Source: FAO, 2012).

1.2.4 Production of *Macrobrachium rosenbergii* in Malaysia

Freshwater prawn farming has a special significance within Malaysia in that this is where Dr Shao-Wen Ling's pioneer studies were made (New & Valenti, 2000). *Macrobrachium rosenbergii* has remained popular in demand since the 1980s when the market price was RM15 (*i.e*. US$ 4.81) per kg until today when the price is now RM45 (*i.e.* US$ 13.81 kg^{-1}). Production in Malaysia although relatively small compared with other Asian countries (**Figure 1.2**) is expanding steadily (**Figure 1.3**). The latest estimates of *M. rosenbergii* production in Malaysia were 457 t in 2013 at a value estimated at US$ 4.7 million (FAO, 2013).

Impediments to the development of this species in Malaysia are said to be an insufficient supply of quality post larvae, the potential high risk of loss, a lack of confidence in the industry,

and, in investment from large private companies (FFRC, 2003; Phuong *et al*., 2003).

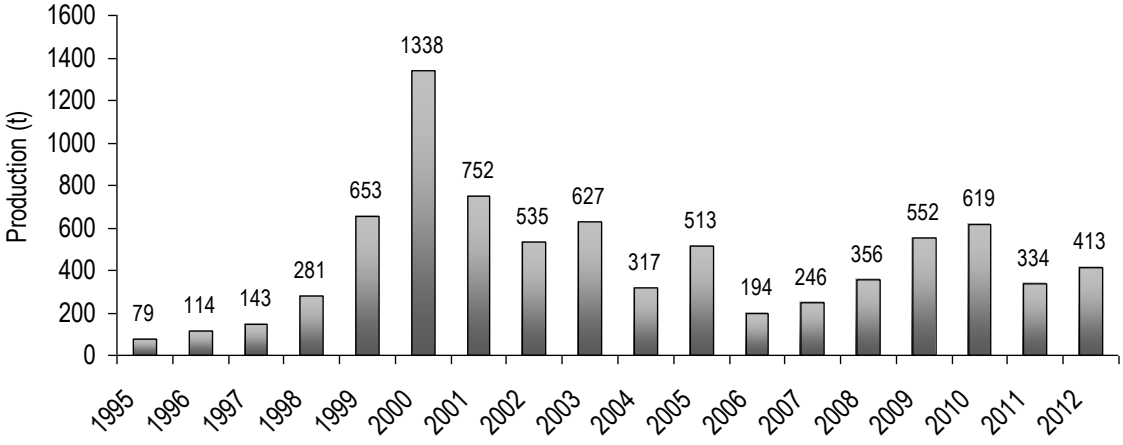


Figure 1.3. Production of *Macrobrachium rosenbergii* in Malaysia over the period of 1995 to 2012 (Source: FAO, 2012).

Nonetheless, realising the importance and value of this species, under the Ninth Malaysian Plan (RMK–9) for 2006–2010, the Malaysian Government had allocated RM4.4 billion (*i.e*. US$ 1.24 billion) for modernising agriculture and for the development of *M. rosenbergii* culture technology (MDOF, 2005). Given this investment, it would appear that the industry within Malaysia is progressing and that the signs are that the production of *M. rosenbergii* will continue to develop in the coming years.

1.3 Nutrition and feeding of *Macrobrachium rosenbergii*

1.3.1 Feeding characteristics

It is important to understand the feeding behaviour of prawns in order to provide feed efficiently. Experimental findings and the natural history of prawns indicate that these crustaceans are omnivorous scavengers that feed on a variety of bottom organisms and organic materials, *e.g*. detritus. Freshwater prawns are omnivorous and their natural diet includes aquatic insects, aquatic plants, algae, very small worms, small molluscs and other crustaceans (Ling, 1969). They are also highly territorial and cannibalistic behaviour may occur when food becomes

insufficient (Brown *et al*., 2009). Freshwater prawns find their feed principally by smell, taste and feel, rather than by eyesight (Soesanto, 1980; New & Valenti, 2000; Tidwell *et al*., 2002).

The food habits of prawns vary during different life stages. Freshwater prawns are fed a series of feed types over the course of cultivation, each satisfying nutritional needs at different points of their life history. During the larval stage, freshwater prawns are fed principally on *Artemia* nauplii (D'Abramo *et al*., 2003). This larval stage lasts between 22 to 30 days after which they develop into post larvae. At this early stage of growth, the PL require a high protein diet (Manik, 1980). As they grow into juveniles, they become increasingly carnivorous and so it becomes more important to provide high quality and nutritionally complete diets (Tidwell *et al*., 2002).

It is suggested that omnivorous decapods possess a full complement of digestive enzymes (Yonge, 1924); *M. rosenbergii*, for example, are capable of digesting a wide range of food items. Dall (1968) described the structure of the gut of shrimp and its division into three sections: an anterior chamber of the proventriculus, a midgut, and, a rectum. Food is picked up by the chelipeds and conveyed to the mouth which then enters a short oesophagus and leads vertically into the anterior chamber of the proventriculus, known as the 'stomach'. This part of the gut serves as a distensible crop, and posteriorly, as a gastric mill. Food is then mixed with digestive secretions and passed forward into the posterior proventriculus. The posterior proventriculus is partly embedded in the digestive gland and is divided into a dorsal channel which leads directly into the long simple midgut, and a ventral filter press which permits only the finest particles to pass into the digestive gland. Digestion is completed in the proximal half of the digestive gland tubules. Characterisation of the activities of the digestive enzymes in the alimentary tract indicates the presence of trypsin, amino peptidases, proteases, amylases, chitinase, cellulose, esterases and lipases (Mitra *et al*., 2005). Indigestible particles are passed

through the midgut towards the posterior diverticulum of the midgut and then to the rectum. Defaecation begins an hour after feeding and ceases about four to six hours after ingestion (Dall, 1968; Guillaume *et al*., 2001).

1.3.2 Nutrient requirement and diets of *Macrobrachium rosenbergii*

A number of key studies on the nutritional requirements of *M. rosenbergii* have been undertaken; see for example the study of Fox *et al*. (1994). Protein, being an important dietary constituent among animals, directly influences the formulation of diets and consequently affects the cost of production. Under laboratory conditions, clear water systems that do not have a supply of natural food, diets with about 35–40% protein were found suitable for growth. Balazs *et al.* (1973) proposed that a diet containing more than 35% protein was appropriate for *M. rosenbergii*. Later, Millikin *et al*. (1980) reported that the growth performance of *M. rosenbergii* post larvae were as equally good from diets with 40% and 49% protein whilst those fed diets containing a 23% or 32% protein fraction showed depressed growth rates.

Macrobrachium rosenbergii effectively utilises carbohydrate as a source of energy, supported by the comparatively high specific activity of amylase. Prawns are also known to utilise diets with high dietary fibre (as high as 30%) and dietary lipid levels of between 3–7% (Mitra *et al*., 2005). The vitamin requirements of *M. rosenbergii* though are probably similar to those of other crustaceans and fish species. Information on the quantitative mineral requirements of freshwater prawn, though, are limited. What is known though is that the dietary supply of calcium seems to improve the growth of freshwater prawns. The optimum levels of zinc are between 50–90 mg kg^{-1} diet but higher dietary doses, *i.e.* >90 mg kg^{-1}, decrease growth and feed conversion efficiency (Wickins & Lee, 2002). The nutrient requirements of *M. rosenbergii* are summarised in **Table 1.1**.

Table 1.1. A summary of the nutrient requirements of the freshwater prawn *Macrobrachium rosenbergii* based on laboratory trials (Source: Mitra *et al*., 2005).

Nutrients	Requirement
Protein (%)	35 – 38
Carbohydrate (%)	25 – 35
Lipid (%)	3 – 7
Calcium: Phosphorus	1.5:1
Zinc (mg kg^{-1})	90
Other minerals	Quantitative requirements not known yet
Energy (kJ g^{-1})	15.7 – 17.0

The types of feed used in freshwater prawn farming vary widely and include 'farm-made feeds' and specifically formulated commercial pellets. Diets used for freshwater prawns range from inexpensive organic fertiliser such as distiller's grain, to expensive and highly refined 40% protein marine shrimp diets costing US$ 500 or more per ton (Tidwell *et al*., 2002; Coyle *et.al*., 2003; Posadas, 2004). Commercially available sinking catfish feed are also effective diets used for the pond culture of freshwater prawns (D'Abramo *et al*., 2003; Posadas, 2004). Commercial feeds may be the most productive and reliable to use but they are expensive, are not always available to the small farmer, and do not take advantage of locally available ingredients (FAO−FIMA, 2009).

Generally, attention is given to include locally available ingredients to reduce costs. Soybean meal, tapioca, groundnut meal, copra meal, broken rice and palm oil are among some of the local ingredients that have been used (Manik, 1976; Boonyaratpalin & New 1982; Thong, 1985; Hari & Kurup, 2001). Low cost diets containing meat and bone meal, mustard oil cake, sesame meal, rice bran, for example, have been used to feed *M. rosenbergii* reared in Bangladesh (Hossain & Paul, 2007). The use of these ingredients are generally for semi intensive culture where natural food supplies are also available (D'Abramo & New, 2000). Natural productivity can be a significant food source in freshwater prawn production (Lilyestrom *et al*., 1987).

Freshwater prawns are able to increase the level of predation on natural pond biota to supplement their nutritional intake when expensive nutrients, *i.e.* vitamins, are deleted from the diet (Corbin *et al*., 1983). Tidwell *et al*. (1995) reported that freshwater prawns are able to adjust to reductions in the nutritional value of prepared diets by increasing predation on natural fauna, *i.e*. macro-invertebrates, in the pond.

The farm feeding schedule used also influences the amount of protein needed in the diet (Galitzine & Morgan, 2009). *Macrobrachium rosenbergii* are slow, continuous feeders that chew food to a suitable size before swallowing (New & Valenti, 2000). The pace at which animals reach and consume their feed is important when artificial pellets are presented to them, because they can rapidly disintegrate in water (Felix & Sudharsan, 2004). By feeding stock multiple times daily, the breakdown of food pellets and subsequent nutrient leaching minimises feed losses (Tidwell *et al*., 2002). In addition, the use of feeding attractants in aqua feeds has received considerable attention in recent years (Harpaz, 1997). The rationale for their use is to improve feed intake and feed efficiency (Nakajima *et al.,* 1990), and, the addition of an attractant in the water stimulates the prawns to search for feed (Harpaz, 1997; Felix & Sudharsan, 2004). As crustaceans are primarily chemosensory feeders (Akiyama & Chwang, 1989), they are attracted to the artificial feed pellets by following an attractant 'plume' originating from the food source (Atemia, 1988). The ingestion of food and the continuation of feeding can be encouraged through the addition of feeding stimulants (Mackie & Mitchell, 1985). The use of chemoattractants is particularly important when incorporating non-conventional and/or novel plant protein ingredients to encourage ingestion of potentially unpalatable test diets.

Research on feed formulation has mainly concentrated on investigations into the inclusion of

various economical protein sources and in varying the nutrient composition under laboratory experimental and pond conditions (Tidwell *et al*., 1993; Hari & Kurup, 2001; Du & Niu, 2003; Hasanuzzaman *et al*., 2009). The paucity of information concerning particular antagonistic effects of specific novel ingredients and the general lack of information on nutritionally efficient and economic diets resulting in successful crops of stock is still a major constraint in the culture of this species.

1.4 Fishmeal as the main protein source

The expansion of global aquaculture production is increasing the demand for cheaper and affordable diets as feed accounts for between 35 to 60% of aquaculture production costs. While protein in high energy extruded diets presently accounts for about 50% of the cost, this is due to an extensive reliance on premium quality fishmeals (FM) as a source of protein (Higgs *et al*., 1996). Efforts to identify cheaper alternative protein sources, such as plant proteins, by feed manufacturers have therefore increased due to the rising cost of FM.

Fishmeal is a generic term for a nutrient-rich feed ingredient used primarily in aqua feeds made from fish bones and offal from processed fish (Miles & Chapman, 2006). Fishmeal is currently the most important protein source for aquaculture diets because of its high protein quality, digestibility and palatability (Akiyama *et al*., 1989). In addition to this, FM serves as an effective feed attractant, contains significant levels of polyunsaturated fatty acids (PUFA), highly unsaturated fatty acids (HUFA), minerals and phospholipids (Fox *et al*., 2004).

In freshwater prawn farming that uses commercially manufactured shrimp feeds, the primary protein sources used within the pellets contains between 30–50% protein derived from marine animal sources such as fish, shrimp and squid meals. Commercial freshwater prawn feeds

have been reported to contain variable levels of fishmeal, *e.g.* from 23.8 to 38.5% in diets from Hawaii (Corcin *et al*., 1983), from 28 to 36% in diets from Taiwan (Hsieh *et al*., 1989), from 22 to 30% in diets from Thailand (New, 1990), 13% FM (55% crude protein FM) in diets from Malaysia (Chow, 1984), and, from 20–30% FM in commercial diets from Indonesia (Manik, 1976).

The main species used globally for the manufacture of FM are small pelagic species such as anchoveta (*Engraulis ringens*), sand eels (*Ammodytes* spp.), Atlantic menhaden (*Brevoortia tyrannus*), capelin (Family *Osmeridae*, *e.g. Mallotus* spp.), Atlantic herring (*Clupea harengus harengus*), Norway pout (*Trisopterus esmarkii*), European sprat (*Sprattus sprattus*), Chilean jack mackerel (*Trachurus murphyi*) and chub mackerel (*Scomber aponicus*) (see De Silva & Turchini, 2009).

1.4.1 Future of fishmeal as aqua feed

For decades, the need to provide fish as feed for other fish has been seen as an insurmountable obstacle as the amount of fish available from the wild is finite. Reduction fisheries, refers to those fisheries in which harvest is 'reduced' to FM and fish oil, primarily for agriculture and aquaculture feeds. Within the animal husbandry sub-sector, aquaculture is the largest user of FM using an estimated 68% of the global FM that is produced whilst the pig industry uses approximately 25% of the FM that is produced (IFFO, 2011) (**Figure 1.4**).

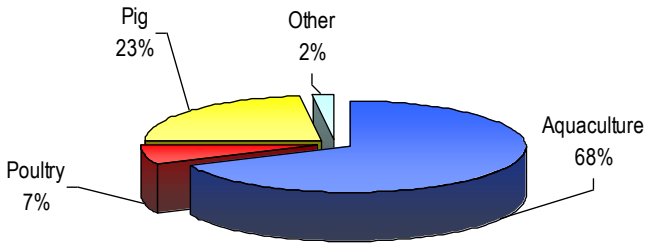


Figure 1.4. The world fish meal production usage by sector (Source: IFFO, 2011).

Crustaceans recorded the highest usage of FM in aquaculture at 30% whereas the remaining 70% is split into other groups, *i.e*. 22% salmonid, 21% marine fish, 6% tilapias, 6% eels, 5% cyprinids and 10% other species (**Figure 1.5**). Fishmeal and fish oil are produced from target small pelagic species that mature quickly, reproduce prolifically, are low on the food chain, and are preyed on by higher trophic level animals such as piscivorous fish, sea birds and marine mammals (Galitzine & Morgan, 2009). These pelagic species play a crucial role in marine ecosystems as they transfer energy from plankton to larger fish species, seabirds and marine mammals (Naylor *et al*., 2000; Watson *et al*., 2006; MATF, 2007). If these species are overfished then this will result in potentially serious impacts on the marine ecosystem (Baraff & Loughlin, 2000; Tasker *et al*., 2000; Furness, 2003; Becker & Beissinger, 2006). Fisheries targeting pelagic species also have the potential to reduce the productivity of other fisheries that rely on these species as prey (Walters *et al*., 2005).

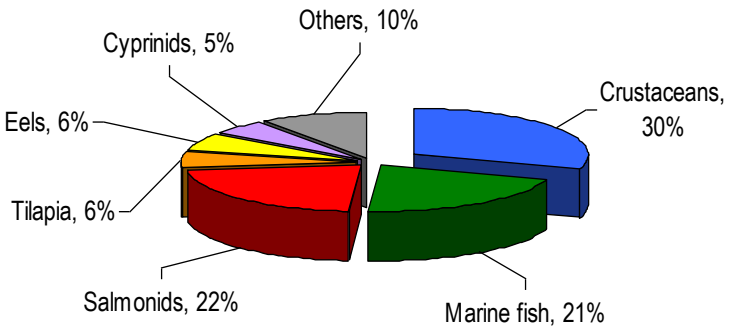


Figure 1.5. The percentage of fishmeal usage in aquaculture (Source: IFFO, 2011).

The quality and price of FM often fluctuates as a consequence of market constraints and natural phenomena such as El Nino (Venero *et al*., 2008). Environmental concerns such as pollution events and over-fishing impose further challenges that will also affect availability and price (Akiyama, 1992; Tacon & Forster, 2000). Pelagic fish are often high in oil and are also nutritious in their own right, and could be used more efficiently by humans, if consumed directly (Tacon & Akiyama, 1997; Watson *et al*., 2006). In addition, FM prices are likely to be higher in

the future because of the increased demands that will be placed upon this important commodity for finfish and crustacean culture, pet foods and speciality livestock feeds (**Figure 1.4**; Rumsey, 1993).

It is generally believed that most forage fish populations are stable over multiple years, though they naturally oscillate with ocean conditions (Hardy & Tacon, 2002; Huntington *et al.,* 2004). Concerns, however, have been raised about the potential for increased demand from expanding industries for farmed carnivorous fish (Weber, 2003), since most populations are currently classified by the FAO as fully exploited (Tacon, 2005).

On account of these constraints, there is a need to replace and to reduce current dependency on FM for inclusion in aqua feeds and this will involve the identification and development of sustainable and less expensive alternative sources of protein such as plant proteins (Tacon & Akiyama, 1997; Forster *et al*., 1999).

1.4.2 Sustainable alternative: Plant proteins and its limitations

Plant proteins are sustainable compared to FM as they are produced in large quantities, there is a stable production, are less expensive and are not an overexploited resource (Davis *et al*., 2004; Venero *et al*., 2008). Common protein ingredients used in crustacean diets include soybean meal, cottonseed meal, rapeseed/canola meal, wheat meal, peanut meal and rice bran (**Table 1.2**) (NRC, 1993; Hertrampf & Piedad-Pascual, 2000; D'Abramo & New, 2000; Davis *et al*., 2004).

The utility of plant proteins as an ingredient within feeds, however, can be limited due to a variety of reasons such as an imbalance (or lack) of essential animo acids (EAAs), reduced levels of minerals and limited levels of HUFA (Davis *et al*., 2004). Whilst the unpredictability of

factors such as feed/ingredient prices, the nutritional values of each ingredient and their availability are disadvantages, there are also specific negative factors that are as equally important that need to be considered in the formulation of aqua-feeds. One key negative consideration are the anti-nutritional factors (ANFs) which are found in most plant protein sources.

Table 1.2. Plant origins feed ingredients (% as fed basis) used in various diets for freshwater prawn *Macrobrachium rosenbergii* (Source: http://affris.org/prawn/tables/table_4.htm).

Feed ingredients	Crude protein
Cotton seed cake	37.0
Rapeseed cake	35.9
Rice bran	12.6
Sesame cake	29.0
Soybean meal, full fat	37.5
Soybean meal (defatted, dehulled)	48.0
Wheat	14.5
Wheat gluten	79.5

Soybean meal (*Glycine max*) is the most commonly used ingredient in aquatic diets (Hertrampf & Piedad-Pascual, 2000; Venero *et al.,* 2008). Soybean meal is readily available, is not prohibitively priced and is rich in protein (Lim & Dominy, 1990). Soybean meal (SBM) contains most of the required essential amino acids with a balanced profile among many of the plant proteins that have been considered, however, it does have a deficiency in methionine (NRC, 1993; Guillaume *et al.,* 2001; Venero *et al*., 2008). Tidwell *et al.* (1993) and Hari & Kurup (2001) suggested that the FM component of diets could be partially replaced by SBM and a second ingredient, distillers' by-product, in the formulation of diets for the pond production of freshwater prawns. It is known, however, that high levels of SBM appear to affect growth, result in lower food consumption rates and feed efficiency in marine shrimp (Lim & Dominy, 1990;

Tacon & Akiyama, 1997). To investigate this, Du & Niu (2003) conducted a 42-day trial to evaluate the response of juvenile freshwater prawns to the replacement of FM derived dietary protein with SBM derived dietary protein at levels of 20, 50, 75 and 100%. The study found that substituting FM with an increasing inclusion of SBM in *M. rosenbergii* diets resulted in a decreased growth rate, lower food conversion efficiency, a lower whole body dry matter and crude lipid content. The potential disadvantages of using SBM are the presence of a wide variety of ANFs such as phytic acid (PA), lectin, protease inhibitors and saponins (Venero *et al*., 2000; Francis *et al.,* 2001) all of which limit its potential. Another common problem encountered when replacing FM with SBM is that the availability of phosphorus (P) decreases due to the presence of phytin-bound phosphorus (NRC, 1993; Hertrampf & Piedad-Pascual, 2000).

Cottonseed meal (*Gossypum hirsitum*) is another potential plant protein that could be used as a replacement for FM in diets as it is also readily available and has a competitive price. It contains good levels of protein (26–54%) and has a balanced amino acid profile but this may be negatively affected by processing conditions (Lovell, 1998). High levels of crude fibre and low lysine availability in addition to the presence of ANFs such as gossypol and PA also limits the amount of cottonseed meal used in diets (Hertrampf & Piedad-Pascual, 2000; Francis *et al*., 2001; Venero *et al*., 2008). Reduced growth and high mortality were observed for *Litopenaeus vannamei* (formerly *Penaeus vannamei*) when cottonseed meals were substituted with FM above 26.5% in the diets (Lim, 1996). Lawrence & Castille (1989) reported that cottonseed meal could partially substitute FM in diets of penaeids shrimp but at a lower level than that used with SBM.

Rapeseed meal (*Brassica napus*) / canola meal (*Brassica campestris*) have also been used in

replacing FM in aquatic diets. It is rich in protein and is one of the most complete ingredients among protein sources based on its essential amino acid (EAA) profile (NRC, 1993; Venero *et al*., 2008). The presence of indigestible components (*e.g.* fibre) and ANFs such as PA, protease inhibitors, and saponins, however, limits the inclusion of rapeseed meal in diets (Venero *et al*., 2008). Special processing has the potential to improve the quality of rapeseed meal such as the use of dephytinised canola protein concentrates. As with other plant protein sources, 60–90% of the total P is in the form of PA, *i.e*. 3.1 to 3.7% which has low P availability (Venero *et al*., 2008). An earlier study by Rique-Marie *et al.* (2005) reported that dephytinised canola protein concentrate can replace up to 50% of the FM protein in the diet of *L. vannamei* without affecting growth.

Other sources of plant proteins that are commonly utilised include sesame meal (*Sesamum indicum*), sunflower meal (*Helianthus annuus*), wheat meal and corn meal (Guillaume *et al.,* 2001; D'Abramo & New, 2000; Venero *et al*., 2008).

1.5 Anti-nutritional factors in plant protein

As discussed above, most alternate protein sources used in aquafeeds contain ANFs that can produce adverse effects and decrease productivity. The different tolerance limits of individual species to the presence of anti-nutrients needs to be considered before deciding on reducing or eliminating the levels of ANFs in diets. Feeding experiments, therefore, using purified individual anti-nutrients are necessary to determine the threshold limits at which they do not affect the productivity of commonly cultured prawn or shrimp species.

The use of plant protein materials such as legumes, legume seeds, oilseeds and leaf protein concentrates, as ingredients in aquafeeds is limited by the presence of a variety of ANF

substances (**Table 1.3**), which poses obvious difficulties and challenges when analysing their effects individually. Each ANF has a different chemical structure, a mechanism of activity and potential toxic effect on different species which can vary under different culture conditions. Commonly found ANFs are gossypol, PA, protease inhibitors, saponins and tannins (NRF, 1993; Francis *et al.,* 2001). Anti-nutritional factors are typically divided into four categories: 1) factors affecting protein utilisation and digestion; 2) factors affecting mineral utilisation; 3) antivitamins; and, 4) miscellaneous substances such as cynogens, mycotoxins and saponins (Francis *et al.,* 2001).

Table 1.3. Important anti-nutrients present in some commonly used alternative aqua feed ingredients. (Source: Francis *et al.*, 2001).

Plant-derived nutrient source	Anti-nutrients present
Cottonseed meal	Phytic acid, phytoestrogens, gossypol,
Rapeseed meal	Phytic acid, protease inhibitors, tannins, glucosinolates
Sesame meal	Phytic acid, protease inhibitors
Soybean meal	Phytic acid, protease inhibitors, saponins
Wheat meal	Phytic acid, protease inhibitor, tannins

Subtle effects are commonly observed when anti-nutrients are consumed over long periods of time. Previous studies have reported that: anti-nutrients can suppress growth through interfering with protein utilisation and/or protein availability; can impair proper nutrient intake or metabolism; can act as inhibitors diminishing the bioavailability of protein and certain amino acids; can decrease food intake because of their inpalatability; they can also diminish the bioavailability of certain minerals such as calcium, phosphorus, iron, magnesium, manganese or zinc; and, they can cause damage to body tissues and organs resulting in mortality (Liener, 1980; Suguira *et al*., 1983; Richardson *et al*, 1985; Liener, 1989; NRC, 1993; Tacon, 1995;

Francis *et al*., 2001).

Studies on the effects of several anti-nutrients on *M. rosenbergii* have been reported. The effects of ANFs such as gossypol, saponins, tannins and protease inhibitor in animals, for example, are briefly summarised in the following text.

1.5.1 Gossypol

Gossypols are polyphenols contained in the pigment glands of plants (NRC, 1993; Guillaume *et al*., 2001; Francis *et al*., 2001). Gossypol is found in both a bound and free form. The bound form of gossypol is non-toxic and of little significance as it is unavailable to the host and passes through the gastro-intestinal tract unabsorbed; the free form of gossypol, however, is highly toxic (Evans, 1985; Tanksley, 1990; Ogunji, 2004).

Free gossypol is tolerated at varying amounts by different fish species but excessive concentrations can depress growth and cause damage to the tissues of various body organs (NRF, 1993; Guillaume *et al*., 2001). Free gossypol at a low level (*i.e.* 0.1 g kg^{-1}) in the diet of rainbow trout, *Oncorhynchus mykiss*, was reported to cause damage (necrosis and ceroid deposition) to the liver (Herman, 1970). Channel catfish, *Ictalurus punctatus*, fed a diet containing 0.9 g kg^{-1} free gossypol were observed to have a lower growth rate compared to their counterparts receiving a normal, control diet (Robinson & Li, 1994). Higher inclusion rates, *e.g.* 1.2 g kg^{-1} of free gossypol, also affected the growth rate of blue tilapia, *Oreochromis aureus* (see Robinson *et al*., 1984). *Macrobrachium rosenbergii*, however, fed diets containing 1.0–10 g kg^{-1} gossypol did not affect their growth performances (Basso, 2003).

1.5.2 Saponins

Saponins are steroid or triterpenoid glycosides found in many plants, notably legumes (Guillaume *et al.,* 2001; Francis *et al.,* 2001). Saponin in various legume seeds ranged from 0.018–0.041 g kg^{-1} whereas defatted roasted soybean flour, by comparison, contains 0.067 g kg^{-1} saponin (Francis *et al*., 2001). Saponins are anti-nutrients as they interfere with the digestibility and absorption of nutrients due to the formation of sparingly digestible saponin-nutrient complexes (Potter *et al*., 1993), as well as reducing the palatability of the diet due to their bitter taste (Guillaume *et al.,* 2001).

Generally, fish, however, are able to tolerate saponins below 1 g kg^{-1} in the diet without affecting growth (Francis *et al*., 2001). Higher levels of saponin, *i.e.* 1.5 g kg^{-1}, have been observed to cause extensive damage to the intestinal mucosa in *O. mykiss* (see Bureau *et al.,* 1998). Saponin is toxic and has reported to result in the mortality of tilapia mossambica (*Oreochromis mossambica*) 5–6 h after tea seed cake (*Camellia sinensis*) containing 7–8% saponins was added to the water at a dose of 100 ppm (De *et al*., 1987). Chen *et al.* (1996) reported that saponin as low as 0.5 mg l^{-1} decreased the feeding rate, growth and moulting frequency in *Marsupenaeus* [syn. *Penaeus*] *japonicus*, whilst a study by Yeh *et al.* (2006) reported that saponin at 0.9 mg l^{-1} was found to decrease respiratory protein levels and the acid-base balance, and therefore modulated the immune system of *M. rosenbergii.*

1.5.3 Tannins

Tannins have been extensively studied due to their presence in a wide range of plant materials, for example, they have been studied in cereals, legumes and legume seeds (Liener, 1989; Francis *et al.,* 2001; Mueller-Harvey, 2001). Tannins are defined as polyphenolic compounds of a high molecular weight which form complexes with proteins (Gupta & Haslam, 1979). Tannins

are regarded as an anti-nutrient because of their ability to interact with and to precipitate proteins and their potential as ion chelators (Hagerman, 1998).

The negative effects of tannins from protein–tannin complexes are reported to result in poor growth performances and feed intake in fish species such as common carp, *Cyprinus carpio* (see Becker & Makkar, 1999; Francis *et al.*, 2001). In contrast to this latter study, however, protein–tannin complexes in the experimental diets fed to *M. rosenbergii* did not reduce protein digestibility or growth performance (Basso, 2003).

1.5.4 Protease inhibitors

Protease inhibitors are crystalline globular proteins that reduce the activity of trypsin and chymotrypsin which are pancreatic enzymes involved in protein digestion (Liener & Kakade, 1980). Protease inhibitors are commonly encountered in many plant-based ingredients, particularly legume seeds and cereals (Norton, 1991; Guillaume *et al.,* 2001; Francis *et al.,* 2001). In soybeans, there are two distinct protease inhibitors: Kunitz factor and Bowman-Kirk factor. First, there is inhibition of trypsin activity, as well as chymotrypsin, and also of elastase through the formation of stable complexes, which results in a marked decrease in protein digestibility (Norton, 1991; Guillaume *et al.,* 2001).

As with the other ANFs discussed here, protease inhibitors have been reported to reduce growth performance, feed efficiency and the survival of certain fish species such as common carp (Abel *et al*., 1984; Makkar & Becker, 1999), channel catfish (Wilson & Poe, 1985), rainbow trout (Dabrowski *et al.,* 1989; Krogdahl *et al.,* 1994), Atlantic salmon, *Salmo salar* (see Olli *et al.,* 1994), tilapia mossambicus and Nile tilapia (Jackson *et al.,* 1982; Wee & Shu, 1989). From these studies, it would appear that different fish species differ in their tolerances to dietary

protease inhibitors; carp and catfish, for example, are able to tolerate higher levels than salmonids are able to deal with (NRC, 1993). In a contrast to this though, the study of Rumsey (1991) found little effect on the growth and feed intake in rainbow trout when protease inhibitor levels in the diet were below 5 g kg^{-1}, whereas a study by Wee & Shu (1989) observed that inclusion rates of 1.6 g kg^{-1} or higher affected growth in Nile tilapia. Li *et al.* (2008) reported that a Kazal family serine protease inhibitor, a male reproduction-related peptidase inhibitor Kazal-type (MRPINK), has been identified in *M. rosenbergii,* and found to have an inhibitory effect on sperm gelatinolytic activity.

In general, it could be concluded that different fish and crustacean species differ in their ability to tolerate different levels of ANFs. A greater understanding of the species-specific effects of particular anti-nutrients, therefore, is essential in the formulation of new diets with acceptable levels of ANFs. Phytic acid is an anti-nutritional component that will be focused upon in this study because of its presence in a number of potentially useful plant ingredients used in the formulation of diets used in commercial freshwater prawn culture.

1.6 Anti-nutritional factor: Phytic acid

Since its discovery by Pfeffer in 1872, when working on wheat endosperm, PA has been categorised as an anti-nutritional component. Research has traditionally focused on its unique structure that gives it the ability to bind minerals and proteins which in turn can result in an array of detrimental effects (Oatway *et al*., 2001). To date, extensive literature exists on the nutritional significance and effects of PA on poultry, swine, rats and fish (for example see Davies & Nightingale, 1975; Civera & Guillaume, 1989; Pallauf & Rimbach, 1997; Francis *et al*., 2001; Rapp *et al*., 2001; Yonekura & Suzuki, 2003). Much less information, however, is available on studies relating to its effects in crustaceans. To date, no study has been carried

out on the effects of PA on freshwater prawn species.

Collectively, the developments of phytases have offered a solution to overcome the adverse effects of PA. The assay for phytase activity is based on the release of inorganic phosphate from the hydrolysis of sodium phytate by phytase (Engelen *et al*., 1994). While the primary role of phytase feed enzymes was to increase the availability of phytate bound phosphorus, their use has provided new insights into the anti-nutritive properties of phytate (Selle *et al*., 2000). Thus far, no studies on the effects of phytase on freshwater prawn species have been undertaken.

The aim of this PhD programme of research, therefore, was to investigate and elucidate the effects of the anti-nutritional factor PA and the role of phytase in hydrolysing PA in the diets of the freshwater prawn, *M. rosenbergii*.

1.6.1 General description and chemical structure

The correct chemical description of phytic acid is *myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (IUPAC-IUB, 1977). The salts of PA are described as phytates and more accurately, phytate is a mix of potassium-, magnesium- and calcium salts of phytic acid that are present as a chelate in cereals, legumes and oilseeds (Liener, 1980; Pallauf & Rimbach, 1997).

As already mentioned, PA was first reported by Pfeffer in 1872 whilst working with sub-cellular particles in wheat endosperm which contained a calcium/magnesium salt of organic phosphate. Two structures for PA were proposed. Neuberg (1908) proposed a structure that contained three cyclic pyrophosphate moieties, whereas Anderson (1914), proposed a structure in which the six hydroxyl groups of *myo*-inositol are esterified with orthophosphate moieties (Cosgrove, 1980a; Lasztity & Lasztity, 1990; Harland & Morris, 1995; Rickard & Thompson, 1997). Fifty

years later in 1969, nuclear magnetic resonance (NMR) spectroscopy confirmed that Anderson's proposed configuration was correct (Johnson & Tate, 1969). The chemical formula of PA is $C_6H_{18}O_{24}P_6$ and its structure is shown in **Figure 1.6**.

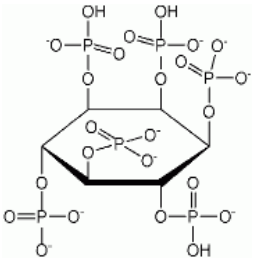


Figure 1.6. Structure of fully protonated phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis phosphate). (Source: Graf, 1986).

1.6.2 Biological activity and physiological effects

Phytic acid is regarded as an anti-nutrient because of its direct and indirect ability to bind and form complexes with nutrients altering their solubility, functionality, digestibility and absorption, which affects their bioavailability (Bilgiçli *et al*., 2006).

Phytic acid is the major form of phosphorus (P) in seeds and accumulates in other protein tissues and organs as well (Cosgrove, 1980; Raboy, 1997; Lott *et al*., 2000). One clear function for PA metabolism in these tissues is in the storage and retrieval of P and minerals during development and germination (Raboy, 2003). The PA molecule has a high P content of 282 g kg^{-1} and chelating potential to form a wide variety of insoluble salts with di- and trivalent cations (Selle *et al.,* 2000).

Phytic acid strongly interacts with proteins in a pH-dependent manner. Of the 12 proton dissociation sites on the PA molecule, six are strongly acidic with an approximate pKa value of 1.5 (**Figure 1.6**) (Lott *et al*., 2000; Maenz, 2001). Phytic acid forms electrostatic linkages with basic lysine, arginine and histidine residues resulting in neutral insoluble complexes (Maenz, 2001). Three sites of the remaining six sites are weakly acidic with pKa values of between 5.7 and 7.6. The phytate and proteins have a net negative charge that leads to their virtually complete dissociation from each other, whilst the remaining three sites are very weakly acidic,

with pKa values greater than 10, where precipitation of phytate occurs (Graf, 1986; Maenz, 2001). This leaves the molecule with several negative charges which may attract positively charged molecules and thus confers on PA a high chelation capacity for multivalent cations and proteins when the pH is conducive (Cosgrove, 1966; Selle *et al*., 2000). Chelates formed can exist either as soluble or insoluble complexes that precipitate out of solution based on pH and concentration (Cheryan *et al*., 1983; Selle *et al*., 2000; Maenz, 2001). Okubo *et al.* (1976) studied the pH range at which the glycinin component of soy proteins binds to PA and observed no binding occurred above pH 4.9, with the extent of binding increasing with decreasing pH.

Protein digestion inhibited by PA–protein interactions will vary between proteins due to differences in the total number of cationic groups available to participate in binding with PA (Omosaiye & Cheryan, 1979). These bindings render protein unavailable for absorption (Maga, 1982; Reddy *et al.,* 1982). The pH range in the stomach is therefore vital in facilitating the formation of PA–protein complexes.

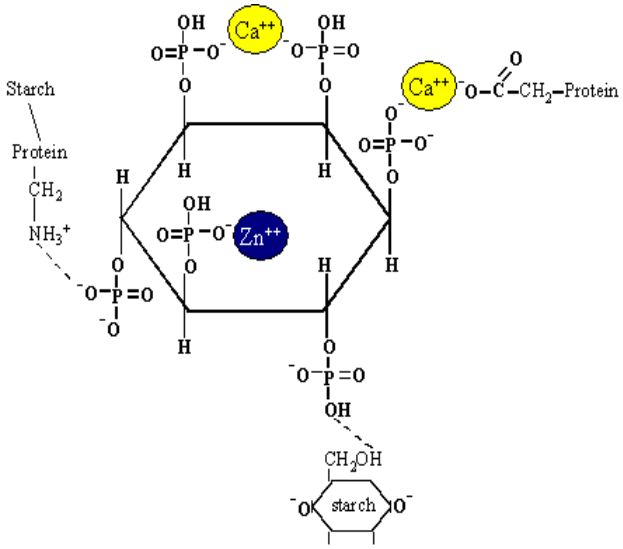


Figure 1.7. The protein, mineral and starch complexing potential of phytic acid (Source: Sutton *et. al*., 2004).

Phytic acid also acts as an anti-oxidant and is reported to suppress lipid oxidation (Graf *et al.,* 1987). Phytic acid forms an iron chelate which greatly accelerates Fe^{2+}, mediated oxygen reduction yet blocks iron-driven hydroxyl radical generation and suppresses lipid peroxidation (Graf *et al*., 1987).

There are several factors responsible for the differences in carbohydrate digestion and absorption include the anti-nutrients (phytic acid, enzyme inhibitors and tannins), the nature and amount of dietary fibre, starch, fat and protein, the extent of processing and the form in which the food is presented (Graf, 1986). Alpha amylase activity from wheat, maize, barley and peanut were suppressed by the present of PA (Sharma *et al*., 1978). The mechanism whereby PA affects starch digestion is unclear, however, PA may affect starch digestibility through its chelation of Ca which is required for the activity of amylase (Cheryan, 1980; Reddy *et al.,*1982) or through its direct binding with starch through phosphate linkages (**Figure 1.7**) (Badenhuizen, 1959).

Since the discovery of PA about 150 years ago, the bioavailability of minerals in plant products has received much attention. The capacity of PA to bind minerals reduces the digestion and absorption of phosphorus, calcium, zinc and ferum from plant-derived ingredients by fish (**Figure 1.7**) (Liener, 1980; Graf, 1983, 1986; Adeola & Sands, 2003). In animal nutrition, the interest in PA has centred around its effect on P utilisation (Selle *et al*., 2000). Formation of insoluble phytate makes both Ca and P unavailable. The low availability of P typically results in the need to add inorganic P supplements in the diets. The cost of inorganic P can be quite high (*e.g*. £365 per t; Strutt & Parker Farm Ltd, 2012), depending on the supply situation as global reserves are limited (BASF, 2012).

Phytic acid readily forms complexes with multivalent cations, with Zn^{2+} forming the most stable

complex, followed by Cu^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+} (**Figure 1.7**) (Graf, 1986). This may lower the bioavailability of minerals and there have been a number of studies that have shown interference with intestinal absorption of Zn^{2+} and Fe^{2+} by phytate (Davies & Nightingale, 1975; Morris, 1985) while numerous other studies have reported no inhibitory effects and even found enhancement of intestinal iron absorption by phytate (Wetter *et al*., 1984).

The degree of solubility of PA–mineral complexes depends on the concentration of PA, cations and the pH of the solution (Cheryan, 1980). When the concentration of divalent cations exceeds the concentration of PA, insoluble chelates of PA and minerals are formed that precipitate out of solution at neutral and basic pH (Adeola & Sands, 2003). This chelation process, therefore, is likely to have a profound influence on the efficiency of the digestive utilisation of nutrients (Adeola & Sands, 2003).

The digestive physiology of crustaceans has been reported by Ceccaldi (1990; 1997). Although the digestion process and metabolism among crustaceans varies, the main digestive enzymes that are found are similar to those in stomachless fish (Guillaume, 1997). Stomachless species, such as the cyprinids, do not secrete hydrochloric acid or pepsin for the digestion of protein (Steffens, 1989). The lack of an acid-based digestion system in *M. rosenbergii* (see Guillaume, 1997) is particularly important because the specificity of PA binding with protein and minerals is pH dependent. In most crustaceans, the foregut fluid is reported to be pH 5–7 (Dall & Moriarty, 1983).

Extensive research has been carried out on the anti-nutritive effects of PA in fish. Growth performances of commonly cultured fish species such as carp, tilapia, rainbow trout and salmon are negatively affected by the inclusion of PA containing ingredients in their diets (Spinelli *et al*., 1983; Richardson *et al*., 1985; Hossain & Jauncey, 1993; Denstadli *et al*., 2006)

and also in the marine shrimp species *L. vannamei* and *M. japonicus* (see Civera & Guillaume *et al*., 1989; Davies *et al.*, 1993). Little is known, however, about the effects of PA in freshwater crustacean species like *M. rosenbergii*.

1.6.3 The effects of phytic acid

Based on the molecular structure and the results emanating from a wide range of trials investigating the responses in various aquatic species, PA has been shown to have a strong anti-nutritive effect (Pallauf & Rimbach, 1996). The inclusions of PA in experimental diets presented to *M. rosenbergii*, therefore, are expected to:

1) Decrease the utilisation of protein

As detailed above (see **Section 1.6.2** above), the charged phosphate groups on PA can form electrostatic associations with the terminal amino groups on proteins or with the free amino groups on lysine and arginine residues within protein molecules (Cheryan, 1980). In addition, phytate–mineral–protein complexes can form with multivalent cations acting as a bridge between phosphate groups on the phytate molecule and the terminal carboxyl group of proteins and the free carboxyl groups on aspartate and glutamate residues within protein molecules (Cheryan, 1980). In theory, protein in the phytate–bound form may be less susceptible to protease activity during intestinal passage. In addition, phytate binding to proteins and minerals in the digesta has the potential to impair the activity of digestive enzymes as phytate has been shown to inhibit trypsin activity (Singh & Krikorian, 1982).

2) Decrease the utilisation of phosphorus

Phytic phosphorus represents 40–85% of the total P presented in plant sources (Cheryan, 1980; Reddy *et al*., 1989; Pallauf & Rimbach, 1997; Guillaume *et al*., 2001). Phytic phosphorus

is poorly digested by monogastric animals due to the deficiency of endogenous phytase (NRC, 1993; Harland & Morris, 1995; Jackson *et al*., 1996).

3) Decrease the utilisation of mineral elements

Undigested PA is known to decrease mineral bioavailability. In general, an inverse relationship exists between the phytate content of the diet and multivalent cations. Phytic acid effectively binds different mono-, di- and trivalent cations and their mixtures to form insoluble complexes (Reddy *et al.,* 1989). The formation of insoluble phytate-mineral complexes prevents mineral absorption, thus, reduces the bioavailability of essential minerals (Davies, 1982).

4) Increase feed cost

Phosphorus is the primary macro mineral for which dietary supply must cover the greatest part of the requirements (Guillaume *et al*., 2001). Additional supplements of highly utilised inorganic P have been added to diets because of the poor utilisation of plant source P. Phosphorus, however, is the third most expensive nutrient (behind fish meal and fish oil) that is added to feed.

5) Environment pollution

Undigested phytate–P is the primary source of P in faeces resulting in P pollution in agriculture practices (Sugiura *et al*., 1999). This is caused by excess P accumulation in water which stimulates algae and phytoplankton growth and can lead to reduced dissolved oxygen and potential P loading in lakes and rivers causing environmental pollution (Sugiura *et al.,* 1999; Cheng & Hardy, 2003).

1.6.4 Phytic acid in plant proteins

Phytic acid is an abundant plant constituent comprising 5–30 g kg^{-1} of all nuts, cereals, legumes, oilseeds and pollen (Graf & Eaton, 1990; Reddy, 2002) and serves as the storage form of P, representing 65–85% of the total P (Cheryan, 1980; Reddy *et al*., 1989). Phytic acid is found widely in eukaryotic cells (Sasakawa *et al*., 1995).

Phytic acid occurs primarily as salts of mono- and divalent cations (*e.g.* K and Mg salts in rice and Ca and Mg and K salts in soybeans) in discrete regions of cereal grains and legumes.

The site of PA in several important cereal grains and oilseeds has been reviewed in a study by Cheryan (1980), however, most is concentrated in the bran (aleurone layer, testa and pericarp) and germ of small grains whereas in legume seeds, PA accumulates in the cotyledons (Pallauf & Rimbach, 1995). In wheat and rice, PA is primarily found in the outer layer (the pericarp and the aleurone) and is absent within the endosperm (Adeola & Sands, 2003). In maize, 90% of the PA is distributed in the endosperm and concentrated in the germ (O'Dell & Boland., 1976), whilst in oilseeds and other legume seeds, PA accumulates in globoid crystals that are evenly dispersed within protein bodies (Erdman, 1979). Phytic acid in soybean meal though is closely associated with protein bodies and distributed evenly with no specific site of localisation (Adeola & Sands, 2003). Phytic acid in cottonseed and sunflower seeds are concentrated within globoids which may serve as storage sites (Adeola & Sands, 2003). The structure, form and site of PA in grains, oilseeds and legume seeds is essential as it may determine the extent of interactions with other nutrients and thus, could be a significant factor in the digestive utilisation of PA (Adeola & Sands, 2003).

Table 1.4. The estimated content of phytic acid in a range of crop cereals and legumes (Selle *et al*., 2000; Cao *et al*., 2007).

Plant protein sources	Phytic acid content (g kg^{-1})
Rapeseed meal (*Brassica napus*)	6.34 – 6.65
Cottonseed meal (*Gossypum hirsitum*)	7.72 – 9.04
Sunflower seed (*Helianthus annus*)	7.48
Soybean meal (*Glycine max*)	3.88 – 4.52
Wheat meal (*Triticum* spp.)	6.85 – 8.36
Rice bran (*Oryza sativa*)	14.17 – 15.93
Corn glutton meal (*Zea mays*)	2.67

The PA content in some potential alternative plant protein ingredients that are used in the formulation of diets presented to aquatic species are shown in **Table 1.4**. The range of PA in cereal grains is estimated to be 8.6–10.6 g kg^{-1}, in legumes it is 5.5–17 g kg^{-1}, whilst in cottonseed the range is reported to be between 10–47 g kg^{-1} (Lott *et al*., 2000).

1.7 Phytase

The use of enzymes can inactivate ANFs and enhance the nutritional values of plant-based protein in feeds by providing a natural means of transforming complex feed components into absorbable nutrients. The addition of enzymes into diets can improve nutrient utilisation, reducing feed costs and the excretion of nutrients into the environment.

Since the hydrolysis of PA is of great importance, as PA is ubiquitous in plant products, enzymes (*i.e.* phytases) capable of hydrolysing this have co-evolved (Maenz, 2001). Phytase can be found from various sources such as fungi, yeast, bacteria, plants and animals (Pallauf & Rimbach, 1997; Liu *et al*., 1998) (**Table 1.5**). Phytase is widespread in nature and fungal species are the most widely used micro-organisms in research for the expression of phytases (Liu *et al.,* 1998). Phytase produced by *Aspergillus niger*, for example, is the most extensively

studied as *A.niger* strains are among the best producers of extracellular phytase (Wodzinski & Ullah, 1996).

The efficiency of phytase in dephosphorylating PA in plant derived ingredients and thereby improving its nutrient and minerals availability for fish is well established (Jackson *et al*., 1996; Papatryphon *et al*., 1999; Sugiura *et al*., 2001; Robinson *et al*., 2002; Debnath *et al*., 2005; Yoo *et al*., 2005; Denstadli *et al*., 2007; Liebert & Portz, 2007; Cao *et al*., 2008). To date, however, no studies have been conducted looking at phytase in experimental diets and their effects when presented to *M. rosenbergii*.

1.7.1 General description and chemical structure

Phytase (*myo*-inositol hexaphosphate hydrolase) catalyses the partial or complete hydrolytic removal of PA (*myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate) to *myo*-inositol and orthophosphoric acid via *myo*-inositol penta to monophosphates (Cosgrove, 1980; Nayini & Markakis, 1986; Loewus & Murthy, 2000; Selle *et al*., 2000). The complete hydrolysis of phytate results in the production of one molecule of inositol and six molecules of inorganic phosphate (**Figure 1.8**). Two kinds of phytase have been recognised that initiate the hydrolysis of phytate at either the third carbon or sixth carbon position on the inositol ring of PA (Selle *et al*., 2000). The Enzyme Nomenclature Committee of the International Union of Biochemistry recognises these two forms of phytase as 3−phytase(s) (EC 3.1.3.8) and 6−phytase(s) (EC 3.1.3.26) (IUPAC-IUB, 1984). This classification is based on the first phosphate group that is hydrolysed by the enzyme. Generally, 3–phytases are of microbial origin and commence hydrolysis at C3 atom of the inositol ring, whereas 6–phytases are of plant origin and commence phosphate cleavage at the C6 atom of the inositol ring (Dvorakova, 1998). Both phytase eventually dephosphorylate PA completely (Nayini & Markakis, 1986). Most phytase are hitherto

characterised as monomeric enzymes, such as the fungal phytases (Ullah & Gibson, 1987; Dvorakova *et al*., 1997). *Aspergillus niger* (3–phytase, E.C.3.1.3.8), for instance, is a non-specific phosphomonoesterase, which catalyses the reaction of *myo*-inositol hexakisphosphate (**Figure 1.8**) (Cosgrove, 1980; Ullah & Phillippy, 1988).

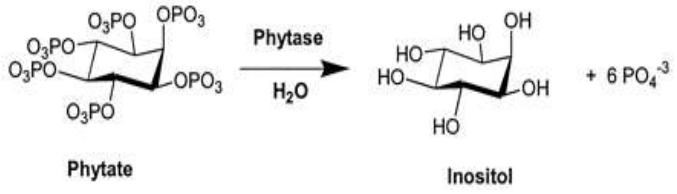


Figure 1.8. The complete hydrolysis of phytate by phytase (Source: Garrett *et al*., 2004).

The assay for phytase activity is based on the release of inorganic phosphate from the hydrolysis of sodium phytate by phytase (Engelen *et al*., 1994). The definition of one unit of phytase is the activity of phytase that generates 1 micromole of inorganic phosphorus per minute from an excess of sodium phytate at pH 5.5 and 37°C (BASF, 1993). Phytase activity is expressed as phytase units or FTU per unit of feed (FTU kg^{-1}).

1.7.2 Biological activity and physiological effects

The mechanism of action by phytase (**Figure 1.9**) is when a 3–phytase (EC.3.1.3.8) first hydrolyse phytate at the 3 position in a reaction written as:

myo-inositol hexakisphosphate + H_2O = 1D–*myo*-inositol 1, 2, 4, 5, 6-pentakisphosphate + phosphate

A 6-phytase (EC 3.1.3.26), however, hydrolyse phytate at the 6 position in a reaction as:

myo-inositol hexakisphosphate + H_2O = 1D–*myo*-inositol 1, 2, 3, 4, 5-pentakisphosphate + phosphate

There are, however, conflicting reports as to the final reaction product of phytase acting on

phytate. In theory, the inositol pentakisphosphate can rebind to the enzyme releasing a further phosphate group and the inositol tetrakis phosphate. Further sequential reactions would ultimately release inositol. A detailed investigation using NMR as to the structure of the products resulting from the phytase reaction indicates that the end product is inorganic phosphorus and *myo*-inositol monophosphate including metal divalent and trivalent ions, proteins and peptides (**Figure 1.9**) (Agranoff, 2009).

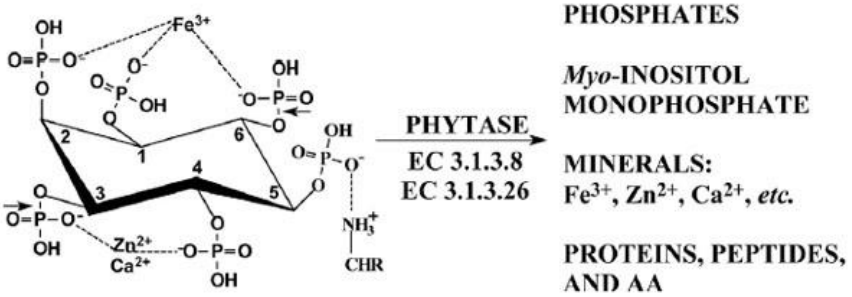


Figure 1.9. Summary of the reactions of phytic acid catalysed by 3–phytase (EC 3.1.3.8) and 6–phytase (EC3.1.3.26). The products released beside inorganic phosphorus and *myo*-inositol monophosphate include metal divalent and trivalent ions, proteins, peptides, and amino acid (NH^{3+}–CHR). The arrows indicate that the ester bond is initially attacked by the 3– and 6–phytase, respectively (Source: Agranoff, 2009).

In general, microbial derived phytases have advantages over their plant counterparts. Microbial phytases (3–phytase) exhibit considerable enzymatic activity over a wide pH range whereas plant phytases (6–phytase) are considered to be more susceptible to inactivation by gastrointestinal enzymes (Konietzny & Greiner, 2002). Phillippy (1999) reported that the phytase enzyme from *A. niger* was more stable in the presence of pepsin or pancreatic enzymes than the corresponding enzyme from wheat. Limited stability and the extended germination time involved in the purification process of plant phytase enzymes in comparison with microbial enzymes further renders the purification process from plant sources (Konietzny & Greiner, 2002). Another major problem in the purification of enzymes from plants is the

separation of the enzymes from contaminating non-specific acid phosphatases (Konietzny *et al*., 1995).

Phytase has been found to exist in monogastric animals (Bitar & Rienhold, 1972; Yang *et al*., 1991) but intestinal phytase is found to be inadequate and does not play a significant role in food-derived phytate digestion (Pallauf & Rimbach, 1997).

The optimal temperature and pH are the two most important elements in phytase activity. Microbial phytase are not thermo stable and the optimal temperature is 45 to 50°C (Kerovuo, 2000). Wyss *et al*. (1999) reported 70 to 80% of enzyme activity is lost at temperatures between 50–55°C for *A. niger.* The pH optima of phytases vary from 2.2 to 8.0 with most microbial phytases, especially those of fungal origin, with a pH optimum between 4.5 and 5.6 (Kerovuo, 2000). *Aspergillus niger* differs from other phytases as it possesses two separate pH optima, one at 2.5 and one at 5.5 (Ullah & Gibson, 1987).

Phytase has also been detected in various bacteria but the only bacteria producing extracellular phytases are those belonging to the genera *Bacillus* and *Enterobacter* whereas *E.coli* phytase is a periplasmic enzyme. In addition, some yeasts have also been shown to produce phytase species such as *Saccharomyces cerevisiae, Candida tropicalis* and *Torulopsis candida* (see Nayini & Markakis, 1986; Lambrechts *et al*., 1992). All agronomic species of cereals, legumes and oilseeds contain some phytase activity but only cereals such as wheat, rye and barley possess appreciable amounts of phytase activity (Eeckhout & De Paepe, 1994).

Table 1.5. Phytase from various sources (Source: Kerovuo, 2000).

Microbial phytase and plant source	Reference
Fungi:	
Aspergillus niger	Shieh *et al.* (1969)
Aspergillus ficuum	Gibson & Ullah (1990)
Penicillium spp.	Shieh & Ware (1968)
Yeast:	
Saccharomyces cerevisiae	Nayini & Markakis (1984)
Candida tropicalis	Lambrechts *et al.* (1992)
Torulopsis candida	Lambrechts *et al*. (1992)
Bacteria:	
Bacillus subtilis	Powar & Jagannathan (1982)
Echerichia coli	Greiner *et al*. (1993)
Pseudomonas sp.	Irving & Cosgrove (1971)
Enterobacter sp.	Yoon *et al*. (1996)
Plants:	
Maize (germinated) (*Zea mays*)	Laboure *et al*. (1993)
Soybean seeds (*Glysine max*)	Gibson & Ullah (1988)
Legume seeds	Scott (1991)
Animals:	
Rat (intestinal mucosa)	Yang *et al.* (1991)
Paramecium sp.	Freund *et al*. (1992)

1.7.3 Benefits of phytase in aquafeeds

Although phytase activity was first detected in rice bran over a century ago (Suzuki *et al.,* 1907), attempts to develop a phytase feed enzyme did not commence until 1962 in North America (Wodzinski & Ullah, 1996).

The benefits of supplementation of phytase in feed (Felix & Selvaraj, 2004) are:

1) Improved utilisation of nutrients;
2) Reduction in feed costs from the reduced additional supplementation of P in the diet;
3) Environmental improvement from the reduced excretion of waste and the threat of pollution.

The effects of phytase on nutrient utilisation:

1) Influence of phytase on protein bioavailability;
2) Improving bioavailability of P, decreasing the need for inorganic P (Harland & Morris, 1995);
3) Effects on mineral utilisation.

The supplementation of phytase to PA containing diets neutralises the negative effects of PA (Francis *et al*., 2001). The use of phytase has been shown to increase P availability in some fish (Jackson *et al.,* 1996; Hughes & Soares, 1998; Suguira *et al*., 1999) and shrimp species (Civera & Guillaume, 1989). Phytase supplemented in rainbow trout diets containing various plant proteins based ingredients, for example, significantly increased the availability of P (Riche & Brown, 1996). Atlantic salmon fed diets of soy protein concentrate incubated with phytase improved protein utilisation, apparent digestibility coefficients and body levels of Ca, Mg and Zn (Storebakken *et al.,* 1998; Vielma *et al.,* 1998).

The use of fungal phytases as feed supplements have proven effective in alleviating the negative effects of phytate in livestock diets (Konietzny & Greiner, 2002). Commercial phytases are largely sourced from *Aspergillus* sp., as they are the most prolific extracellular producers of this enzyme and generally relatively heat- and acid-stable (Wodzinski & Ullah, 1996). From a zero starting point in the early 1990s, the annual sales value of commercial supplemental phytase was estimated at US$ 50 million within the decade, representing approximately one third of the entire feed enzyme market (Sheppy, 2001). The use of phytase as a feed additive has been approved in 22 countries with the FDA (The Food and Drug Administration) approving the preparation of phytase under GRAS (Generally Regarded As Safe) (Wodzinski & Ullah, 1996). Microbial phytase sourced from *A. niger* (3–phytase), therefore, appears

appropriate for study here when included into experimental diets presented to *M. rosenbergii*.

1.8 Aims and outline of this thesis

The principle aim of this programme of research, therefore, was to obtain a greater understanding on the nutritional implications of ANFs, and in particular PA, when incorporated into experimental diets presented to the freshwater prawn, *M. rosenbergii*. To investigate this, a series of trials looking at the effect of using PA and microbial phytase on the growth, feed efficiency, digestibility and utilisation, prawn tissue proximate compositions, moult frequency and mineral composition of the commercially important freshwater prawn *M. rosenbergii* were conducted. The experimental approaches addressed in this thesis and their interconnectivity are summarised in the schematic presented in **Figure 1.10**. The objectives, however, can be briefly summarised as:

1) Chapter 3 - To investigate the long term effects of including graded levels of phytic acid into experimental diets on the growth performance, feed efficiency, apparent digestibility, nutrient utilisation and whole body proximate composition of *M. rosenbergii*;
2) Chapter 4 – To evaluate the potential use of dietary microbial phytase in experimental diets presented to *M. rosenbergii* to improve the utilisation of phytic acid;
3) Chapter 5 - To elucidate the interactive effects of graded levels of phytic acid and minerals (calcium, phosphorus, zinc, magnesium, copper, iron, manganese, potassium and sodium) in the diets of *M. rosenbergii* on moulting and the compositions determined in whole body, muscle tissue and the carapace; and,
4) Chapter 6 - To investigate the effect of dietary microbial phytase supplements presented to *M. rosenbergii* in hydrolysing phytic acid and their interaction with minerals in the whole body, muscle tissue and the carapace.

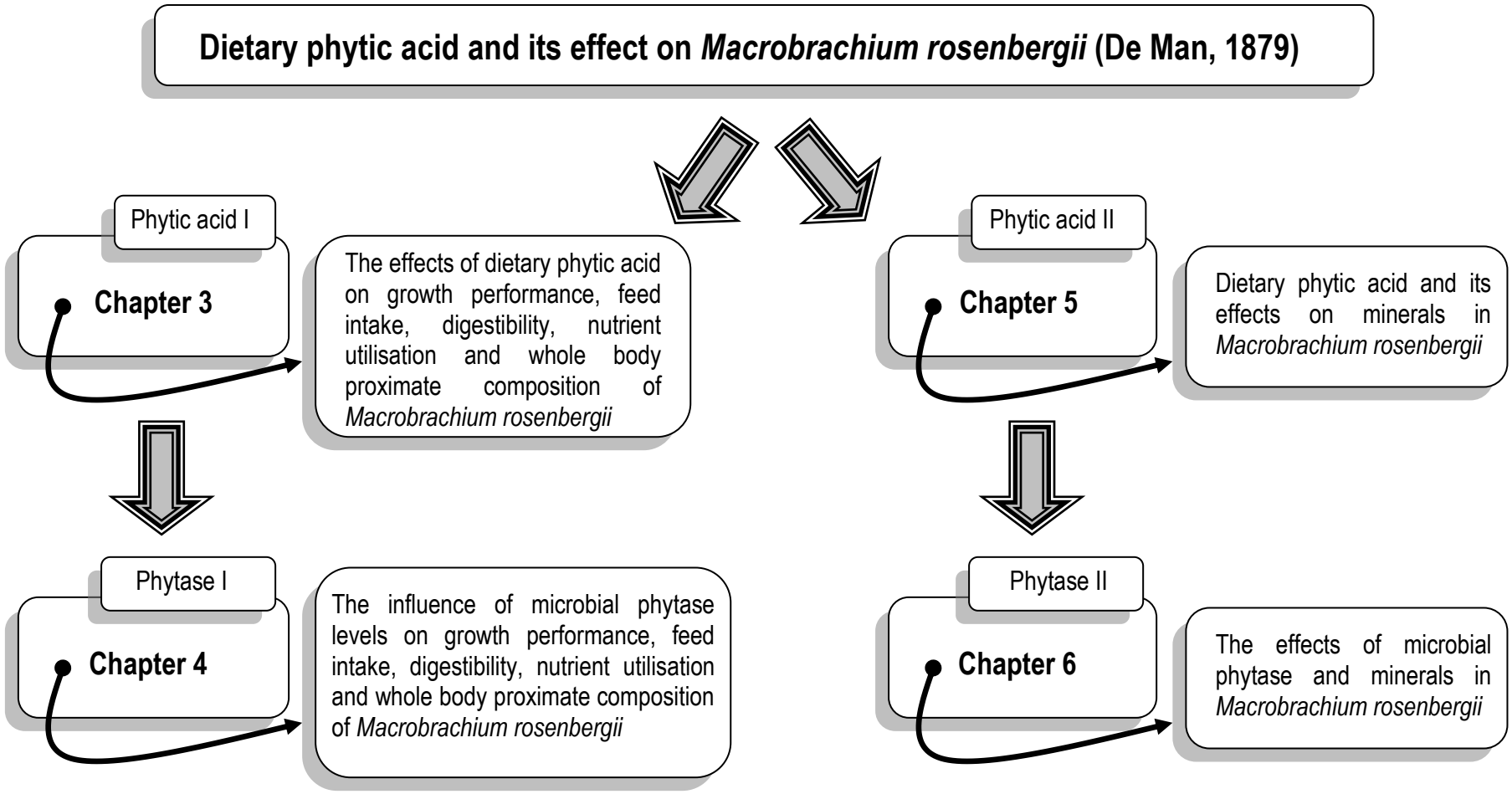


Figure 1.10. Schematic representation of the research frame.

Chapter 2 - General materials and methodology

the giant Malaysian freshwater prawn

Photograph by | rasina rasid ©

2.1 Experimental facilities

All experiments were conducted in a re-circulating water system in an indoor facility within the Prawn Unit Tropical Aquarium of the Institute of Aquaculture, University of Stirling, UK.

2.2 Experimental system

The re-circulating water system used in these study consisted of rectangular covered tanks each measuring 40 × 30 × 40 cm (l, w, d) (0.12 m^2, 26.4 L) (**Figure 2.1**). Each tank was maintained with a water column depth of 17 cm giving 20.4 L per tank at a 77.27% capacity with a constant water flow rate of 1 L min^{-1}. Tanks were aligned sequentially across two levels in a rack system in the aquarium and each tank was covered with a black plastic lid to minimise any disturbances from passing shadows.

To prevent cannibalism, individual juvenile *Macrobrachium rosenbergii* (De Man, 1879) were placed in 2 mm thick nylon mesh cylinder 18 cm tall ×10.5 cm diameter with a volume of 1.53 L. Nylon 250 µm mesh was used for the base to prevent feed from falling through the cylinder. In order to avoid waste accumulating and to facilitate the cleaning of each cylinder and tank, each cylinder was constructed with a double base unit 1.5 cm high (**Figure 2.2**). Each cylinder had its own individual lid made of the same mesh material. Each tank contained 5 mesh cylinders housing individual animals.

Each tank unit was fitted internally with an overflow stand drain pipe to maintain water level and a centrally positioned air stone to provide sufficient aeration. Water flow from all experimental tanks passed through the drain pipe into a central treatment unit fitted with mechanical and biological filtration before the water recirculated into the system. Filters were backwashed and rinsed every two weeks and approximately 10% of the water was exchanged daily with clean

fresh water to maintain water quality. Uneaten feed and faecal material were siphoned out daily. Water quality was monitored on a weekly basis before the morning feed ration by technical personnel working within the Prawn Unit to ensure the system was within acceptable limits for maintaining freshwater prawns (New & Valenti, 2000) over the entire experimental period. Parameters measured included temperature, pH, dissolved oxygen, ammonia (NH_3), nitrite (NO_2), nitrate (NO_3), general hardness and calcium hardness ($CaCO_3$). **Table 2.1** below shows the average values of the water quality parameters throughout the experimental period.

Table 2.1. Physicochemical water quality parameters that were measured during the experimental period. The recommended optimal conditions, as described by Boyd & Zimmerman (2000) are also provided.

Parameters	Water quality	Optimum
Temperature	27.5 – 30.0°C	28 - 31°C
pH	7.2	7.0 – 8.5
Dissolved oxygen	7.0 mg L^{-1}	3 - 7 mg L^{-1}
Ammonia (NH_3)	<0.25 mg L^{-1}	< .3 mg L^{-1}
Nitrite (NO_2)	<0.25 mg L^{-1}	<0.25 mg L^{-1}
Nitrate (NO_3)	<0.25 mg L^{-1}	<0.25 mg L^{-1}
General hardness	108 mg L^{-1}	>60 <160 mg L^{-1}
Calcium hardness ($CaCO_3$)	65 mg L^{-1}	30 - 150 mg L^{-1}

Temperature was maintained at 28.0 ± 0.4°C with the aid of a thermostatically controlled immersion heater. A 12 h:12 h (0800:2000) light:dark regime was maintained using a timer (Sangano, UK) and artificial light was provided from fluorescent tubes (58 W, 240 V, General Electric Hungary).

2.3 Experimental animals

The *M. rosenbergii* used in all the nutrition trials in this programme of research were originally obtained from Malaysia and Thailand and were reared and bred in the tropical aquarium and Prawn Unit of the Institute of Aquaculture, University of Stirling, UK.

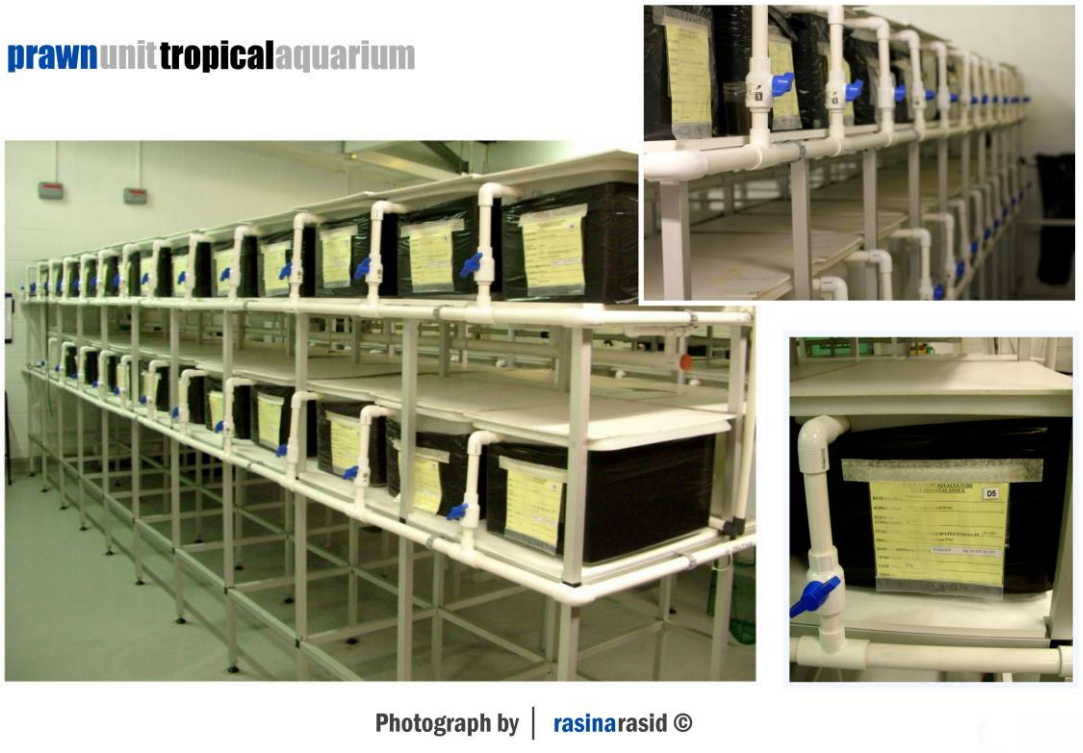


Figure 2.1. Set up system used for the experimental trials.

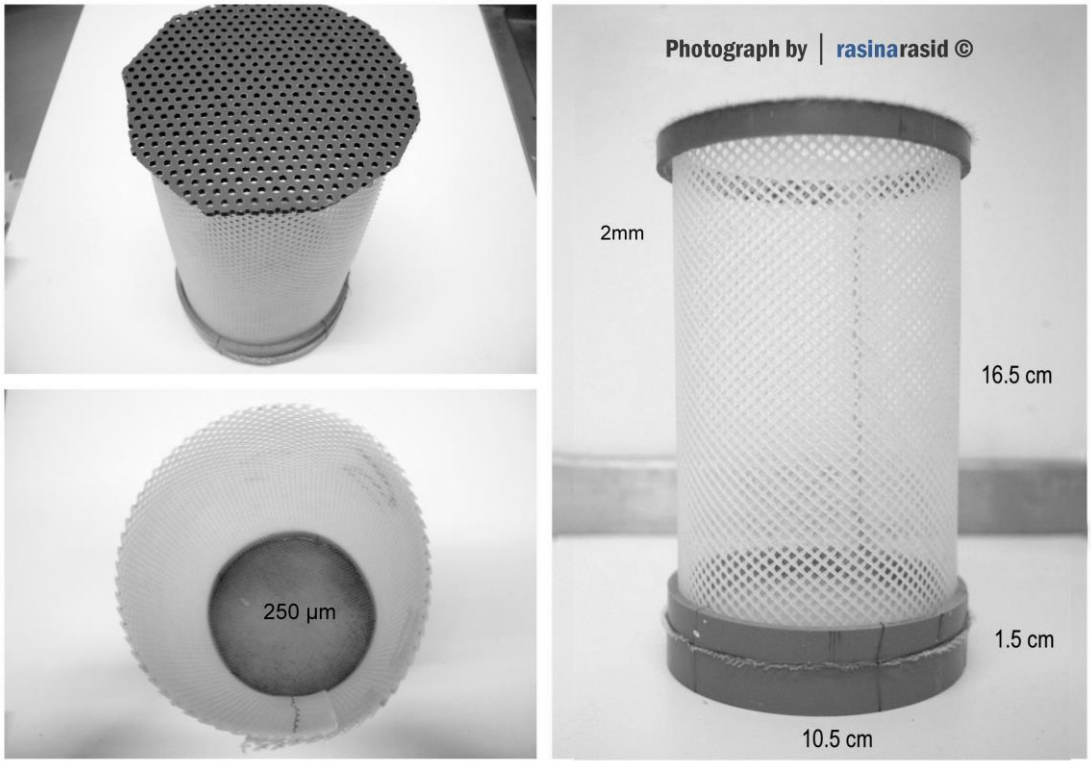


Figure 2.2. Nylon mesh cylinders used to house the individual animals used in the experiment.

Experimental animals for each trial were derived from single batches of eggs fertilised from the broodstock. When hatched, larvae were fed enriched *Artemia* until metamorphosis to post larvae (PL). Thereafter, they were transferred to two recirculating freshwater system, fibreglass tanks measuring 200 cm × 45 cm × 17 cm (l, w, d) (0.9 m^2 area, 108 L water volume) maintained at a temperature of 28 ± 0.5°C. Post larvae were fed *ad libitum* using a diet consisting of high protein 'Golden Pearls' pellets (Artemia International LLC, Texas, USA), frozen squid, fresh mussels and blended green beans until the *M. rosenbergii* reached the required size for each trial.

All the experimental animals were individually weighed and graded prior to the start of each trial in order to obtain a uniform size. Due to the large variation in the growth rate of *M. rosenbergii*, pre-selection of individual animals based on size and weight at the start of each experiment was necessary to exclude the largest and smallest animals (D'Abramo & Castell, 1997). Sex was not considered as a variable and animals were randomly selected.

2.3.1 Acclimation and sampling procedures

Experimental animals were randomly assigned to an experimental tank and to an individual mesh cylinder pot seven days prior to the start of each trial to allow the prawns to acclimate and to adapt to the research aquaria. Prior to each feeding trial the prawns were fed frozen squid, fresh mussels and blended green beans. During the acclimation period, prawns were fed the control diet (detailed in each chapter) to adjust to a pellet diet and standardised experimental conditions.

After the acclimation period, each experimental animal was weighed and the length of the carapace (CL) measured. Excess water was gently blotted with soft paper towel from each

animal which was then weighed using a top pan balance (Combics 1, Sartorius) to the nearest 0.01 g. The carapace length was measured from the posterior margin of the eye orbit to the base of the carapace using a calibrated vernier calliper to the nearest 0.1 mm. The same procedure was used throughout the entire experimental period to reduce sampling error.

At the start of each trial, several experimental animals were randomly sacrificed as initial samples as a basis for whole body proximate and mineral composition. Throughout the experimental period, prawns were sampled, weighed and the CL measured every 20 days unless stated otherwise. Data collected were used to calculate and adjust the daily food ration to approx. 10% body weight day^{-1}. Dietary treatments were randomly assigned to each of the tanks. Moulting and mortality were checked twice a day and recorded daily. Exuviae were returned to the animal to allow consumption.

On the termination of each trial, the experimental animals were starved for 24 h to allow for the evacuation of waste before prior to sacrifice and subsequent analysis. Only hard exoskeleton animals were euthanised by submerging them in ice water for 1 min. They were then plastic packed, labelled and frozen at -20°C until analysed.

2.3.2 Feeding regime

All experimental animals were hand fed individually, twice daily to apparent satiation representing approximately 10% of body weight. Diets were given at 09:00 and 16:00 h. The ration was adjusted every 20 days after sampling and the weighing of animals to avoid underfeeding or overfeeding.

2.3.3 Faecal collection

Faecal collection was performed on a daily basis during the trial period in order to conduct *in vivo* digestibility studies. Immediate pipetting (basic siphon system) was used as the most suitable method of faecal collection from prawns as the frequent handling and other methods of faecal collection have been reported to be more stressful (Merican & Shim, 1995).

All experimental trials were carried out in a recirculating freshwater system. Water flow was kept to the minimum 1 L min^{-1} to facilitate settling of faecal materials. The temperature within the experimental system was maintained at 28.0 ± 0.5°C. Tanks were covered with black plastic sheets to reduce the possible effects of stress induced by shadows cast by general husbandry operations within the research aquarium (**Figure 2.1**). Individual experimental animals were placed in 2 mm nylon mesh cylinders similar to those used in the growth trials. An additional piece of 250 µm mesh was placed under the 1 mm nylon mesh forming the base to prevent the faecal material from falling through.

Prior to the start of the trial, the experimental animals were fed experimental test diets for 7 d to allow evacuation of all previously ingested feed materials before commencing faecal collection. At the start of each faecal collection, each mesh pot was siphoned clean of overnight faeces and uneaten feed. During the collection period of 20 d, experimental animals were fed twice daily at 09:00 and 16:00. At each feeding, prawns were given 1 h to consume their ration after which uneaten diets were siphoned from each pot. Faeces were collected by siphoning the bottom of the pot as soon as possible after being excreted into the water in line with those used by others (see Merican & Shim, 1995). Faeces were collected by sieving onto filter paper. Faeces collected were then centrifuged at 2268 × g for 10 min (MSE Centaur 2, Sanyo Gallenkamp) and the supernatants were discarded. Pooled faecal materials were then stored at

-20°C until analysed.

2.4 Experimental diets

The experimental diets for each trial were prepared within the Nutrition Lab of the Institute of Aquaculture, University of Stirling. Diets were formulated to contain the required nutritional composition corresponding to levels compatible with those suggested to meet *M. rosenbergii*'s requirements (**Table 1.1**).

2.4.1 Diet formulation and preparation

The primary objective in diet formulation is to provide a nutritionally balanced mixture of ingredients to support maintenance, growth and health as well as to facilitate the manufacturing process to produce a diet with desirable physical properties (NRC, 1993). Taking this objective into account, experimental diets in this programme of research were not formulated using purified ingredients such as casein and gelatine but a production type reference diet was formulated to serve as a basis. It has been suggested that purified ingredients lack palatability and may present poor amino acid balance (NRC, 1993; De Silva & Anderson, 1995).

Ingredients such as fishmeal, soy protein concentrate, soybean meal, wheat meal and wheat gluten were used to formulate the diets. The fishmeals were supplied by Ewos UK Ltd (Bathgate, Edinburgh) while the high soy protein concentrate, fish soluble and wheat meal were provided by BioMar UK Ltd. All the ingredients were stored in a cool dry environment. The proximate composition of each ingredient is shown in **Table 2.4**.

Fish solubles is a highly fine powder of flavoured fish protein concentrate which is an excellent binder, highly digestible and is rich in dissolved proteins, water-soluble vitamins and micro

minerals (Soares *et al*., 1973). Fish solubles is a by-product that is produced from pressed liquids, *i.e*. a mixture of water and oil from processed fish, which are processed firstly by centrifugation to remove the oil component and then the water fraction is condensed and dried within a vacuum evaporator to produce a concentrated form of fish soluble concentrate (Soares *et al*., 1973; Nilsang *et al*., 2005; Augustiner, 2013). Wheat gluten is wheat protein concentrate and is a by-product from wet milling of wheat flour to remove the starch from the wheat flour and then by carefully drying the remaining high protein gluten (Yuan *et al*., 2010). Wheat gluten is commonly used in fish diets due to its high protein contents, low amounts of fibre and anti-nutritional factors, and its high apparent digestibility (Pfeffer *et al*., 1995; Sugiura *et al*., 1998; Robaina *et al*., 1999; Bonaldo *et al*., 2011).

All the dry feed ingredients were first ground to a small particle size (approximately 750 µm) in an electric hammer mill to obtain a homogenous mixture. The dry ingredients were then weighed using a Mettler PM6000 balance according to the formulation required before being placed into the bowl of a Hobart A200 Industrial Food Mixer (Hobart Co Ltd, London, England) and mixed until uniformly blended. Approximately 20–30% of the water and the fish oil needed were added slowly to the mixture with continuous stirring until a dough was formed. The mixtures were then steam pelleted using a California Pellet Mill (CL2 Model, San Francisco, California) with a 1.0 mm die. An electric fan convector heater was used to air dry the pellets at 35–40°C in a drying cabinet for 24 h. Once cooled, the pellets were then packed in labelled polythene bags and stored in a freezer at -20°C until required. Prepared diet samples were subject to proximate composition, gross energy, phosphorus and other mineral analyses to ensure that the target inclusion rates were achieved.

Yttrium oxide was used as an inert marker to allow indirect determination of digestibility and it

was included in all diets at 2.0 g kg^{-1}. Carboxymethylcellulose (Sigma C5013) was added as a binder and α-cellulose (Sigma C8002) was added as a bulking agent in the vitamin premix.

The vitamin and mineral pre-mixes were prepared according to the standards of the Nutritional Laboratory of the Institute Aquaculture and the compositions of the prepared diets are shown in **Table 2.2** and **Table 2.3** respectively.

Table 2.2. Composition of the vitamin pre-mixes used in the experimental diets (Source: Jauncey & Ross, 1982).

Vitamins	Amount (g kg^{-1})*
Vitamin A as retinol palmitate	1.00
Vitamin D as cholecalciferol	0.004
Vitamin E as tocopherol acetate	7.00
Vitamin K	1.50
Vitamin C as ascorbic acid	37.50
Vitamin B_{12} as cyanocobalamin	0.00125
Vitamin B_1 as thiamine hydrochloride	4.25
Vitamin B_2 as riboflavin	3.00
Vitamin B_6 as pyridoxine hydrochloride	1.25
Calcium pantothenate	5.25
Niacin	12.50
Biotin	0.09
Folic acid	1.00
Choline chloride	74.05
Inositol	25.00
Ethoxyquin†	0.20

†Antioxidant to prevent rancidity
*The mixture was made up to 1 kg with α-cellulose

Table 2.3. Composition of the mineral pre-mixes used in the experimental diets (Source: Jauncey & Ross, 1982).

Mineral mix	Chemical formula	g kg^{-1}
Calcium carbonate	$CaCO_3$	472.77
Calcium iodate	$CaIO_3,6H_2O$	0.30
Chromic chloride	$CrCl_3,6H_2O$	0.13
Cobalt sulphate	$CoSO_4,7H_2O$	0.48
Copper sulphate	$CuSO_4,5H_2O$	0.75
Iron sulphate	Fe $SO_4,7H_2O$	25.00
Magnesium sulphate	$MgSO_4,7H_2O$	127.50
Manganese sulphate	$MnSO_4,4H_2O$	2.54
Potassium chloride	KCl	50.00
Sodium chloride	NaCl	60.00
Zinc sulphate	$ZnSO_4,4H_2O$	5.50

Table 2.4. Biochemical composition of the ingredients used in the formulation of the experimental diets.

	Fish meal	Fish soluble	High protein soybean meal	Soy Protein Concentrate (SPC)	Wheat meal	Wheat gluten	Corn starch
Proximate composition (g kg-1, as fed)							
Dry matter	904.9	959.7	891.7	906.6	858.0	948.8	880.0
Crude protein	723.6	716.9	485.4	646.0	109.7	749.2	2.0
Crude lipid	87.4	129.0	5.8	1.10	11.0	12.2	0.0
Crude fibre	11.6	0.52	33.0	32.4	6.0	0.03	0.8
Ash	126.1	119.8	57.4	34.1	13.1	9.0	1.6
Nitrogen free extracts	-	61.8	368.0	227.1	718.2	187.3	875.6
Gross energy (kJ g^{-1})	20.7	21.2	17.94	17.99	17.2	22.8	15.9
Phosphorus	19.2	110.0	6.5	6.0	3.4	0.5	-
Phytic acid (g kg^{-1})	-	-	3.48	5.62	4.75	Traces	0.38

2.4.2 Attractant

Chemoreception is an important mechanism governing the process of food location in many aquatic animals. Earlier work on the attraction of *M. rosenbergii* to food items has shown that while the larvae do not seem to respond to waterborne stimuli, however, the post larvae actively swim towards the apparent source of the chemical attractant (Moller, 1978). The fact that several secondary metabolites produced during post mortem changes (adenosine monophosphate, AMP; inosine monophosphate, IMP; hypoxanthine, Hx; ammonia; trimethylamine, TMA; lactic acid) have proven to be effective attractants (Costa-Pierce & Laws, 1985; Harada, 1986; Harpaz *et al,.* 1987) suggests that like many crustaceans, *M. rosenbergii* are scavengers that often eat decayed and decaying materials.

A positive attractive behavioural response was elicited in about 50% of the adult *M. rosenbergii* tested with trimethylamine (TMA), betaine, arginine, glycine and the amino acid taurine (Harpaz, 1997). Kanazawa (1970) and Meyers & Zein-Eldin (1972) observed that caridean prawns exhibit a marked preference for more odoriferous pellet formulations. Using this information, and based on the occurrence of coprophagy among decapod crustaceans, Costa-Pierce (1985) reported that the incorporation of trimethylaminehydrochloride (TMAH) into a low grade feed for adult *M. rosenbergii* brought about a 30–38% increase in food consumption due to the faecal odour produced by TMAH. This compound has a faecal odour and the attraction may be partially attributed to the coprophagus nature of freshwater prawns (New & Valenti, 2000).

The attractant (TMAH), therefore, was added to all the experimental diets following the inclusion rates detailed in Costa-Pierce & Laws (1985). TMAH, an anhydrous 99% pure product (Sigma Aldrich, 243205), was diluted with distilled water to give a 15% aqueous solution. The

solution was then sprayed (misted) evenly onto dietary pellets until they required a "sheen" of moisture and had a distinct faecal odour (Costa-Pierce & Law, 1985), *i.e.* approximately 15 ml of TMAH per 100 g of pellets. Pellets were then stored in air tight plastic containers and kept cool at 4°C until they were required. Spraying of attractant was done on a weekly basis to ensure that that the diets being presented to the prawns remained fresh.

2.5 Biochemical evaluation of diets, carcasses and faeces

The chemical compositions of the dietary ingredients, experimental diets, experimental animals and faeces were determined by proximate analyses based on AOAC (1995) protocols as follows (see **Sections 2.5.1** to **2.7**).

2.5.1 Moisture

The moisture content of the diets was determined by thermal drying to a constant weight in an oven at 105°C for 24 h. Specifically, samples were ground to a powder using a mortar and a pestle or by using an electric grinder (FOSS). The samples were then placed into a pre-weighed dish and dried in the oven as described. After removing the samples from the oven, they were placed in a desiccator to cool and then were reweighed. The moisture contents of the samples were calculated as:

$$\text{Moisture \%} = \frac{\text{sample weight (g)} - \text{dried sample weight (g)}}{\text{sample weight (g)}} \times 100$$

2.5.2 Crude protein

The crude protein content of each diet was determined by the Kjeldahl method according to the AOAC (1995) protocols using a Kjeltec Autoanalyser (FOSS 2300). Triplicate samples of 200 mg were weighed into a Kjeldahl digestion tube and two mercury Kjeltabs and 5 ml

concentrated sulphuric acid were added. The tubes were then placed into a digestion block at 300ºC and left for 1 h and then left to cool inside a fume cupboard for at least 30 min. Once cool, 20 ml de-ionised water and 5 ml 40% sodium thiosulphate were added to each digestion tube and mixed thoroughly. The tubes were distilled using a Kjeltec Autoanalyser and titration values were recorded. Three urea standard tubes and three blanks were also prepared and analysed. The protein content of the samples were calculated as:

$$\text{Protein \%} = \frac{(\text{sample titre} - \text{blank titre}) \times 1750.875}{\text{sample weight (mg)}}$$

where 1750.875 is a multiplication factor to convert the titre volume to % protein based on standardised protein factor.

2.5.3 Crude lipid

The crude lipid content in each diet was determined by exhaustive Soxhlet extraction using petroleum ether on a Tecator Soxtec System (FOSS 2050). Triplicate samples (1.0 g) were weighed into thimbles and loosely plugged with cotton wool. Then, 80 ml petroleum-ether (40–60°C) was added to a pre-weighed cup with ten glass balls (2 mm). The thimbles were placed into the Soxtec system by fixing the metal adapters in the system to the magnetic rings at the bottom of each condenser and the corresponding cups were placed into the unit underneath the thimbles. Samples were boiled in the solvent for 20 min and then rinsed (80 ml petroleum ether) for 2 h. After the extraction, the solvent was evaporated off; the extraction cup was removed and placed into an oven at 105°C for at least 1 h. The extraction cup was removed from the oven and left to cool inside a desiccator before reweighing. The crude lipid contents of the samples were calculated as:

$$\text{Lipid \%} = \frac{\text{lipid weight (g)}}{\text{sample weight (g)}} \times 100$$

2.5.4 Crude fibre

Crude fibre content of each diet was determined using a FOSS fibercaps system according to the AOAC (1995) protocols. Triplicate samples (0.5–1.0 g) were weighed into fibercap capsules and each secured with a lid. The samples are then de-fatted in a series of three beakers each containing 120 ml petroleum ether (30 second) and then allowed to drain and air-dried in a fume cupboard (10 min). The samples were then submerged into 350 ml of a 1.25% sulphuric acid solution in the extraction vessel and allowed to boil (100°C) for 30 min and then removed and rinsed twice gently in fresh hot water (100°C). The samples were then submerged in 350 ml of boiling 1.25% sodium hydroxide solution (100°C) in the extraction vessel for 30 min and then rinsed using the same protocol detailed above. Once hydrolysed, the samples were dried in an oven for at least 5 h at 105°C. The capsules containing samples were then removed from the oven and allowed to cool in a dessicator before reweighing. The oven-dried samples were then placed in pre-weighed porcelain crucibles and ashed at 600°C for 4 h. Once cooled to room temperature in a dessicator, the crucibles were reweighed. The crude fibre content of the samples were calculated as:

$$\text{Fibre \%} = \frac{\text{(initial capsule weight – ash weight)}}{\text{sample weight}} \times 100$$

2.5.5 Ash

The ash content of each diet was determined as the amount of total inorganic matter left after incineration (AOAC, 1995). Approximately 1 g of sample was weighed into a pre-weighed porcelain crucible and incinerated in a muffle furnace (Gallankamp) overnight at 600°C (*i.e*. min 12 h). After ashing, the samples were then removed and cooled to room temperature in a dessicator and then reweighed. The ash content was calculated as:

$$\text{Ash \%} = \frac{\text{ash weight}}{\text{sample weight}} \times 100$$

2.5.6 Nitrogen free extractives (NFE)

Nitrogen-free extract, which here in this context refers to the carbohydrate portion, was determined by subtracting the sum of moisture, crude protein, crude lipid, crude fibre and ash from 100.

2.5.7 Gross energy

The gross energy content of each experimental diet was determined using an adiabatic bomb calorimeter (Parr 6200, USA) and benzoic acid as a standard. Triplicate samples of 1 g were weighed and moulded into a tablet using a briquette press and placed into a crucible. Nickel firing wire was fixed between the electrodes and placed in the calorimeter bomb which was then pressurised with oxygen. The water bucket of the bomb was filled with water until it weighed exactly 2812.8 g. Once the calorimeter bomb was placed in the water bucket and the electrode assembly was put in place, the bomb was then fired and the energy content of the sample was automatically determined by the calorimeter. The gross energy content of the samples were calculated as:

$$\text{Energy kJ g}^{-1} = \frac{[(\text{final temp.} - \text{initial temp.}) \times 10.82] - 0.0896}{\text{sample weight}}$$

where 10.82 is the factor of heat capacity of the system and 0.0896 represents the combined energy values (expressed in kJ) for the wire and the cotton thread used in the analysis.

2.5.8 Yttrium oxide (Y_2O_3)

Yttrium oxide (Y_2O_3) was used as a digestibility marker in the studies presented here as it can be used at low dietary concentrations and has a very low solubility under neutral conditions

(Budavari *et al*., 1989; Refstie *et al*., 1997; Austreng *et al.,* 2000). The diet and faecal samples were digested in 5 ml of 69% nitric acid using a MarsXpress microwave (CEM Corporation, USA) (for 30 min) and then allowed to cool. Once cool, the samples were diluted to 10 ml with distilled water and were prepared for analyses by inductively coupled plasma-optical emission spectrometry (ICP-OES). The yttrium oxide content of each sample was calculated as:

$$\text{Yttrium oxide } \mu g\ mg^{-1} = \frac{(\text{sample volume} / 1000)}{\text{sample weight (mg)}} \times \text{ppb result}$$

2.5.9 Mineral

The mineral content (calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc) of each experimental diet were determined using a Thermo XSeries 2 inductively coupled plasma mass spectrophotometer (ICP MS) (Thermo Scientific, USA). Triplicate samples each weighing approximately 70 mg were digested in 5 ml of 69% nitric acid for 1 h in a MarsXpress microwave (CEM Corporation, USA) and allowed to cool at room temperature. Once cool, the samples were then diluted to 10 ml with distilled water and were ready for quantification by inductively coupled plasma-optical emission spectrometry (ICP-OES).

2.5.10 Phosphorus

The phosphorus (P) content of each of sample (*i.e*. separate dietary ingredients, whole diets, whole body, muscle tissue and carapace) were determined using the protocol detailed by Allen (1989). The principle of the method is that phosphorus is converted to soluble inorganic phosphorus by digestion with nitric acid and perchloric acid by reacting with molybdate to form molybdophosphoric acid. This method is based on the formation of a heteroploy-acid complex (phosphomolybdic acid) when an acid molybdate reagent is added to orthophosphate. Reduction of this complex gives a characteristic molybdenum blue colour (Allen, 1989).

Triplicate samples of 10 mg were added into conical flasks and then 25 ml of concentrated nitric acid (65%) added to each and left at room temperature overnight for extraction (*i.e*. min. 12 h). The samples were then subjected to digestion on a hot plate in 25 ml concentrated nitric acid (65%) until boiled dried and then allowed to cool completely. Two ml perchloric acid was then slowly added to each flask before returning them to the hotplate to allow the acid to simmer. Thick fumes are produced by the process and the flasks were removed once the production of fumes ceased. Then, 25 ml of distilled water was added to each sample and these were boiled until white fumes appeared. Thereafter, 5 ml of ammonia solution (dilute 100 ml concentrated ammonia solution (S.G.0.88) to 500 ml with distilled water) was then added and the samples boiled until crystals were formed. The crystals were then dissolved by adding 20 ml of acidified water (dilute 2 ml HCl (12M) to 1 L of distilled water) and 80 ml of distilled water. Afterwards, 20 ml of a mixed reagent consisting of 250 ml sulphuric acid, 250 ml sodium molybdate, 500 ml distilled water and 2 g L^{-1} ascorbic acid were added for colour development. The samples were then left for 30 min to allow the colour to develop. The quantity of phosphorus was then determined using a spectrophotometer (Cecil Elegant Technology, UK) at an absorbance of 690 nm against a distilled water blank. A calibration curve from a series of standards (0, 5, 10, 15, 20 and 25 ml) were prepared and used to determine P in the sample aliquot. The phosphorus content of the samples were calculated as:

$$\text{Phosphorus} = P / \text{Sample weight}$$

where P was obtained from standard curve graph at y = f(x).

2.6 Phytic acid analysis

Phytic acid (PA) or total phosphorus measured as phosphorus released by phytase and alkaline phosphatase from each sample was determined using a commercially available assay

procedure (Megazyme, K-Phyt 05/07). This method involves acid extraction of inositol phosphates followed by treatment with a phytase that is specific for PA (IP_6) and the lower *myo*-inositol phosphate (*i.e*. IP_2, IP_3, IP_4, IP_5). Subsequent treatment with alkaline phosphatase ensures the release of the final phosphate from *myo*-inositol (IP_1) which is relatively resistant to the reaction of phytase. The total phosphate released was measured using a colorimetric method (Fiske & Subbarow, 1925; Lowry & Lopez, 1946).

The principle of this procedure is that phytase hydrolyses PA (phytate; *myo*-inositol hexakisphosphate) into *myo*-inositol $(phosphate)_n$ and inorganic phosphate. Alkaline phosphatase further hydrolyses *myo*-inositol $(phosphate)_n$ producing *myo*-inositol and inorganic phosphatase. Inorganic phosphatase and ammonium molybdate reacts to form 12-molybdophosphoric acid, which is subsequently reduced under acidic conditions to molybdenum blue. The amount of molybdenum blue formed in this reaction is proportional to the amount of inorganic phosphatase present in the sample and is measured by an increase in absorbance at 655 nm. Inorganic phosphatase is quantified as phosphorus from a calibration curve (CurveExpert version 1.4) generated using standards of known P concentration. The assay is specific for the measurement of P released as “available phosphorus” from PA, *myo*-inositol $(phosphatase)_n$ and monophosphate ester by phytase and alkaline phosphatase (Megazyme International, 2007).

Triplicate samples weighing approximately 1.0 g were placed into a 75 ml beaker and 20 ml of a 0.66M hydrochloric acid was added. To avoid spillage, the beaker was covered with foil and placed on a shaker overnight for extraction. After extraction, 1 ml of the extract was transferred into a 1.5 ml microfuge tube and centrifuged at 18,928 × g (RCF) for 10 min. Thereafter 0.5 ml of the resulting extract supernatant was immediately transferred to a fresh 1.5 ml microfuge

tube and 0.5 ml of a 0.75 M sodium hydroxide solution was added to neutralise the sample. The neutralised sample extract was subjected to enzymatic dephosphorylation reaction. Samples were split into two 1.5 ml microfuge tubes (free P and total P), to determine free P and total P, respectively. To each microfuge tube the following was added: 0.62 ml (0.60 ml for total P) distilled water, 0.20 ml sodium acetate buffer (25 ml, 200 mM, pH 5.5), 0.05 ml sample extract and 0.02 ml phytase (for microfuge tube total P only) accordingly. The contents in each tube were then mixed by vortex and incubated in water bath set at 40°C for 10 min. Thereafter, 0.02 ml (free P only) distilled water, 0.02 ml glycine buffer (25 ml, 400 mM, pH 10.4) and alkaline phosphatase (for microfuge tube total P only; 1.2 ml, 80 U/ml) were added to each tube before they were mixed by vortex again. The samples were then incubated in water bath set at 40°C for 15 min. The reactions were then stopped by adding 0.30 ml trichloroacetic acid (50 % w/v) and then centrifuged at 18,928 × g (RCF) for 10 min. 1 ml of sample (and standard P) is then added 0.5 ml colour reagent (1 part of ammonium molybdate (5% w/v) to 5 part of ascorbic acid (10% w/v) sulphuric acid (1M)$^{-1}$) and mixed by vortex. Next, samples were incubated in water bath set at 40°C for 1 h. Thereafter, the samples were mixed by vortex again and then transferred into 1 ml semi-micro cuvettes and the absorbance read at 655 nm. A calibration curve was prepared using phosphorus prepared at 0, 0.5, 2.5, 5.0, 7.5 µgml^{-1} (Megazyme International, 2007).

A standard curve was then plotted using CurveExpert (version 1.4) against which the phytic acid content of each sample was determined. The Δ^A phosphorus content was determined by subtracting the absorbance of the free P sample from the absorbance of the total P sample. The concentration of P is then calculated as:

$$= \frac{\text{mean M} \times 20 \times F \times \Delta^A \text{phosphorus}}{10\,000 \times 1.0 \times v}$$

where mean M is mean value of P standards; F is the dilution factor; v is sample volume.

PA content is calculated as:

Phytic acid = phosphorus concentration / 0.282

where the calculation of PA content assumes that the amount of P measured is exclusively released from PA and that comprises 28.2% of PA.

2.7 Phytase analysis

The method used to analyse the phytase content of each sample is based on the determination of inorganic orthophosphate released from the hydrolysis of sodium phytate at pH 5.5 (Engelen *et al.,* 1994).

To investigate this, a buffer solution containing 1.76 g acetic acid (100 %) dissolved in 1 L of distilled water was prepared. Then 30.02 g of $Na_2C_2H_3O_2.3H_2O$ and 0.147 g of $CaCl_2.2H_2O$ were dissolved in 900 ml distilled water and the pH adjusted to pH 5.5 with 100% acetic acid and then the final volume made up to 1 L with distilled water. A substrate solution of 8.40 g sodium phytate from rice (Sigma Aldrich P8810) was dissolved in 900 ml of the buffer solution, adjusted to pH 5.5 with acetic acid (4 mol L^{-1}) and diluted to 1 L with distilled water. A nitric acid solution was then prepared by carefully adding 70 ml nitric acid (65%) to 130 ml distilled water whilst continually stirring.

An ammonium heptomolybdate stock solution was prepared by first dissolving 100 g ammonium heptamolybdate in 900 ml distilled water, and then by adding 10 ml ammonia (25%) and then making the final volume up to 1 L with distilled water. An ammonium vanadate stock solution was prepared by first dissolving 2.35 g ammonium vanadate in 400 ml distilled water at 60°C and then, whilst continuously stirring, slowly adding 20 ml nitric acid solution (prepared

earlier, details mentioned above). The solution was then allowed to cool to room temperature before the final volume was made up to 1 L with distilled water.

A colour-stop mix was prepared by adding 250 ml of the ammonium heptamolybdate stock solution and 250 ml ammonium vanadate stock solution. Then whilst continuously stirring, 165 ml concentrated nitric acid (65 %) was added. The solution was then allowed to cool to room temperature before making it up to a final volume of 1 L with distilled water.

Preparation of standard solution: Commercial phytase (Sigma Aldrich, P1259) was used as a standard phytase solution. First, 0.2 g of phytase (Sigma Aldrich, P1259) was diluted with buffer solution (prepared earlier, mentioned above) to prepare 200 FTU ml^{-1} as a standard stock solution. Next, dilute working standards solutions labelled as solution A and B were prepared. Solution A was prepared by dilution of 0.02, 0.06, 0.1 FTU 2 ml^{-1} phytase standards in buffer solution whilst solution B was diluted to 0.04 and 0.08 FTU 2 ml^{-1} phytase standard in buffer solution. The final dilutions were prepared in duplicate (standard and blank).

Samples were weighed and diluted in duplicate (sample and blank) with buffer solutions. Samples and standard solutions were transferred into tubes and equilibrated for 5 min. Then, 4 ml substrate solutions at 37.0 ± 0.1°C were added and mixed. After 65 min, the incubation was terminated by adding 4 ml of colour-stop solution and mixed. The blanks were treated in the same way as the samples mentioned above except for the 4 ml substrate solution that was added to all blank tubes and mixed after adding the colour-stop solution. The samples, standards solutions and blanks were then centrifuged 18,151 × g (RCF) for 5 min. The absorbance of each solution was then measured at 415 nm with a spectrophotometer (Uvikon 860, Kontron Instruments) after zeroing the instrument with a distilled water blank. The corrected difference in absorbance was calculated by substracting the absorbance of the blank

from that of the corresponding standard sample. A linear calibration curve was then plotted using CurveExpert (Microsoft Corporation, Version 1.4) and the enzyme concentration of each sample determined by reading values from the graph at y = f(x). The derived enzyme activities are expressed as activity units (FTU).

2.8 Biological evaluation: growth and diet performances (*in vivo* studies)

Experimental data collected throughout the trials and the biological evaluation of the test diets, the carcasses and faeces were used to determine various biological parameters particularly growth and diet performance and nutrient utilisation. The determination of each of these is briefly discussed in the following sections.

2.8.1 Growth performance

Prior to the start and then throughout each experimental trial, the length and weight of all experimental animals were periodically determined. Growth can be expressed as the increase in length, volume or weight against time (Hartnoll, 1982). The following parameters were applied to evaluate growth performance in terms of weight and carapace length measurement in this study. The length of the carapace was measured from the posterior margin of the eye orbit to the base of the carapace with a calibrated vernier scale calliper (**Figure 2.3**).

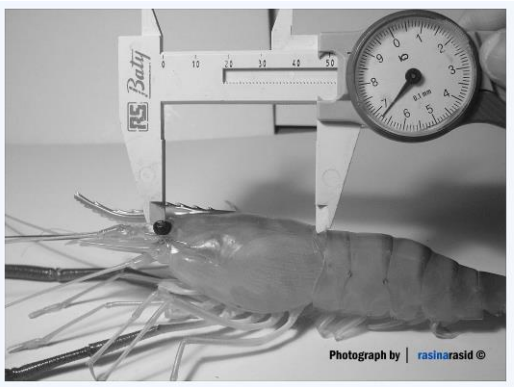


Figure 2.3. Carapace length measurement using a calibrated vernier scale calliper.

The additional performance measurements were taken:

1. Weight gain (g) = mean final body weight – mean initial body weight

2. Carapace increment (mm) = mean final carapace length – mean initial carapace length

3. Specific growth rate (SGR) (% day^{-1}) = $\frac{[\ln(\text{final weight}) - \ln(\text{initial weight})]}{\text{no. of days}} \times 100$

SGR represents the average weight change per day between any two measurement times and is based on the natural logarithm of weight.

4. Survival (%) = $\frac{\text{(final no. of prawns)}}{\text{initial no. of prawns}} \times 100$

2.8.2 Moults

Moults events were recorded on a daily basis and the exuviae from each animal was immediately returned to allow the animal to feed on them to recover components within the moult. Growth in terms of the number of moults was evaluated as follows:

1. Total number of moults = $\sum$ moults per animal per treatment
2. Average number of moults = total number of moults / number of animals per treatment
3. Moulting frequency = total number of moults / number of animals / time (days)

2.8.3 Feed utilisation

A food conversion ratios (FCR) calculates the efficiency of the diet which measures the degree of gross utilisation of food for growth. The more suitable the diet, the less food is required to produce a unit weight gain. This can be determined by:

1. Feed intake (g prawn^{-1} day^{-1}) = total feed intake per prawn / number of days
2. Feed conversion ratio (FCR) = dry feed intake / wet weight gain

The protein efficiency ratio (PER) indicates the efficiency with which protein is utilised, where

the higher the value, the more efficient protein utilisation is.

3. Protein Efficiency Ratio (PER) = wet weight gain / crude protein intake

2.8.4 Apparent digestibility coefficient

Digestibility is a relative measure of the extent by which ingested food and its nutrient components have been digested and absorbed by the animal (Guillaume *et al*., 2001) and is most commonly measured in aquatic animals by indirect methods using inert marker materials. Yttrium oxide (Y_2O_3) was selected as a digestibility marker as it can be used at low dietary concentrations and has a very low solubility under neutral conditions (Budavari *et al*., 1989; Refstie *et al*., 1997; Austreng *et al.,* 2000).

The marker concentrates in the faeces relative to the digestible material and therefore, digestibility is determined by the relative quantities of the marker in feed and faeces. Apparent crude protein digestibility (ACPD) and apparent crude lipid digestibility (ACLD) was estimated by the digestibility coefficient of both ingredients and diets using the formula for apparent digestibility coefficient (ADC) as described by Maynard & Loosli (1969):

$$\text{ADC \%} = 100 \times 1 - \left(\frac{F}{D} \times \frac{D_i}{F_i} \right)$$

where D is the concentration of the nutrient (or kJ g^{-1} for gross energy) in the diet, F is the concentration of the nutrient (or kJ g^{-1} for gross energy) in the faeces, D_i is the concentration of Y_2O_3 in the diet and F_i is the concentration of Y_2O_3 in the faeces.

2.8.5 Apparent net nutrient utilisation

Apparent net protein utilisation (ANPU) indicates the ingested protein retained by deposition in the whole body composition of prawns. Apparent net lipid utilisation (ANLU) indicates the ingested lipid retained by deposition in the whole body composition of prawns. Apparent net

energy utilisation (ANEU) indicates the ingested total energy retained by deposition in the whole body composition of prawns and is given by:

1. Apparent net protein utilisation (ANPU) % = $\frac{\text{(final prawn body protein – initial prawn body protein)}}{\text{crude protein intake}} \times 100$

2. Apparent net lipid utilisation (ANLU) % = $\frac{\text{(final prawn body lipid – initial prawn body lipid)}}{\text{crude lipid intake}} \times 100$

3. Apparent net energy utilisation (ANEU) % = $\frac{\text{(final prawn carcass energy – initial prawn carcass energy)}}{\text{gross energy intake}} \times 100$

2.8.6 Hepatosomatic index (HSI)

At the termination of each experimental trial and prior to killing the test animals, the prawns were starved for 24 h. Wet weight of the hepatopancreas of the experimental animals were evaluated in relation to the body weight (wet weight). Hepatosomatic indexes (HSI) of the samples were calculated as:

HSI % = (weight of hepatopancreas / total weight of animal) × 100

2.8.7 Whole body composition

Whole body proximate analyses were carried out to determine whole body composition following the methods as described in **Section 2.5** and the moisture, crude protein, crude lipid and ash contents and gross energy were analysed and expressed as percentage of wet weight.

2.9 Histopathology

A histological assessment of the digestive glands in *M. rosenbergii* was undertaken to investigate whether there were any differences or abnormalities in the glands of those animals receiving phytic acid in their diets. Prawns were sampled prior to the start and at the

termination of the trials where upon they were weighed, the CL measurement of each recorded before they were fixed in Davidson's fixative. Additional handling was kept to a minimum to limit the amount of handling stress that each experimental animal was subjected to prior to fixation.

2.9.1 Fixation and preparation

To ensure proper fixation in Davidson's, a fresh batch of solution was prepared on the day of sampling and stored at room temperature. Prawn tissues were fixed in a ratio of 1 part tissue (or equivalent volume there of) to approximately 10 parts fixative and then kept in fixative for 24-72 h, depending on the size of the animals fixed, to ensure proper fixation before were transferred into 70% ethyl alcohol and stored until they could be processed for wax embedding and histological sectioning.

A. Davidson fixative:

a) 330 ml 95% ethyl alcohol
b) 220 ml 100% formalin
c) 115 ml glacial acetic acid
d) 335 ml distilled water

2.9.2 Cassetting, blocking, sectioning, staining and examination

Following fixation, appropriate pieces of prawn tissue were dissected out from each animal and placed in cassettes and then placed in an autoembedder (Shandon Excelsior, Thermo) for dehydration, clearing and wax impregnation. For the dissection step, all appendages were removed and whole prawns were bisected longitudinally through the cephalothorax, just lateral to the midline, using a new scalpel blade. The trimmed samples were then placed into labelled cassettes and immersed in 70% ethanol until they were placed in the autoembedder.

After wax embedding, the pieces of wax-embedded tissues were placed in embedding moulds that were half filled with molten wax. The samples were placed into solidifying wax, carefully positioning each piece so that it was cut side down and centred before the mould was topped up with wax. Moulds were placed onto a cold plate to solidify. After 5 min, the wax blocks were removed from the stainless steel mould base and the excess wax trimmed off to form blocks that were ready to be sectioned.

The first surface layer of wax was removed to expose the surface of the tissues using a Leica 2035 Biocut microtome. The exposed surface tissues were then decalcified by placing the trimmed blocks face down in a layer of rapid decalcifying solution (RDC) for 1 h. The blocks were blotted dry after washing thoroughly under running water and cooled on the cold plate prior to sectioning. Ribbon 5 µm thick sections were floated out onto the surface of warm water and picked up on glass slides before drying in the oven at 60°C before staining.

The cut sections were then stained with Mayer's haematoxylin and eosin following standard methods, as follows;

1) Dewaxing (xylene) for 5 min
2) Alcohol I for 2 min
3) Methylated spirit for 1.5 min
4) Rinse in tap water
5) Mayer's haematoxylin for 5 min
6) Rinse in tap water
7) 3 quick dip in 1% acid alcohol
8) Rinse in tap water
9) Eosin for 5 min
10) Rinse in tap water
11) Methylated spirit for 30 sec
12) Alcohol II for 2 min

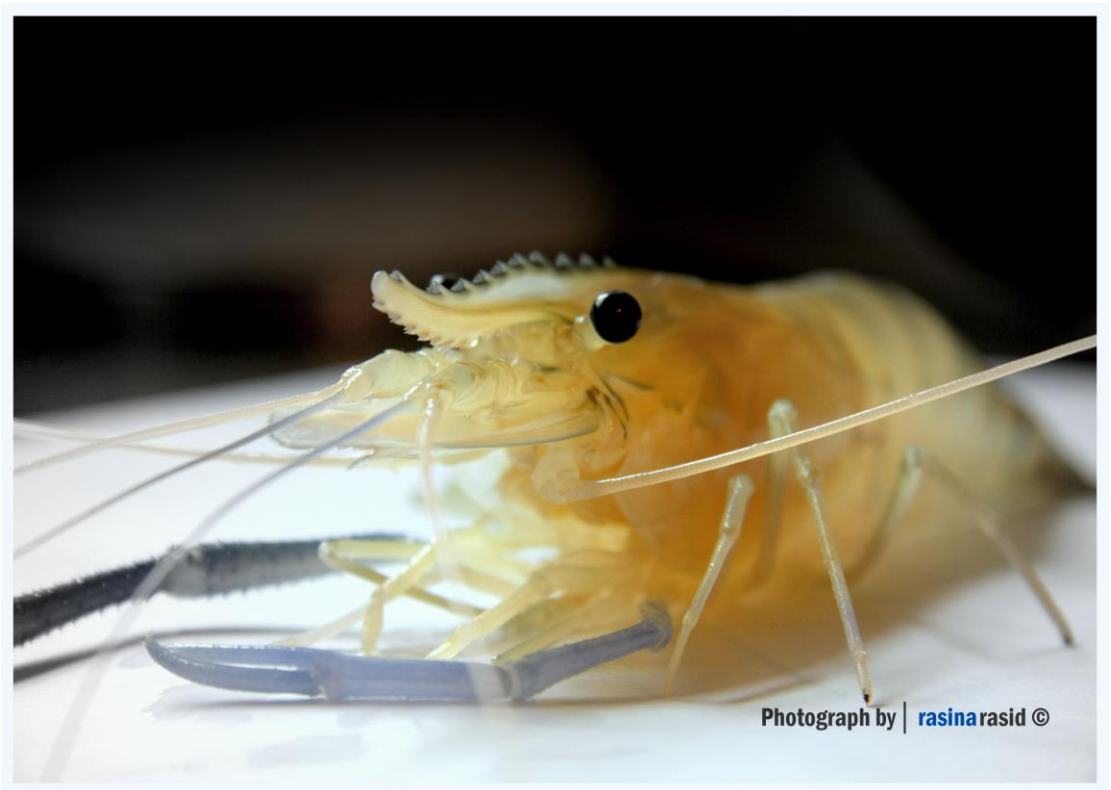
13) Alcohol I for 1.5 min
14) Clearing (xylene) for 5 min
15) Coverslip (xylene) using Pertex mounting medium (Cellpath)

The haematoxylin component stains the cell nuclei blue-black whilst the eosin counter stain demonstrates the general histological architecture (cytoplasm and muscle fibres: deep pink; collagen: light pink; eosinophil granules and red blood cells: bright orange-red). The slides were left to dry prior to examining under light microscopy at ×4 - ×100 oil immersion on an Olympus BX51 compound microscope.

2.10 Statistical analysis

Statistical analyses were performed using SPSS (version 18.0, SPSS Inc, 2010). All data are presented as means ± SD. All data were subject to normality test using Kolmogorov-Smirnov and homogeneity of variance using Levene's test. Data which were identified as non-homogeneous (Levene's test) were subjected to square root, log or arcine transformation before analysis (Zar, 1999). The main statistical hypothesis tested for this study was 'no significant difference between dietary treatment means'. Significant differences between dietary treatments means were determined by one-way ANOVA and differences were regarded as significant when $p<0.05$ (Zar, 1999). *Post-hoc* analysis (Tukey's Honest Significant Difference Test) was performed where significant differences existed between treatment means. Regression analyses were conducted using the Excel regression analyses (Microsoft Office) to examine the relationship between two variables, *i.e*. samples in each treatment group and dietary treatment inclusion. The linear fits and the comparison slopes were tested at a significance level of $p=0.05$.

Chapter 3 - The effects of dietary phytic acid on the growth, feed intake, whole body composition, digestibility and utilisation of *Macrobrachium rosenbergii*



The effects of dietary phytic acid on the growth, feed intake, body composition, digestibility and utilisation of *Macrobrachium rosenbergii*

3.1 Introduction

Over the last four decades, attention has been devoted to the possibility of increasing the inclusion of plant protein sources into the artificial diets given to aquaculture species because of the unpredictable and limited supply of fish meal (Tacon & Akiyama, 1997). The latest figures available reported a significant but declining proportion of the world fisheries production processed into fish meal (FM) and fish oil (FO), *i.e.* fishmeal production in 2011 was 19.4 M tons which had declined to 16.3 M tons in 2012 (FAO, 2014). Over the period of 1995 to 2008, for example, the volume of FM used in aquaculture diets has increased from 1.87 M tons to 3.73 M tons (FAO, 2012). The International Fish Meal and Fish Oil Organisation (IFFO, 2011) reported that the crustacean diets had the highest usage of FM, *i.e*. 30% in aquaculture compound aquafeeds (see **Figure 1.5**). Anti-nutritional factors (ANFs) such as phytic acid (PA), *i.e*. *myo*-inositol hexaphosphate within plant proteins, however, are a major impediment in the efforts towards the increased use of plant protein ingredients in aquafeeds (Storebakken *et al*., 2000).

In 1988, the First International Workshop on ANFs in Legume Seeds convened in Wageningen, The Netherlands, with subsequent workshops in 1993 and 1998. At these, methods for the chemical analysis of ANFs were discussed alongside the ANF content in legume seeds, their mode of action and their effects on the animal species to which they were fed (Poel *et al*., 1993). At a subsequent workshop in Toledo, Spain (2004), however, focus was placed on the presence, effects and inactivation of ANFs in legume seeds and other oilseeds and their effects on monogastric animals, principally broiler chickens, pigs and rats.

Studies on ANFs in aquatic farmed animals have only begun fairly recently and mostly done on

fish species and of these only very few on the effects of ANFs on freshwater prawns. One particular important ANF is PA, which is closely associated with proteins and minerals. To date, there has been no published work on the effects of PA on the freshwater prawn, *Macrobrachium rosenbergii*. The nutritional implications and interference of PA on the diets, growth and health of *M. rosenbergii*, therefore, are still not fully known.

Phytic acid is commonly found in most plant protein ingredients (Reddy, 2002). The unique structure of PA of six negatively charged phosphate groups facilitates strong binding with di- and trivalent anions (Nolan *et al*., 1987). Phytic acid interacts either directly with the positively charged group or indirectly with the positively charged group of proteins mediated by a positively charged mineral ion such as calcium (Thompson, 1993). Phytic acid can also bind with starch either directly by hydrogen bonding with the phosphate group or indirectly through the proteins to which it is associated with (Thompson, 1993). Studies have shown a reduction in protein digestibility by PA due to the formation of these complexes, which is thought to reduce the solubility and digestibility of proteins (Singh & Krikorian, 1982; Spinelli *et al*., 1983).

The review of Francis *et al.* (2001) on the effects of ANFs indicated that the growth and feed efficiency in commonly cultured fish species such as Chinook salmon, *Oncorhynchus tshawytscha*, rainbow trout, *Oncorhynchus mykiss*, and common carp, *Cyprinus carpio* were negatively affected by the inclusion of phytate containing ingredients in the diets (Spinelli *et al*., 1983; Richardson *et al*., 1985; Hossain & Jauncey, 1993). Mixed results, however, are reported on the effects of PA on the growth and feed efficiency for several other fish species including species such as channel catfish, *Ictalurus punctatus*, cobia, *Rachycentron canadum*, Nile tilapia, *Oreochromis niloticus*, and Atlantic salmon, *Salmo salar* (see Satoh *et al*., 1989; Storebakken *et al*., 2000; Riche & Garling, 2004; Sajjadi & Carter, 2004; Lin, 2006; Denstadli *et al*., 2006). Likewise, mixed results have also been reported on the effects of PA for marine

shrimp. Civera & Guillaume (1989) reported poor feed utilisation and growth rate in *Litopenaeus* [syn. *Penaeus*] *vannamei* was strongly depressed by phytic phosphorus and sodium phytate in the diet (*i.e*. 15 g kg^{-1}). Sodium phytate, however, had no detrimental effect on the growth rate or survival of *Marsupenaeus* [syn. *Penaeus*] *japonicus* (see Civera & Guillaume, 1989) and *L. vannamei* (see Davis *et al*., 1993) when incorporated into diets at higher levels of 15 to 20 g PA kg^{-1}.

The interactions between PA and protein have been discussed in a number of studies (Spinelli *et al.,* 1983; Pallauf & Rimbach, 1997). Under acidic conditions, a negative influence on the solubility of protein occurs because of the ionic binding between the basic phosphate groups by PA and protonised amino acids (De Rham & Jost, 1979; Fretzdorff *et al.,* 1995). Protein digestibility has been shown to decrease with the inclusion of 5, 10 and 26 g sodium phytate kg^{-1} in diets fed to rainbow trout (Spinelli *et al*., 1983), Atlantic salmon (Sajjadi & Carter, 2004) and Chinook salmon (Richardson *et al*., 1985). Denstadli *et al*. (2006) reported that the inclusion of 20 g PA kg^{-1} significantly reduced lipid digestibility in Atlantic salmon.

The aim of the present study was to investigate the effects of graded levels of PA included in experimental diets presented to *M. rosenbergii* on their growth performances, feed intake, apparent nutrient digestibility, nutrient utilisation and whole body chemical composition. The potential effects of ANFs on the normal structure of the digestive gland, determined by a subsequent histopathology study, were also investigated.

3.2 Materials and methodology

3.2.1 Experimental system

The experimental feed trial was conducted over a period of 140 days within the Prawn Unit, Tropical Aquarium at the Institute of Aquaculture, University of Stirling, Scotland. Twenty four

tanks (26.4 L) were used with each tank containing five prawns, four tanks per treatment, which were randomly assigned to one of the six experimental groups as described in detail in **Section 2.1**. The recirculating system was supplied with well aerated water regulated at 28.7 ± 0.4°C at a fixed flow rate of 1 L min^{-1} such that a water depth of 17 cm was maintained in all experimental tanks. Water samples from the experimental tanks were taken on a weekly basis at 09:00 am and checked to ensure that the water quality parameters were maintained within the acceptable levels for the culture of *M. rosenbergii* using mechanical and biological filters (see **Table 2.1**). All experimental tanks and prawns were subjected to a photoperiod regime of 12 h light: 12 h dark.

3.2.2 Experimental animals

Macrobrachium rosenbergii (mean initial weight of 0.29 ± 0.02 g and mean initial carapace length of 6.03 ± 0.30 mm) originating from a single batch of fertilised eggs were randomly distributed between the individual cylinder mesh pots (see **Figure 2.2**). During the 7-day acclimation period, all experimental animals were fed the control diet (PA0). Moulting events and mortalities were recorded on a daily basis. Exuviae were noted and left to allow the animal to naturally feed on throughout the experiment.

3.2.3 Sampling procedure

Prior to the distribution of the prawns to the experimental tanks, 120 animals were selected at random and taken as an initial sample. The prawns were killed by submerging them in iced water for 1 min. The samples were frozen at -20°C and kept until they could be analysed. All prawns were individually weighed and measured (carapace length) (see details in **Section 2.3.1**) every 20 days. All experimental animals were individually hand fed to apparent satiation twice a day at 09:00 am and 16:00 pm. The daily intake of feed was recorded and uneaten food

was collected by siphoning each pot after each feed and used to precisely calculate feed intake and their feed conversion ratio (FCR). Apparent digestibility was measured over the last 14 days of the growth experiment. Faecal matter was collected by siphoning twice per day and was pooled per treatment. Faeces were centrifuged (Centaur 2 Sanyo) at 2268 × g for 10 min to separate and discard the supernatant and then frozen at -20°C and kept until the samples could be analysed. At the end of the experimental period, similar to the initial samples, the prawns were starved for 24 h before they were euthanased by immersion in iced water. Only those animals with a hard exoskeleton were used for subsequent analysis, these were placed in individual bags with an identifier tag and then frozen until analysed. Histology of the prawn's digestive glands were performed as described in **Section 2.9**.

3.2.4 Experimental diets

Experimental diets were prepared within the feed processing room at the Institute of Aquaculture, University of Stirling. A reference diet based on practical ingredients was formulated to meet the nutrient requirements of *M. rosenbergii* (see **Table 1.1**; D'Abramo & New, 2000). Six experimental diets (control-PA0, PA5, PA10, PA15, PA20 and PA25) differed only in their content of sodium phytate (and corn starch), which was added to obtain nominal inclusion rates of 0, 5, 10, 15, 20 and 25 g PA kg^{-1} in the diet. Diets were formulated to provide approximately 420.0 g kg^{-1} of protein, 70.0 g kg^{-1} of lipid and 20 kJ g^{-1} total energy. Natural ingredients such as fishmeal, soy protein concentrate and wheat meal were selected as the main ingredients in the present study. Yttrium oxide (Y_2O_3) was used as an inert marker in the diets included at a rate of 2.0 g kg^{-1}. The compositions of the vitamin and mineral pre-mixes used in the experimental diets are shown in **Table 2.2** and **Table 2.3**, respectively.

The dry ingredients were ground to a powder, using a hammer mill prior to mixing them in a

A200 Hobart Ltd mixer and adding PA, fish oil and water. The mixture was steam-pelleted using a California Pellet Mill (Model CL2) with a 1.0 mm die. Pellets were dried for 24 h at 40°C and then once cooled, the diets were packed in labelled polythene bags and frozen (details provided in **Section 2.4.1**). A chemo-attractant *i.e.* a 15% trimethylamine (TMAH) aqueous solution, was then sprayed onto the dried pellets weekly prior to feeding (see **Section 2.4.2**). Formulations of the experimental diets are shown in **Table 3.1**.

3.2.5 Chemical analyses

Proximate composition analyses of moisture, crude protein, crude lipid, crude fibre and the ash contents of the experimental ingredients, diets, whole body samples and faeces were conducted according to AOAC procedures (1995). Each analytical method is described in detail in **Chapter 2**, General Materials and Methodology (see **Section 2.5**). The PA content of the ingredients and diets were analysed according to the method described in **Section 2.6**.

3.2.6 Calculations and statistical analyses

All experimental animals were weighed and measured at the beginning of the trial and then every 20 days until the end of 140 day trial producing eight sets of data in total. Growth performance and feed utilisation such as specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), protein efficiency ratio (PER), nutrient utilisation and apparent digestibility coefficient (ADC) were calculated using the formulae presented in **Section 2.8**. Statistical analyses were performed using SPSS 18 (SPSS Inc, 2010) as described in detail in **Section 2.10**. All data are presented as means ± SD (n = 4; unless stated otherwise) and were analysed by a one-way ANOVA, followed by Tukey's *post hoc* test when appropriate. Differences were regarded as significant when $p<0.05$ (Zar, 1999).

Table 3.1. Feed formulations and proximate composition (g kg^{-1}, as fed) of the experimental diets presented to *Macrobrachium rosenbergii*.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Ingredients	PA0	PA5	PA10	PA15	PA20	PA25
Fishmeal[1]	320.0	320.0	320.0	320.0	320.0	320.0
Soy protein conc.[2]	285.0	285.0	285.0	285.0	285.0	285.0
Wheat meal	210.0	210.0	210.0	210.0	210.0	210.0
Corn starch	88.0	80.2	72.4	64.6	56.8	50.0
Fish oil[3]	40.0	40.0	40.0	40.0	40.0	40.0
Vit. premix[4]	15.0	15.0	15.0	15.0	15.0	15.0
Min. premix[5]	15.0	15.0	15.0	15.0	15.0	15.0
Yttrium oxide[6]	2.0	2.0	2.0	2.0	2.0	2.0
Carboxymethyl-cellulose[7]	25.0	25.0	25.0	25.0	25.0	25.0
Phytic acid[8]	0.0	6.9	13.8	20.7	27.6	34.5
Proximate composition (g kg^{-1}, as fed)						
Dry matter	908.5	908.3	908.1	908.4	907.5	907.4
Crude protein	429.4	426.7	425.9	428.8	427.3	428.5
Crude lipid	69.8	70.0	70.5	71.9	72.0	72.2
Crude fibre	20.6	20.4	19.5	20.1	18.8	19.0
Ash	68.5	69.2	69.7	70.2	70.7	71.1
NFE[9]	320.6	320.7	319.7	312.5	311.5	307.3
Gross energy[10]	19.7	19.6	19.6	19.6	19.5	19.0
Phytic acid	0.26	6.48	11.28	16.53	21.45	26.16

[1]Ewos Ltd; [2]Soy protein concentrate, BioMar UK Ltd; [3]Herring oil; [4]Vitamin premix (**Table 2.2**); [5]Mineral premix (**Table 2.3**); [6]Sigma Aldrich, 205168; [7]Sigma Aldrich, C5013; [8]Phytic acid sodium salt hydrate from rice, Sigma Aldrich, P8810; [9]Nitrogen free extract; [10](kJ g^{-1}).

3.3 Results

3.3.1 Chemical composition of diets

Five experimental diets termed PA5, PA10, PA15, PA20 and PA25, which indicate the amount of PA (g kg^{-1}) included in each diet, were assessed against a control, *i.e.* PA0, which had no

additional PA added (*i.e*. 0 g kg^{-1}). The analysed proximate compositions of the experimental diets are shown in **Table 3.1**. The content of the main nutrients were consistent among diets with approximately 908.0 g kg^{-1} dry matter, 427.8 g kg^{-1} crude protein, 70.9 g kg^{-1} crude lipid and 19.5 kJ g^{-1} gross energy. The inclusion of PA did not cause significant changes to the dry matter, crude protein, crude lipid, crude fibre and energy contents of the test diets. The graded inclusions of PA did, however, result in consistent gradual increases in the ash and lipid content of the diets from 68.5 g kg^{-1} to 71.1 g kg^{-1} of ash and 69.8 g kg^{-1} to 72.2 g kg^{-1} of lipid. The NFE values decreased slightly with increasing PA inclusions from 320.6 g kg^{-1} to 307.3 g kg^{-1}. The dietary PA content of the six experimental diets when analysed were slightly higher than the nominal values and were determined to be 0.26, 6.48, 11.28, 16.53, 21.45 and 26.16 g PA kg^{-1}, respectively.

3.3.2 Growth performance

The growth performance in terms of carapace length increase and weight gain, average daily weight gain and specific growth rate (SGR) of the *M. rosenbergii* fed the diets over the 140 day trial period is shown in **Table 3.2**. There was a rapid increase in the length of the carapace and weight gain over the first 40 days in all test groups except for the highest treatment (*i.e.* PA25, 26.16 g PA kg^{-1}; **Figure 3.1**; **Figure 3.2**). This growth pattern was maintained for the next 100 days. In general, the dietary inclusion of PA up to 26.16 g kg^{-1} had no significant effects on the growth of *M. rosenbergii* when compared to the controls. Specifically, the final carapace length increase varied from 12.00 to 14.72 mm and final weight gain between 5.44 to 6.53 g. The highest increase and weight gain was recorded for the dietary treatment containing 16.53 g PA kg^{-1}. SGR did not differ significantly between treatment groups and control with overall range from 2.12–2.23. Dietary treatments containing PA did not show any significant effect on the hepatosomatic index (HSI), which ranged from 3.30–4.34%. The lowest HSI was observed in

those test animals receiving the diet containing the highest PA inclusion (*i.e.* nominally PA25, actually 26.16 g PA kg^{-1}). Survival was relatively high at 85.0–95.0% regardless of dietary PA inclusion.

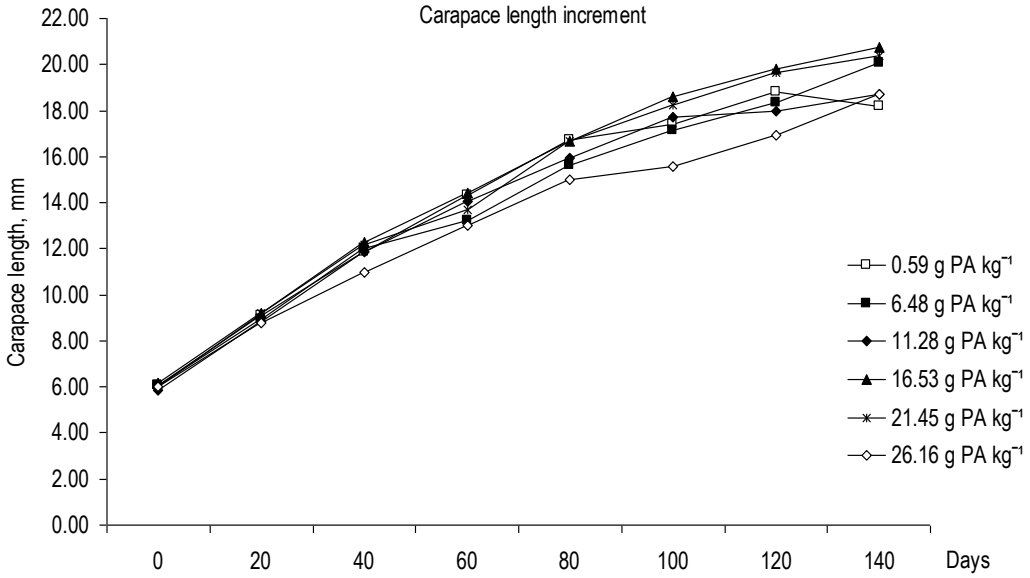


Figure 3.1. Increase in the length of the carapace of the *Macrobrachium rosenbergii* fed the experimental diets containing graded inclusion levels of phytic acid (0–26.16 g PA kg^{-1}) over 140 days. Given that the differences in the growth curves at each point are not significant, no error bars are shown for clarity.

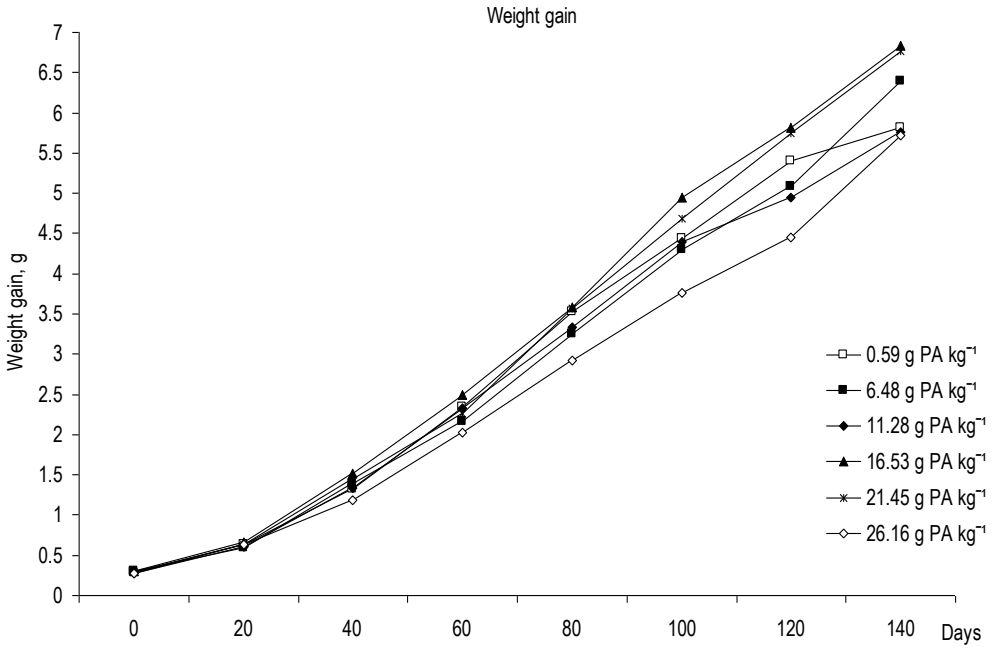


Figure 3.2. Weight gained by *Macrobrachium rosenbergii* fed the experimental diets containing graded inclusion levels of phytic acid (0–26.16 g PA kg^{-1}) over 140 days. No error bars are shown for clarity as differences in the growth curves at each point are not significant.

3.3.3 Feed efficiency

The feed efficiency, *i.e*. feed intake (FI), feed conversion ration (FCR) and protein efficiency ratio (PER), is shown in **Table 3.2**. No significant overall effects and interactions of dietary PA level on FI and FCR were identified. FI ranged from 0.054 g prawn^{-1} day^{-1} to 0.068 g prawn^{-1} day^{-1} and FCR varied from 1.39 to 1.57. One-way ANOVA comparing the different dietary treatments in each sample point showed no significant effects and interactions of dietary PA and PER, which varied from 1.25 to 1.52.

3.3.4 Apparent nutrient utilisation

The apparent nutrient utilisation of protein (ANPU), lipid (ANLU) and energy (ANEU) of the *M. rosenbergii* fed the experimental diets with graded inclusion levels of PA for 140 days are shown in **Table 3.3**. Increasing dietary PA inclusions significantly decreased the nutrient utilisation of protein, lipid and energy. Specifically, increasing dietary PA from 0.26 g PA kg^{-1} to 26.16 g PA kg^{-1} in the diet significantly reduced (p<0.05) protein utilisation from 13.53% to 4.64%. Prawns fed diets containing high levels of dietary PA at 21.45–26.16 g PA kg^{-1} demonstrate significant lower ANPU and ANLU values compared to prawns in the other groups and those in the controls. Similar trends were found for ANLU with a decrease from 4.25% to −27.27%. Prawns fed the PA enriched experimental diets resulted in drastically lower ANLU values where negative values indicate lower levels of lipid that were retained in the carcass. Likewise, energy utilisation showed a similar trend, where an increasing inclusion of dietary PA decreased ANEU from 13.51 to −2.74 kJ kg^{-1}. A dietary inclusion rate of 26.16 g PA kg^{-1} resulted in the lowest, negative values of ANEU.

3.3.5 Apparent digestibility coefficient

The apparent digestibility coefficients of dry matter (ADDM), crude protein (ADCP) and crude

lipid (ADCL) of the *M. rosenbergii* fed the experimental diets for 140 days are shown in **Table 3.4**. The increase in dietary PA levels from 0.26 g PA kg^{-1} to 26.16 g PA kg^{-1} decreased ADDM from 82.37% to 69.09%. An interaction between PA levels and ADCP were also observed where ADCP decreased from 66.54% to 45.24% with increasing inclusions of PA of 0.26 g PA kg^{-1} to 26.16 g PA kg^{-1}. Equally, ADCL decreased with increasing inclusion levels of PA of 0.26 g PA kg^{-1} to 26.16 g PA kg^{-1} from 93.10% to 40.99%.

3.3.6 Whole body proximate composition

The whole body proximate compositions of the *M. rosenbergii* at the start and at the end of the 140 day feeding trial are presented in **Table 3.5**. The initial composition of the carcass was 79.13% moisture, 12.39% protein, 0.51% lipid, 2.10% ash and 3.49 kJ g^{-1} gross energy. After 140 days being fed on the dietary treatments, moisture, protein, lipid, ash and gross energy content in the carcass varied between 73.55–76.05%, 14.21–17.35%, 0.07–0.66%, 2.21–6.09% and 3.95–4.67 kJ g^{-1}, respectively. At the end of the trial, statistical analysis using ANOVA confirmed that there were significant effects on the protein, lipid, gross energy and ash content between the treatment groups while the moisture content was not affected by the varied inclusion rates of PA. Specifically, the protein content of the prawn receiving the experimental diet containing 26.16 g PA kg^{-1} were significantly affected ($p<0.04$), with lower protein contents than those prawns receiving the diet containing 6.48 g PA kg^{-1}. Likewise, the lipid content of the prawns receiving between 16.53 to 26.16 g PA kg^{-1} in their diets decreased significantly ($p<0.01$) and had lower levels of lipid than the prawns in the other test and control groups. Prawns fed diets containing high levels of dietary PA at 21.45–26.16 g PA kg^{-1} demonstrated significant lower ($p<0.05$) gross energy content in the final carcass. In a contrast to these results, the ash content of the whole body was found to increase with an increasing inclusion of PA. Prawns fed the diet containing 16.53 to 26.16 g PA kg^{-1} had a significantly

higher ash content (p<0.01) than those prawns fed the diets containing between 6.48 to 11.28 g PA kg^{-1}.

3.3.7 Histology

Histological examination of the digestive gland of the experimental *M. rosenbergii* at the end of the trial indicated functional healthy organs with no apparent deviations to normal tissue when compared between the control and other treatment groups.

Table 3.2. Growth performance and feed efficiency of the experimental *Macrobrachium rosenbergii* fed graded levels of phytic acid in their diets for 140 days.

	Dietary phytic acid (g kg^{-1})					
	0.26 (Control)	6.48	11.28	16.53	21.45	26.16
Initial carapace length (mm)	6.17 ± 0.32	6.20 ± 0.13	5.70 ± 0.40	6.04 ± 0.11	6.12 ± 0.30	5.95 ± 0.27
Final carapace length (mm)	18.17 ± 2.56	20.07 ± 2.12	18.71 ± 2.40	20.76 ± 2.33	20.38 ± 1.74	18.73 ± 3.00
Carapace length increase (mm)	12.00 ± 2.65	13.87 ± 2.09	13.01 ± 2.59	14.72 ± 2.32	14.26 ± 1.51	12.79 ± 3.20
Initial weight (g)	0.30 ± 0.02	0.30 ± 0.03	0.28 ± 0.03	0.31 ± 0.01	0.30 ± 0.02	0.28 ± 0.02
Final weight (g)	5.81 ± 1.07	6.40 ± 1.01	5.76 ± 1.49	6.84 ± 0.84	6.77 ± 1.11	5.72 ± 1.24
Weight gain (g)	5.52 ± 1.07	6.10 ± 1.03	5.48 ± 1.48	6.53 ± 0.84	6.48 ± 1.12	5.44 ± 1.24
Av. daily weight gain	0.039 ± 0.01	0.044 ± 0.01	0.039 ± 0.01	0.047 ± 0.01	0.046 ± 0.01	0.039 ± 0.01
Specific growth rate (% day^{-1})	2.12 ± 0.16	2.18 ± 0.17	2.14 ± 0.20	2.21 ± 0.09	2.23 ± 0.15	2.14 ± 0.16
Feed intake (g $prawn^{-1}$ day^{-1})	0.060 ± 0.00	0.061 ± 0.01	0.057 ± 0.01	0.068 ± 0.01	0.066 ± 0.01	0.054 ± 0.01
Feed conversion ratio	1.57 ± 0.29	1.40 ± 0.05	1.48 ± 0.16	1.48 ± 0.18	1.42 ± 0.15	1.39 ± 0.28
Protein efficiency ratio	1.29 ± 0.25	1.42 ± 0.24	1.27 ± 0.34	1.52 ± 0.20	1.50 ± 0.26	1.25 ± 0.29
Hepatosomatic index (HSI) (%)	4.16 ± 0.37	3.35 ± 0.18	3.96 ± 0.17	4.34 ± 0.71	4.18 ± 0.42	3.30 ± 0.20
Survival (%)	85.0 ± 0.50	95.0 ± 0.50	90.0 ± 0.60	95.0 ± 0.50	95.0 ± 0.50	90.0 ± 0.60

Average daily weight gain (g $prawn^{-1}$).
Values are the means ± SD of four replicates.
Statistical analysis found no significant differences between the different diets for any of the measured parameters.

Table 3.3. Apparent nutrient utilisations (%) in the *Macrobrachium rosenbergii* fed the experimental diets for a period of 140 days.

	Dietary phytic acid (g kg^{-1})					
	0.26 (Control)	6.48	11.28	16.53	21.45	26.16
Protein utilisation (ANPU)	13.53 ± 1.22^{a}	13.00 ± 1.43^{a}	11.77 ± 0.77^{a}	11.47 ± 0.92^{a}	6.98 ± 3.22^{b}	4.64 ± 1.99^{b}
Lipid utilisation (ANLU)	4.25 ± 0.93^{a}	3.53 ± 1.57^{a}	2.43 ± 0.90^{a}	−14.77 ± 4.61^{b}	−21.25 ± 1.26^{bc}	−27.27 ± 3.60^{c}
Energy utilisation (ANEU)	10.30 ± 0.72^{a}	13.51 ± 0.04^{b}	11.35 ± 0.66^{ab}	10.45 ± 0.72^{ab}	4.05 ± 0.07^{c}	−2.74 ± 0.96^{d}

Negative values indicate lower values of retained nutrient in the carcass when compared to the initial sample of *M. rosenbergii* at T0.
Values are the means ± SD of three replicates.
Values within the same row with different letters are significantly different (p<0.05).

Table 3.4. Apparent nutrient digestibility (%) in the *Macrobrachium rosenbergii* fed the experimental diets for 140 days.

	Dietary phytic acid (g kg^{-1})					
	0.26 (Control)	6.48	11.28	16.53	21.45	26.16
Dry matter (ADDM)	82.37	74.49	74.30	74.90	72.48	69.09
Crude protein (ADCP)	66.54	67.56	54.47	47.19	46.89	45.27
Crude lipid (ADCL)	93.10	91.25	87.32	57.19	49.58	40.99

Values are derived from a single sample and therefore, no statistical analysis was possible.

Table 3.5. Whole body proximate composition (% wet weight) of the *Macrobrachium rosenbergii* fed graded levels of phytic acid in the experimental diets presented to them over a period of 140 days.

		Dietary phytic acid (g kg^{-1})					
	Initial[1]	0.26 (Control)	6.48	11.28	16.53	21.45	26.16
Moisture content	79.13 ± 0.61	74.05 ± 2.26	73.55 ± 1.88	74.05 ± 1.31	75.79 ± 2.18	74.40 ± 0.45	76.05 ± 1.59
Crude protein	12.39 ± 1.03	16.98 ± 1.82^{a}	17.35 ± 1.32^{a}	16.42 ± 0.63^{ab}	15.30 ± 0.99^{ab}	15.61 ± 0.10^{ab}	14.21 ± 0.81^{b}
Crude lipid	0.51 ± 0.03	0.66 ± 0.04^{a}	0.61 ± 0.01^{a}	0.63 ± 0.08^{a}	0.31 ± 0.07^{b}	0.17 ± 0.03^{bc}	0.07 ± 0.07^{c}
Ash	2.10 ± 0.15	2.21 ± 0.48^{a}	2.37 ± 0.22^{a}	3.92 ± 0.69^{b}	5.01 ± 0.37^{c}	5.42 ± 0.12^{c}	6.09 ± 0.35^{c}
Gross energy (kJ g^{-1})	3.94 ± 0.30	4.67 ± 0.43^{a}	4.40 ± 0.24^{b}	4.54 ± 0.45^{abc}	4.61 ± 0.30^{ac}	3.97 ± 0.11^{d}	3.95 ± 0.03^{e}

Values are the means ± SD of three replicates.

Values within the same row with different letters are significantly different (p<0.05). For parameters where there were no significant differences no superscript values are provided.

[1]Values not included in the one-way Anova.

3.4 Discussion

The interest in finding sustainable alternatives to the use of fish meal in diets fed to aquatic species has been growing rapidly in recent years leading to investigations regarding the utility of proteins derived from plant sources. Greater understanding of their contents, and in particular anti-nutrients, is necessary before a substitute can be regarded as a successful alternative. The present study, therefore, aimed to investigate the effect of including various levels of PA within the experimental diets presented to *M. rosenbergii* and the potential impacts on growth performance, feed and nutrient utilisation as well as on digestibility. In addition to investigating the latter, there was also an interest in looking at the resultant changes in whole body proximate composition as a consequence of including different levels of PA within the diets.

3.4.1 Growth performance

The present study indicates that juvenile *M. rosenbergii* are able to tolerate up to 26.16 g PA kg^{-1} in their diet without adverse effects on growth, survival or on SGR and HSI. In the first 40 days of the trial, the *M. rosenbergii* in all but the highest treatment group (*i.e*. PA25, 26.16 g PA kg^{-1}) showed a rapid growth rate based on increase in the length of the carapace and weight gain (**Figure 3.1**, **Figure 3.2**). This growth pattern was maintained for the subsequent 100 days of the trial. The lowest HSI was also found in the test group receiving the highest level of PA (*i.e*. 26.16 g PA kg^{-1}). These results suggest that, although *M. rosenbergii* are able to tolerate high levels of PA, growth rates were slower when fed 26.16 g PA kg^{-1} in their diet. In terms of the effects on growth, the results of various earlier studies regarding the effects of PA are to some extent, controversial. Civera & Guillaume (1989) working with juvenile *L. vannamei* found poor growth when 15 g kg^{-1} sodium phytate was added to their diets for 28 d, whereas the

inclusion of up to 20 g kg^{-1} had no apparent detrimental effect on the growth performances or survival of juvenile *M. japonicus*. In comparison to studies previously performed on fish species, rainbow trout fed purified diets containing casein and gelatine with an inclusion of 5 g PA kg^{-1} for 150 d were found to have a reduced growth rate (Spinelli *et al*., 1983). Depressed growth was also reported for common carp, Chinook salmon and Atlantic salmon when fed 15 g kg^{-1} sodium phytate for 105 d, 28.5 g PA kg^{-1} for 56 d, and 20.7 g kg^{-1} of sodium phytate for 80 d, respectively (Richardson *et al*., 1985; Hossain & Jauncey, 1993; Denstadli *et al*., 2006). It is not surprising that the findings of this study suggest that *M. rosenbergii* used in the current trials are able to tolerate higher levels of PA than are suggested for marine shrimp, *i.e. L. vannamei*, and fish species considering that the natural diets of freshwater species may contain components from terrestrial sources. Hence, it is possible that freshwater species have a greater ability to tolerate and to adapt to higher levels of anti-nutrients, such as PA in this case.

Nonetheless, the possibility of successful replacement of fishmeal with high plant protein based diet in *M. rosenbergii* is still controversial. Du and Niu (2003) conducted a study on *M. rosenbergii* fed diets where 0, 20, 50, 75 and 100% of fishmeal is replaced by soybean meal in tank water and concluded that soybean meal, without supplementation of amino acids or other additives, is not suitable as a major protein source in *M. rosenbergii* diets. Successful replacement of fishmeal with plant protein is, however, feasible in a semi-intensive pond culture with contribution of natural productivity from the pond as has been noted by Tidwell *et al*. (1995) and by Tacon & Akiyama (1997). Tidwell *et al*. (1995) reported that *M. rosenbergii* are able to adjust to reductions in the nutritional value of prepared diets, *i.e*. protein source, vitamin and mineral content, by increasing predation on natural fauna, *i.e*. macro-invertebrates, in the pond. This was also supported by an earlier study by Weidenbach (1980) who reported that *M. rosenbergii* are able to adjust to the absence of feed pellets by increasing consumption of

available vegetation. A better understanding of the role of natural productivity to satisfy nutrient deficiencies particularly, when using high plant protein diets containing high anti-nutrient level, to decrease production cost is needed.

In general, the growth rate (weight gain; **Figure 3.2**) was relatively low for all the treatment groups including the controls when compared to stock in the normal growout phase within a semi-intensive pond (Tidwell & Abramo, 2000). The growth rates are, however, comparable with those documented by Brown *et al*. (1991) where the initial weights of the *M. rosenbergii* were given as 0.25–0.35 g and then after five moult cycles (~30 days), their final weight was 0.29–0.61 g. This is probably a result of the laboratory conditions under which they were reared which could have imposed a level of stress upon the animals (Briggs, 1991). In addition, due to the natural behaviour of *M. rosenbergii*, the experimental animals were individually reared to prevent aggression, cannibalism, dominance and territorialism, which could have restricted normal growth conditions and biased the results.

3.4.2 Feed efficiency

Diets were formulated to contain the optimum required nutritional composition corresponding to levels compatible and recommended to meet the requirements of *M. rosenbergii*. In the present study, the protein sources that were used were fishmeal and plant protein (soy protein concentrate and wheat meal) used in a ratio of 1:1.5. The high concentration levels of protein in the diets possibly allowed sufficient valuable protein to be available for digestion and for metabolic purposes without causing significant deleterious effects. If there is lower protein level in a diet, then PA may complex with high quality dietary protein thereby reducing its availability.

Civera & Guillaume (1989) reported that the feed consumption of *M. japonicus* and *L. vannamei* seemed to diminish markedly and demonstrated poor feed utilisation when fed diets

containing sodium phytate (15–20 g kg^{-1}). The results from previous studies regarding the effects of PA on feed efficiency by fish, however, are contentious. For example, rainbow trout fed 5 g PA kg^{-1} exhibited reduced feed conversion whereas Nile tilapia fed diets supplemented with high levels of PA of up to 25.8 g PA kg^{-1} had no significant effects on feed efficiency (Spinelli *et al*., 1983; Riche & Garling, 2004). The FCRs of juvenile cobia fed diets containing up to 20 g PA kg^{-1} were also unaffected (Lin, 2006). On the other hand, diets containing between 4.7 g PA kg^{-1} and 10 g PA kg^{-1} were reported as the maximum level that Atlantic salmon could tolerate within their normal diet (Sajjadi & Carter, 2004; Denstadli *et al*., 2006). Similarly, a number of studies reported that feed efficiency was reduced when common carp, channel catfish and Chinook salmon were fed diets containing 10, 22 and 25.8 g PA kg^{-1}, respectively (Richardson *et al*., 1985; Satoh *et al*., 1988; Hossain & Jauncey, 1993). In the present study, feed utilisations did not indicate any apparent adaptation to PA during the experimental period. The feed intake pattern was more or less maintained for all groups. Whether or not the lack of effect of PA on feed intake is attributable to palatability or physiological mechanisms remains to be elucidated.

A study by Richardson *et al*. (1985) reported that an inclusion of 25.8 g PA kg^{-1} significantly depressed PER in Chinook salmon. In a marked contrast to this though, the study by Lin (2006) reported that an inclusion of up to 20 g PA kg^{-1} did not significantly affect PER in juvenile cobia. The finding of the present study is in agreement with the latter study. Despite the high inclusion of PA of up to 26.16 g PA kg^{-1}, minor differences were observed for PER in *M. rosenbergii*. There was no clear dose-response relationship between PA levels and the PER, however, the highest inclusion, *i.e.* 26.16 g PA kg^{-1}, resulted in the lowest protein efficiency.

It is also essential to note that the source of protein and therefore the variable inclusion of PA in various diets, as highlighted by Teskeredźić *et al*. (1995), may play a role in the diversified

results that are seen. In the present study, fishmeal was used as the protein source as also used in the earlier studies conducted by Riche & Garling (2004), Denstadli *et al*. (2006) and Lin (2006). In the studies of Spinelli *et al*. (1983), Richardson *et al*. (1985), Hossain & Jauncey (1993), Satoh *et al*. (1989), Civera & Guillaume (1989), Davis *et al*. (1993) and that of Sajjadi & Carter (2004), however, casein was the principal protein ingredient used. Both intact proteins and amino acids differ in their capacity for binding to PA as reviewed by Dendougui & Schwedt (2004). The casein forms "casein micelles" and these structures are linked together with phosphate groups, demonstrating that they may serve as a preferable substrate for binding to IP6 (PA) (Horne, 1998). Therefore, the extent to which protein availability and digestion is inhibited by PA–protein interactions will vary between proteins due to differences in the total number of cationic groups available to participate in binding with phytate (Adeola & Sands, 2003).

3.4.3 Nutrient utilisation

Nutrient utilisation of protein, lipid and energy are indicators of the percentage of ingested protein, lipid or energy that are retained within the carcass. Results from the present study found that the inclusions of dietary PA extensively affected protein (ANPU), lipid (ANLU) and energy (ANEU) utilisations. In general, the graded inclusion of PA in the diets resulted in an apparent decrease in nutrient utilisations. The inclusion of 26.16 g PA kg^{-1} evidently decreased protein utilisations by as much as three-fold when compared to the levels determined for the *M. rosenbergii* within the control group. This is in agreement with the early study of Hossain & Jauncey (1993) who determined that an inclusion of 10 g PA kg^{-1} in the diet presented to common carp depressed protein utilisation.

Interestingly, lipid utilisations were severely affected by the inclusions of dietary PA in

M. rosenbergii. Lipid content in the carcass of the *M. rosenbergii* fed levels of dietary PA of between 16.53 to 26.16 g PA kg^{-1} were found to be particularly low. ANLU values, therefore, were recorded as negative values indicating that dietary PA does have an effect on lipid storage. Energy utilisation was also observed to decrease when the *M. rosenbergii* were fed dietary PA and resulted in negative values when given the diet containing the highest concentrations of PA, *i.e.* 26.16 g PA kg^{-1}. This is likely due to the prolonged consumption of PA, as lipid might have been used to satisfied energy requirement. If *M. rosenbergii* had an additional metabolic requirement for protein under the experimental conditions, lipid reserves may have been used to satisfy energy requirements, explaining both the lower lipid content in the carcass tissue at the end of the the experiment as compared to the intial lipid content (**Table 3.5**) and the lowest growth (**Figure 3.2**).

3.4.4 Nutrient digestibility

Unfortunately, due to practical difficulties associated with the design of the experimental system, it was not possible to obtain adequate amounts of faecal material to permit replicate samples to be analysed. The analysis of the sample that was taken, however, provides some insight into the effects of PA on the digestive ability of *M. rosenbergii*.

Sajjadi & Carter (2004) reported that protein digestibility was significantly lower for Atlantic salmon fed 8 g PA kg^{-1} than the results found in the study conducted by Denstadli *et al*. (2006) who found that an inclusion of 20.7 g PA kg^{-1} significantly increased protein digestibility. In rainbow trout, protein digestibility was negatively affected by a level as low as 5 g PA kg^{-1} (Spinelli *et al*., 1983). The protein digestibility displayed by *M. rosenbergii* in the present study was observed to decrease with increasing inclusion levels of PA in the experimental diets.

Earlier studies conducted with shrimp reported large differences in the nutrient digestibility of

plant proteins (Akiyama *et al*., 1989; Brunson *et al*., 1997) which could partly be attributed to the presence of other anti-nutritional factors such as protease inhibitors, saponins or tannins (Cruz-Suarez *et al*., 2001; Francis *et al*., 2001). *Macrobrachium rosenbergii*, however, are able to enzymatically digest carbohydrates and are more tolerant of plant proteins than their carnivorous counterparts (Tacon, 1995).

Macrobrachium rosenbergii is classified as an omnivore from its digestive enzyme profile (Lee *et al.*, 1980). Additionally, *M. rosenbergii* are able to efficiently digest various kinds of protein as they possess an intricate protease system, which can hydrolyse complex proteins. Various amino acids, *i.e.* arginine, lysine, methionine and tryptophan were found to be dispensable for growth when omitted individually from the diets presented to juvenile *M. rosenbergii* (see Stahl & Ahearn, 1978). In this latter study, it was suspected that the community of bacteria within the gut were responsible for the production of the omitted amino acids in quantities sufficient for growth. The gut bacteria in *M. rosenbergii* may be capable of synthesising extracellular amounts of the omitted amino acids that are available to *M. rosenbergii*. A similar conclusion was reached by Mittler (1971) who when working with aphids was able to rear two generations on a range of diets from which arginine, leucine, lysine, phenylalanine, threonine, tryptophan and valine were selectively omitted. It is not known specifically whether the prawn itself, symbiotic gut microbes or bacterial contamination are responsible for compensating for the reduced protein through PA chelation. This may explain the less marked effects of dietary PA on protein digestibility in *M. rosenbergii* compared to other species.

Phytate is known to form complexes with proteins at both acidic and alkaline pH (Cheryan, 1980). The pH range in the stomach of *M. rosenbergii* would facilitate the formation of PA–protein complexes as the interactions between protein and PA may influence the enzymatic digestion of protein in the stomach. This interaction may affect changes in protein structure that

can decrease enzymatic activity, protein solubility and proteolytic digestibility. Digestive enzymatic activities of *M. rosenbergii* varies during growth, the intermoult cycle and circadian rhythms, physiological phases that at the same time are regulated by environmental factors such as temperature, light and nutrition (Barnabe, 1994), all of which may have played a role.

In addition, the source of protein used in the present study may have influenced the outcome of protein digestibility. Fishmeal was used as the main protein source instead of casein. Protein digestibility, therefore, is most likely to have been less inhibited by the PA–protein interactions as the total number of cationic groups available to participate in binding with phytate were possibly lower (Adeola & Sands, 2003).

To date, there is limited information available on the effects of PA on lipid digestibility in shrimp and fish species. In the present study, the graded inclusions of PA in the diets of *M. rosenbergii* were found to be markedly affected resulting in a decrease in lipid digestibility. This finding is in agreement with previous studies carried out on Atlantic salmon by Denstadli *et al*. (2006) who reported that the digestibility of lipid in Atlantic salmon fed the highest PA level, *i.e* 20.7 g PA kg^{-1}, was significantly reduced although the differences were reported to be minor compared to other treatment group and the control. These latter authors suggested that the significant decrease in lipid digestibility exhibited by Atlantic salmon might have been due to the reduced bile acid concentration. There is, however, no evidence for the production of bile acids by crustaceans (Boonyaratpalin, 1996). This suggests that the metabolic processes of emulsification, digestion and transportation of lipids in crustaceans differ from fish species. Lipid transport in shrimp, however, is accomplished primarily through high density lipoproteins (HDL) (Boonyaratpalin, 1996). The ternary binding between lipase–mineral–PA which could reduce the capacity of lipase to liberate fatty acids and thereby facilitate the digestion of lipids as indicated by Knuckles (1988), is therefore the most probable explanation.

3.4.5 Whole body proximate composition

In this study, the inclusion of graded levels of PA markedly affected the final proximal composition (*i.e*. protein, lipid, gross energy and ash) of the whole carcass. The moisture content of the carcass was almost constant between the dietary treatments and there were only minor differences. The protein, lipid and energy content of the carcass, however, was significantly lower in those prawns fed the diet containing 26.16 g PA kg^{-1}. In a contrast to this, the ash content of the carcass increased with the increasing inclusion of dietary PA. This is in agreement with the findings of Sajjadi & Carter (2004) who reported that PA had no significant effect on Atlantic salmon final carcass dry matter but lowered lipid content and significantly increased the ash content. Hossain & Jauncey (1993) also reported that the inclusion of 10 g PA kg^{-1} led to lower levels of protein and lipid content in the carcass of common carp. Studies carried out by Richardson *et al.* (1985) also found that Chinook salmon fed 25.8 g PA kg^{-1} had consistently lower percentage of lipid and higher percentages of ash in their bodies. On the other hand, PA had no significant effect on the protein content in the carcass of Atlantic salmon while Chinook salmon fed 25.8 g PA kg^{-1} had lower percentages of protein content (Richardson *et al.*, 1985).

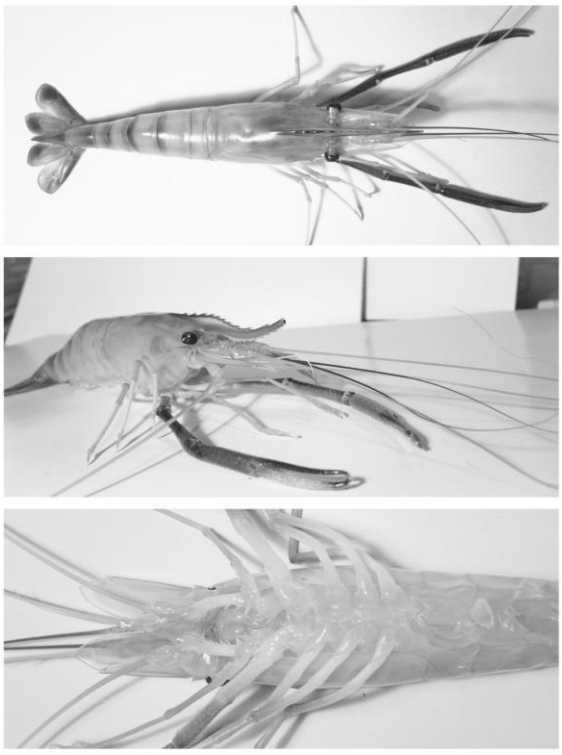
Complexing of PA with protein either through PA–protein or PA–protein–cation types of interaction may alter the protein structure as they closely pack around the negatively charged PA. This may in turn lead to decreased solubility, digestibility and functionality of the proteins (Cheryan, 1980; Cosgrove, 1980; Reddy *et al*., 1982). This possibly explains the decrease in protein content of the carcass, due to the binding of PA–protein making them unavailable. In addition, the reduced lipid content could be correlated with the high levels of PA and reduced feed intake and weight gain which consequentially led to the mobilisation of body lipid reserves to meet protein and energy requirements for vital body functions. Another possible explanation

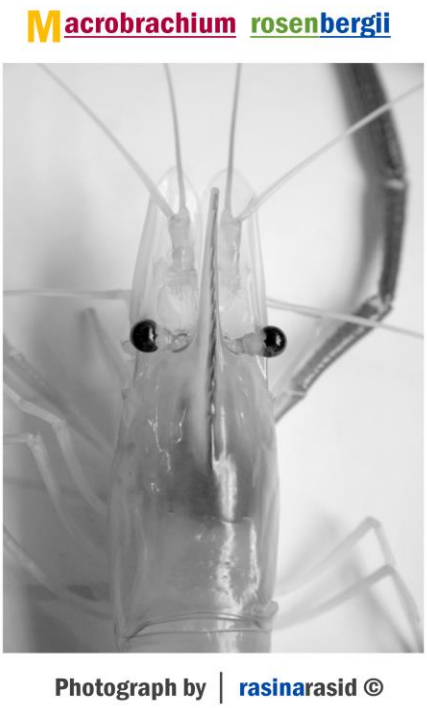
is that at the time of ecdysis, the prawn is inactive and does not feed and so has to utilise reserves, particularly the lipids stored during the prior inter-moult stage. If there is a period of nutrient deficiency or starvation during or after moulting, in the absence of stored lipid, the prawn will use protein for energy (Saravanan *et al*., 2008).

3.5 Conclusion

The findings from the present study, which aimed to investigate the effects of increasing PA levels up to 26.16 g PA kg^{-1}, in diets presented to *M. rosenbergii*, showed no significant negative impacts on growth performance. Feed utilisation (FI, FCR and PER) in *M. rosenbergii* fed various levels of PA also did not differ significantly. The inclusion of PA within the experimental diets, however, did result in significant changes in the protein, lipid, gross energy and ash compositions of the carcass, most notably in those animals fed the diet containing the highest level of PA. Significant changes in the protein, lipid and energy utilisation were also observed in those animals receiving the highest doses. The inclusion of PA in the diets also resulted in changes in protein and lipid digestibility. These changes may have a serious impact on meeting the nutritional requirements of *M. rosenbergii*, particularly when fishmeal is substituted with protein derived from plant sources. Care, therefore, must be taken to ensure that the levels of dietary PA in feeds for *M. rosenbergii* are sufficient to meet these nutritional demands and are appropriate for optimal production.

Chapter 4 - The influence of microbial phytase levels on growth, feed intake, whole body composition, digestibility and utilisation by *Macrobrachium rosenbergii*





The influence of microbial phytase levels on growth, feed intake, body composition, digestibility and utilisation by *Macrobrachium rosenbergii*

4.1 Introduction

Phytic acid (PA), a cyclic inositol compound containing six phosphate groups, is a stable molecule that cannot be effectively digested without enzymatic reactions (Cao *et al*., 2007). Microbial phytase is an enzyme used specifically to hydrolyse PA (Cheng & Hardy, 2003). It commonly occurs in fungi, most notably *Aspergillus* species, and *Aspergillus niger* strains are reported to be among the best producers of extracellular phytase (Wodzinski & Ullah, 1996; Pandey *et al.,* 2001). Supplementation of phytase releases phosphate groups of phytate (Cain & Garling, 1995; Rodehutscord & Pfeffer, 1995) to inorganic phosphorus and *myo*-inositol monophosphate as well as proteins, peptides and metal di- and trivalent ions (see **Figure 1.8**; Agranoff, 2009). Phytase, therefore, has the potential to enhance growth performance and feed conversion efficiency (Sajjadi & Carter, 2004), to improve nutritional efficiency (Forster *et al*., 1999; Suguira *et al*., 2001) and, to improve the digestibility of nutrients (Cheng & Hardy, 2003; Yoo *et al*., 2005).

Research into the supplementation of fish diets with phytase is more advanced than with that for shrimp species and there are numerous studies that focus on its addition. Phytase has, for example, been reported to improve the growth performances of African catfish, *Clarias gariepinus* (see Van Weerd *et al*., 1999), channel catfish, *Ictalurus punctatus* (see Jackson *et al*., 1996, Li & Robinson, 1997), common carp, *Cyprinus carpio* (see Schäfer *et al*., 1995), *Pangasius pangasius* (see Debnath *et al*., 2005), rainbow trout, *Oncorhynchus mykiss* (see Cain & Garling, 1995; Rodehutscord & Pfeffer 1995; Vielma *et al*. 1998), red sea bream, *Pagrus major* (see Biswas *et al*., 2007), and Nile tilapia, *Oreochromis niloticus* (see Cao *et al*., 2008; Abo-State *et al*., 2009). In contrast, there is also a number of studies where the addition

of phytase into the diet has had no discernible effects as was found to be the case in Atlantic salmon, *Salmo salar* (Denstadli *et al*., 2007). In addition to the effects of phytase supplementation on growth performance, the effect on whole body composition has been reported for several penaeids species, *e.g.* for *Penaeus monodon* (see Biswas *et al*., 2007) and for *Litopenaeus vannamei* (see Chen *et al*., 2005; Fox *et al*., 2006). At the time the current study was conducted, there were no known reports regarding the effect of phytase on the freshwater prawn, *Macrobrachium rosenbergii*.

The results determined from several studies investigating feed efficiency after the inclusion of phytase in the diets of several fish species found that there were improvements, for example, channel catfish (Jackson *et al.,* 1995), common carp (Schäfer *et al.,* 1995) and Nile tilapia (Liebert & Portz, 2005; Abo-State *et al*., 2009). While these studies demonstrated improvements in feed efficiency, a study conducted by Chen *et al*. (2005) who supplemented the diet presented to *L. vannamei* with up to 2000 FTU kg^{-1} of phytase was unable to see any improvement in feed efficiency. Whether the addition of phytase to the diet given to *M. rosenbergii* would result in an improved feed efficiency, therefore, needs to be ascertained.

As phytase has been reported to have positive results in fish, there is an interest in whether its addition to prawn diets can improve the digestibility of the diet, and in particular, the digestion of protein. The findings from **Chapter 3** of this thesis indicated that the digestibility of protein as well as lipid were negatively affected by the inclusion of dietary PA. Hence, the interactive effects of phytase on both the digestibility of protein and lipid require further investigation. Furthermore, the optimal phytase supplements in feed formulations are conflicting and there is, therefore, a need to establish what the acceptable supplementation levels for specific species might be.

From the various studies that have already been conducted and published, there appears to be great inconsistency regarding phytase supplementation and protein digestibility. Liebert & Portz (2005), for example, reported that supplementation of the diets presented to Nile tilapia with 750 FTU kg^{-1} was sufficient for protein digestibility, whereas the study of Cao *et al*. (2008) found that diets containing 1000 FTU kg^{-1} also given to Nile tilapia did not affect protein digestibility. An earlier study by Furuya *et al*. (2001), however, reported that a supplementation of 500–1500 FTU kg^{-1} improved protein digestibility in Nile tilapia. Despite these conflicting results for Nile tilapia, improved protein digestibility following supplementation of the diet with phytase has been reported for a number of other fish species including *Pangasius pangasius* (see Debnath *et al*., 2005), rainbow trout (Vielma *et al*., 2004) and red sea bream (Biswas *et al*., 2007). Alongside these studies reporting improvements in protein digestibility, there are also studies that report a decreased protein digestibility following phytase supplementation of the diet, as is the case in the study conducted by Van Weerd *et al*. (1999) on African catfish. Also, a number of other studies report no effect on protein digestibility following phytase supplementation of the the diet as seen in various studies with Atlantic salmon (Storebakken *et al.,* 1998), striped bass (Papatryphon *et al*., 1999), sea bass (Oliva-Teles *et al*., 1998) and rainbow trout (Cheng & Hardy, 2002). For the penaeid species, *L. vannamei*, protein digestibility was found to be higher than that within the control group when given a diet supplemented with 1000 FTU kg^{-1} (Fox *et al*., 2006).

A number of studies investigating the role of supplemental phytase and nutrient utilisation have also been conducted in a range of commonly cultured fish species, including rainbow trout (Rodehutscord & Pfeffer, 1995; Forster *et al.,* 1999), common carp (Schäfer *et al*., 1995), channel catfish (Jackson *et al*., 1995; Li & Robinson, 1997) and Nile tilapia (Furuya *et al*., 2001; Liebert & Portz, 2005; Abo-State *et al*., 2009). Again, at the time that this thesis was conducted

there were no known studies focusing on the role of phytase supplementation and the effects on nutrient utilisation in the freshwater prawn, *M. rosenbergii*.

In a study conducted by Chen *et al.* (2005), it was found that 500–2000 FTU kg^{-1} added to the diet did not affect the whole body contents of *L. vannamei*. In **Chapter 3**, the effects of including PA within the diets presented to *M. rosenbergii* were investigated. The study found that the nutrient utilisations and whole body compositions of the experimental *M. rosenbergii* were affected by the inclusion of various levels of dietary PA. It is anticipated, therefore, that the supplementation of the diet with microbial phytase that is then given to *M. rosenbergii* may reverse these results.

The benefits of phytase supplementation in practical diets containing high levels of plant protein ingredients that are fed to *M. rosenbergii* have yet to be explored. The aim of the present study was, therefore, to investigate the effects of including graded levels of microbial phytase in the diets presented to *M. rosenbergii* on their subsequent growth performance, feed intake, digestibility, nutrient utilisation and whole body chemical composition. The study also set out to determine the optimal dose of microbial phytase appropriate for the culture of *M. rosenbergii*.

4.2 Materials and methodology

4.2.1 Experimental system

The experimental trial was conducted within the Prawn Unit in the Tropical Aquarium of the Institute of Aquaculture, University of Stirling, UK. The trial lasted for 80 days and the digestibility trial was conducted over the last 20 days of this. The experiment was conducted in 12 × 26.4 L recirculating system tanks with three replicates for each diet. Each aerated tank was stocked with five nylon 1.53 L meshed pots with each containing one single juvenile *M. rosenbergii* (**Figure 2.2**). The temperature within the system was maintained at 28.4 ± 0.30

°C and each tank was supplied with freshwater at a flow rate of 1 L min^{-1} such that a constant water depth of 17 cm was maintained. Throughout the 80-day trial, water quality parameters, *i.e*. dissolved oxygen, pH, general hardness, calcium hardness ($CaCO_3$), ammonia (NH_3), nitrite (NO_2) and nitrate (NO_3) levels were monitored daily and maintained by using mechanical and biological filters such that the parameters were within acceptable levels (**Table 2.1**). The animals were subjected to a photoperiod regime of 12 h light: 12 h dark throughout the experiment.

4.2.2 Experimental animals

Sixty juvenile Malaysian freshwater prawns, *M. rosenbergii*, reared from a single batch of fertilised eggs, with an average initial weight of 0.40 ± 0.07 g and average initial carapace length of 8.51 ± 0.52 mm were randomly distributed between the nylon mesh pots in each tank (**Figure 2.2**). The prawns were acclimated for a period of seven days before the start of the trial and fed a basal pellet diet. Moulting and mortality events were recorded on a daily basis and any exuviae within the pots were noted but left for the relevant prawn to naturally feed on throughout the experiment.

4.2.3 Sampling procedures

Prior to the start of the experiment, an initial sample of prawns (n=100) were taken to determine the baseline whole body proximate composition. Prawns were killed by submerging them in ice for one minute, after which the samples were frozen until they were analysed. The prawns were individually weighed and the length of their carapace was recorded every 20 days (details are provided in **Section 2.3.1**). The experimental diets were fed by hand to each prawn to apparent satiation twice daily. Any uneaten food was collected by siphoning each pot after each feed and then weighed to precisely calculate the feed intake and feed conversion ratio

(FCR). Apparent digestibility was measured over the last 20 days of the trial. Faecal material from each pot was also collected by siphon twice per day and then pooled for each treatment group. After collection, the faeces were centrifuged (Centaur 2 Sanyo) at 2,268 × g for 10 min, the supernatant was subsequently discarded and the solid faecal matter frozen at -20ºC until the samples could be analysed. At the end of the experimental trial period, the prawns were starved for 24 h prior and then euthanased in ice. Only prawns with a hard exoskeleton were processed; these were packed in plastic, labelled and then frozen at -20ºC until they could be analysed. For analysis, the experimental animals were subject to whole body proximate composition, histology (as described in **Section 2.9**) and hepatosomatic index (HSI) analysis.

4.2.4 Experimental diets

The experimental diets were formulated using high levels of plant protein based ingredients as the primary protein sources, *i.e.* high protein soybean meal, wheat gluten and wheat meal. Fish soluble concentrate was added as a flavour to make the pellets more palatable to the prawns. The diets were formulated to meet the known optimal nutrient requirements of *M. rosenbergii* (see New & Valenti, 2000; Mitra *et al.,* 2005). Four experimental diets were formulated to produce approximately 420.0 g kg^{-1} of protein, 90.0 g kg^{-1} of lipid and 20 kJ g^{-1} total energy. The experimental diets differed only in their phytase content which was provided through the addition of exogenous microbial phytase (Sigma Aldrich, P1259). Diet “MP0” acted as the control and had no microbial phytase added to it whereas diets “MP500”, “MP1000” and “MP2000” were supplemented with graded inclusion levels of phytase at 500, 1000 and 2000 FTU kg^{-1} phytase, respectively. In order to augment the responses of phytase supplementation and to retain a consistent amount of PA among the dietary treatments, approximately 15 g kg^{-1} PA (Sigma Aldrich, P8810) was added to each dietary treatment, including the control. The compositions of the vitamin and mineral pre-mixes used in the experimental diets are shown in

Table 2.2 and **Table 2.3**, respectively. Yttrium oxide (Y_2O_3) was also incorporated as an inert marker in the diets (see **Section 2.5.8**).

The dry ingredients were weighed and prepared within the feed processing room at the Institute of Aquaculture, University of Stirling. The ingredients were ground to a fine powder using a hammer mill before they were combined in a mixer (A200 Hobart Ltd). Phytic acid and fish oil (BioMar Ltd) were then slowly added to the mixer before finally adding the relative amount of phytase solution. The microbial phytase was diluted in 50 mL distilled water and added to the formulated diet as required. Distilled water was also added to the control diet to achieve an equivalent moisture level in all diets (*i.e.* approximately 86.0 g kg^{-1}). The mixture for each diet was then pelleted using a California Pellet Mill (Model CL2) with a 1.0 mm die. The pelleting temperature measured did not exceed 45ºC (Liebert & Portz, 2005). The pellets were then dried at 35°C for 24 h in a ventilated area. Once the pellets had cooled to room temperature, they were then stored in labelled, sealed, polythene bags and frozen until required (details in **Section 2.4.1**). An attractant, anhydrous trimethylamine (TMA) diluted to 15% with distilled water, was sprayed onto the pellets each week (**Section 2.4.2**). The formulation of each experimental diet is shown in **Table 4.1**.

4.2.5 Chemical analysis

Proximate analysis was conducted to determine the nutrient composition of the diets, the collected faecal matter and the whole body samples. Moisture, crude protein, crude lipid and the ash content were determined according to AOAC (1995) and are described in detail in the General Materials and Methodology chapter of this thesis (see **Section 2.5**). The PA content in the ingredients and the formulated diets were analysed according to the method described in **Section 2.6**. Phytase concentrations in the dietary treatments were analysed according to

procedures described by Engelen *et al.* (1994) and the details are provided in **Section 2.7**.

Table 4.1. Feed formulation and proximate composition (g kg^{-1}, as fed) of each of the experimental diets subsequently fed to juvenile *Macrobrachium rosenbergii* for a period of 80 days.

	Diet 1	Diet 2	Diet 3	Diet 4
Ingredients	MP0	MP500	MP1000	MP2000
High protein soybean meal	355.0	355.0	355.0	355.0
Wheat gluten[1]	300.0	300.0	300.0	300.0
Wheat meal[1]	123.0	123.0	123.0	123.0
Fish soluble[1]	50.0	50.0	50.0	50.0
Fish oil[2]	80.0	80.0	80.0	80.0
Vitamin pre-mix[3]	10.0	10.0	10.0	10.0
Mineral pre-mix[4]	35.0	35.0	35.0	35.0
Yttrium oxide[5]	2.0	2.0	2.0	2.0
Carboxymethylcellulose[6]	29.4	28.9	28.4	27.4
Phytic acid[7]	15.6	15.6	15.6	15.6
Phytase[8] (FTU kg^{-1})	0.0	500	1000	2000
Proximate composition (g kg^{-1}, as fed)				
Dry matter	911.6	915.3	913.5	912.5
Crude protein	436.9	438.1	435.4	442.6
Crude lipid	93.9	91.5	92.3	91.7
Crude fibre	30.9	28.9	27.0	30.7
Ash	72.9	74.7	73.2	75.6
Nitrogen free extract	294.5	297.9	300.1	289.3
Gross energy (kJ g^{-1})	19.7	19.7	19.5	20.5
Phytic acid	15.92	15.05	14.92	13.41
Phytase, FTU kg^{-1}	66	559	1132	2055

[1]BioMar UK Ltd; [2]Herring oil; [3,4] As listed in **Table 2.2** and **Table 2.3**, according to Jauncey & Ross (1982); [5]Sigma Aldrich, 205168; [6]Sigma Aldrich, C5013; [7]Phytic acid sodium salt hydrate from rice, Sigma Aldrich, P8810; [8]Microbial phytase, Sigma Aldrich, P1259.

4.2.6 Calculations and statistical analysis

The experimental *M. rosenbergii* were weighed and measured at the beginning of the trial and then every 20 days thereafter until the end of the 80 day trial thereby producing four sets of data in total. The growth performances, feed utilisation parameters including feed intake (FI), feed conversion ratio (FCR) and protein efficiency ratio (PER), apparent digestibility coefficient (ADC) and nutrient utilisation were calculated using the formulae presented in **Section 2.8**.

Data for all the measured parameters are presented as the mean ± SD from three replicates unless stated otherwise and were analysed and calculated using SPSS 18.0 (SPSS Inc, 2010). Variations in the dietary treatments were compared by a one-way ANOVA. Data were tested for their normality using the Kolmogorov-Smirnov test and data which were identified as being non-homogeneous were tested using Levene's test whilst percentage data were subjected to square root or to an arcsin transformation before analysis. If significant differences were indicated at or less than the p=0.05 level, then Tukey's *post-hoc* test was used to identify the significant differences between specific treatments.

4.3 Results

4.3.1 Chemical composition of diets

The analysed proximate compositions of the experimental diets are shown in **Table 4.1**. Three experimental diets contained graded amounts of microbial phytase, namely 500, 1000 and 2000 FTU kg^{-1}, while one containing no microbial phytase supplement (0 FTU kg^{-1}) was used as the control. The content of main nutrients was fairly consistent among dietary treatments regardless of the microbial phytase supplementation level. All the diets contained approximately 913.2 g kg^{-1} dry matter, 438.3 g kg^{-1} protein, 92.4 g kg^{-1} lipid, 13.1 g kg^{-1} fibre and 74.1 g kg^{-1} ash. The gross energy of all diets was approximately 19.9 kJ g^{-1}.

The inclusion levels of microbial phytase resulted in subtle changes in the dietary PA compositions of the diets as shown in **Table 4.1**. The dietary PA content decreased from 15.92 g kg^{-1} to 13.41 g kg^{-1} PA with increasing levels of microbial phytase supplementation; this is most likely due to enzymatic activity of phytase. Phytase activities were also observed in the control treatment. The analysis of phytase activity levels in the dietary treatments were in close agreement with the intended levels.

4.3.2 Growth performance

In general, the supplementation of microbial phytase showed no significant differences between treatment groups on the growth performances of the *M. rosenbergii*. The initial mean carapace length of the prawns was 8.51 ± 0.52 mm. After feeding the prawns with the experimental diets *i.e.* MP0, MP500, MP1000 and MP2000, for 80 days, the size of their carapace ranged from 12.24 mm to 12.64 mm length, representing progressive increase of 3.60 mm to 4.27 mm with the increasing phytase inclusion (**Table 4.2**). The weight gain varied between 1.09 g to 1.20 g. Although not statistically significant, the SGR varied from 1.62 to 1.84 with increasing microbial phytase supplementation. A similar tendency was also seen in the hepatosomatic index (HSI) values which ranged from 2.60% to 4.20%. The highest carapace length increase, weight gain, SGR and HSI were all recorded in the test group receiving the 2000 FTU kg^{-1} microbial phytase supplement. The survival of the experimental animals was not significantly different among treatments, and ranged from 66.7% to 86.7% with the higher levels of mortality being recorded in the groups receiving the higher level of phytase supplement, *i.e.* 1000–2000 FTU kg^{-1}.

4.3.3 Feed efficiency

The feed utilisations are shown in **Table 4.2**. Feed intake ranged from 0.015 g $prawn^{-1}$ day^{-1} to

0.017 g prawn^{-1} day^{-1} and there were no significant differences among the four treatment groups. With regards to the FCR, the range over the whole experimental period was from 1.13 for the control group and 1.16—1.18 for the experimental groups supplemented with microbial phytase. No significant differences, however, were identified between the inclusion levels of microbial phytase with FCR. The increase in microbial phytase levels in the diet also did not result in a respective increase in PER, which varied from 2.11 to 2.27.

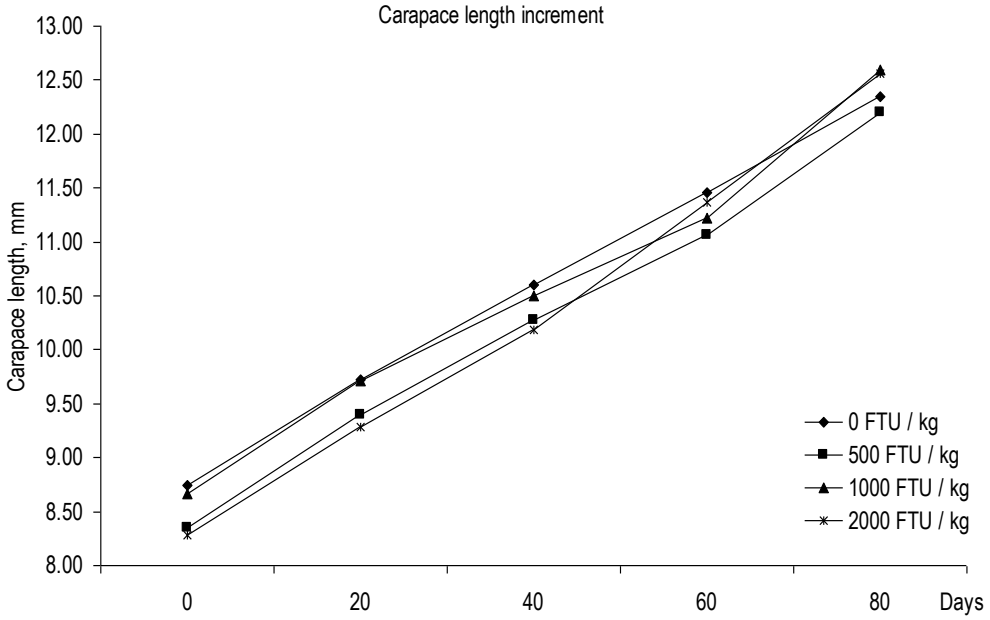


Figure 4.1. The average growth response in terms of increases in the length of the carapace of the juvenile *Macrobrachium rosenbergii* fed the experimental diets containing graded microbial phytase supplementation levels for 80 days. For clarity, no error bars are shown given that the differences in the growth curves at each point are not significantly different.

4.3.4 Apparent nutrient utilisation

The apparent nutrient utilisation of protein (ANPU), lipid (ANLU) and energy (ANEU) of the *M. rosenbergii* fed the microbial phytase supplemented diets is shown in **Table 4.3**. Statistical analysis using ANOVA showed that microbial phytase supplementation resulted in significant differences (p<0.05) in protein and lipid utilisation, while the utilisation of energy was not significantly different. Specifically, inclusion levels of microbial phytase, *i.e.* 1000–2000 FTU kg^{-1}, resulted in a significant increase in protein utilisation where the determined ANPU values

increased from 17.81% to 22.97% with the increasing inclusion of microbial phytase. The ANLU values were also observed to significantly increase from 4.11% to 7.55% with increasing microbial phytase levels. The prawns receiving the diet with the inclusion of 500–2000 FTU kg^{-1} showed a significantly higher (p<0.05) ANLU compared to the control. Energy utilisation did not differ among the dietary treatments regardless of the amount of microbial phytase that was added as a supplement; values ranged between 10.37–10.74 kJ g^{-1}.

4.3.5 Apparent digestibility coefficient (ADC)

The effects of microbial phytase supplementation on nutrient ADCs are shown in **Table 4.4**. In general, the ADC of dry matter, protein and lipid increased with increasing inclusions of microbial phytase. For instance, the ADC of protein increased from 65.50% to 71.99%, for treatment supplemented with 0 FTU kg^{-1} to 2000 FTU kg^{-1}, respectively, while the ADC of lipid followed a similar trend increasing from 61.62% to 68.16% with increasing phytase supplementation. The ADC of dry matter varied from 57.24% to 67.11%. Supplementation of the diet with 2000 FTU kg^{-1} resulted in the highest ADC of dry matter, protein and lipid.

4.3.6 Whole body proximate composition

The whole body proximate composition at the beginning of the dietary trial (*i.e*. day 0) and after 80 days, *i.e.* at the end of the experimental trial, are shown in **Table 4.5**. The initial composition of the carcass was 75.23% moisture, 14.74% protein, 1.39% lipid and 2.12% ash. Supplementations of the diet with microbial phytase did not significantly affect the proximate composition of the whole body of the prawns after 80 days on the experimental diets. Nonetheless, subtle increases were observed in the protein and lipid contents with the increasing inclusion of microbial phytase; the prawns receiving the diet with 2000 FTU kg^{-1} had the highest values. The protein and lipid contents increased from 17.43% to 18.76% and 1.43%

to 1.60%, respectively. At the same time, however, the increasing inclusion of microbial phytase also subtly decreased the ash content in the whole body from 1.55% to 1.45%; this result though was not statistically significant. The moisture and gross energy content of the whole body did not differ among treatment groups and was not influenced by the amount of microbial phytase added to the diet.

Table 4.2. Growth performance and feed efficiency of juvenile *Macrobrachium rosenbergii* fed diets containing different supplements of microbial phytase (0–2000 FTU kg^{-1}) for a period of 80 days.

	Dietary microbial phytase (FTU kg^{-1})			
	0	500	1000	2000
Initial carapace length (mm)	8.74 ± 0.47	8.35 ± 0.42	8.66 ± 0.84	8.28 ± 0.39
Final carapace length (mm)	12.34 ± 0.30	12.24 ± 0.64	12.64 ± 0.58	12.55 ± 0.13
Carapace length increment (mm)	3.60 ± 0.17	3.85 ± 0.35	3.98 ± 0.43	4.27 ± 0.39
Initial weight (g)	0.41 ± 0.05	0.39 ± 0.06	0.43 ± 0.10	0.36 ± 0.07
Final weight (g)	1.50 ± 0.09	1.52 ± 0.19	1.57 ± 0.11	1.56 ± 0.14
Weight gain (g)	1.09 ± 0.08	1.14 ± 0.13	1.15 ± 0.18	1.20 ± 0.21
Av. daily weight gain (g $prawn^{-1}$)	0.014 ± 0.00	0.014 ± 0.00	0.014 ± 0.00	0.015 ± 0.00
Specific growth rate (% day^{-1})	1.62 ± 0.13	1.72 ± 0.05	1.65 ± 0.36	1.84 ± 0.34
Feed intake (g $prawn^{-1}$ day^{-1})	0.015 ± 0.00	0.016 ± 0.00	0.017 ± 0.00	0.017 ± 0.00
Feed conversion ratio	1.13 ± 0.18	1.16 ± 0.01	1.18 ± 0.18	1.16 ± 0.16
Protein efficiency ratio	2.27 ± 0.16	2.21 ± 0.25	2.11 ± 0.33	2.19 ± 0.38
Hepatosomatic index (HSI), (%)	2.60 ± 0.42	3.13 ± 0.84	3.89 ± 1.03	4.20 ± 1.09
Survival (%)	80.0 ± 0.00	86.7 ± 11.55	66.7 ± 11.55	66.7 ± 11.55

Av. =average.
Values are the mean ± SD of three replicates.
Statistical analysis found no significant differences between the different diets for any of the measured parameters.

Table 4.3. Apparent nutrient utilisations (%) in the juvenile *Macrobrachium rosenbergii* fed the supplemented microbial phytase experimental diets for 80 days.

	Dietary microbial phytase (FTU kg^{-1})			
	0	500	1000	2000
Protein utilisation (ANPU)	17.81 ± 0.75^{a}	20.20 ± 0.81^{b}	21.55 ± 0.40^{bc}	22.97 ± 0.99^{c}
Lipid utilisation (ANLU)	4.11 ± 1.06^{a}	5.08 ± 0.73^{ab}	5.90 ± 0.11^{ab}	7.55 ± 1.01^{b}
Energy utilisation (ANEU)	10.44	10.58	10.74	10.37

Values are the mean ± SD of three replicates, except for the figures presented for ANEU which are derived from a single value.
Values within the same row with different letters are significantly different (p<0.05).

Table 4.4. Apparent nutrient digestibility (%) in the *Macrobrachium rosenbergii* fed the microbial phytase supplemented diets for 80 days.

	Dietary microbial phytase (FTU kg^{-1})			
	0	500	1000	2000
Dry matter	58.38	58.29	65.23	67.11
Crude protein (ACPD)	65.50	69.54	70.81	71.99
Crude lipid digestibility (ACLD)	61.62	64.20	65.50	68.16

Values are based on a single sample.

Table 4.5. Whole body proximate composition (% wet weight) of the *Macrobrachium rosenbergii* fed diets containing different supplements of microbial phytase (0–2000 FTU kg^{-1}) for a period of 80 days.

	Dietary microbial phytase (FTU kg^{-1})				
	Initial[1]	0	500	1000	2000
Moisture content	75.74 ± 0.72	73.34 ± 0.88	73.45 ± 1.43	73.09 ± 0.39	73.00 ± 0.17
Crude protein	14.74 ± 0.05	17.43 ± 0.54	18.22 ± 1.06	18.62 ± 0.30	18.76 ± 0.14
Crude lipid	1.39 ± 0.00	1.43 ± 0.03	1.48 ± 0.08	1.53 ± 0.03	1.60 ± 0.02
Ash*	2.12 ± 0.08	1.55 ± 0.16	1.48 ± 0.06	1.47 ± 0.18	1.45 ± 0.28
Gross energy(kJ g^{-1})**	4.40	5.41	5.41	5.35	5.21

Values are the mean ± SD of three replicates.
*Values are the mean ± SD of two replicates.
**One replicate due to limited availability of samples.
Statistical analysis found no significant differences between the different diets for any of the measured parameter.
[1]Values not included in the one-way Anova.

4.4 Discussion

4.4.1 Growth performance

Phytase is the requisite enzyme to degrade PA and notionally has the capacity to hydrolyse PA thus reducing detrimental effects due to dietary PA. This study, therefore, aimed to investigate the effect of supplementing the diets presented to *M. rosenbergii* with microbial phytase. There was some justification in conducting this experiment, in that fish receiving phytase supplemented diets were reported to exhibit improvements in growth. Improved growth performance due to phytase supplementation of the diet, has also been reported for African catfish (Van Weerd *et al*., 1999), channel catfish (Jackson *et al*., 1996, Li & Robinson, 1997), *P. pangasius* (see Debnath *et al*., 2005), common carp (Schäfer *et al*., 1995), Nile tilapia (Liebert & Portz, 2005), rainbow trout (Rodehutscord & Pfeffer, 1995; Vielma *et al*., 2002), red sea bream (Biswas *et al*., 2007), striped bass (Papatryphon *et al*., 1999) and seabass (Olivia-Teles *et al.,* 1998). The study conducted by Sajjadi & Carter (2004) found that diets containing 10 g kg^{-1} of sodium phytate supplemented with 2000 FTU kg^{-1} did enhance weight gain in Atlantic salmon. The inclusion of phytase into a diet containing canola protein concentrate fed to rainbow trout (Forster *et al.,* 1999), soybean meal based diets given to juvenile Korean rockfish (Yoo *et al.,* 2005) and Atlantic salmon fed plant protein based diets (Denstadli *et al*., 2007), however, did not significantly improve growth. The results of the present study are in agreement with the latter studies, as phytase supplementation in the diets did not result in significant differences in growth performance in terms of increase in carapace length, weight gain, average daily weight gain or SGR. However, the highest increase of carapace length, weight gain, SGR and HSI were recorded for the highest inclusion level, *i.e.* 2000 FTU kg^{-1}. This is in accordance with the studies performed with *L. vannamei* where 500–2000 FTU kg^{-1}

did not significantly affect weight gain but the group of animals receiving a dose of 2000 FTU kg^{-1} were recorded as having the highest weight gain (Chen *et al*., 2005). The short period of the experimental trial, *i.e*. 80 days, may explain the lack of significant differences in growth that were observed.

Phytase research in finfish species is comparatively more advantageous with lower levels of supplement needed when compared to the suggested levels required for marine shrimp and freshwater prawns; this may be due to finfish possessing a more acidic gut pH. The optimal pH for the microbial phytase from *A. niger* used in the present study is 5.5 pH (Ullah & Gibson, 1987). In most crustaceans, the foregut fluid is reported to be pH 5–7 (Dall & Moriarty, 1983), while the guts of marine shrimp are slightly more basic at 7.2–7.4 pH (Fox *et al*., 2006). Perhaps the slightly basic pH digestive system of *M. rosenbergii* is not sufficiently acidic to provide the optimal pH for microbial phytase activity. Pre-treatment or acidifying the diets by adding 3% citric acid to reduce the intestinal digesta (Radcliffe & Kornegay, 1996), however, could possibly provide a better environment for phytase function.

4.4.2 Feed efficiency

As previously discussed, several studies have shown that the supplementation of phytase can result in improved feed efficiencies. For example, Rodehutscord & Pfeffer (1995) recorded increased feed intake in rainbow trout fed diets supplemented with phytase. Higher feed intake was also observed in channel catfish fed diets supplemented with at least 250–500 FTU kg^{-1} (Jackson *et al.,* 1996; Li & Robinson, 1997) while 500 FTU kg^{-1} recorded the lowest FCR for *P. pangasius* (see Debnath *et al.,* 2005). In addition, the supplementation / pre-treatment of a diet with 750–1000 FTU kg^{-1} were reported adequate to improve the FCR in Nile tilapia (Liebert & Portz, 2005; Cao *et al.,* 2008). Papatryphon *et al.* (1999) reported significant improvements in

the FCR of striped bass fed diets with increasing doses of phytase added to them. Vielma *et al*. (2002) also reported better growth and feed utilisation in rainbow trout fed diets by pre-treatment with phytase and this could be attributed partially to more efficient PA hydrolysis in comparison to top spraying phytase onto the finished diet. A dose of 2000 FTU kg^{-1} added into the pelleted diet equally enhance feed efficiency in trials using sea bass (Oliva-Teles *et al*., 1998) and Atlantic salmon (Sajjadi & Carter, 2004). In contrast to this, a diet containing 4500 FTU kg^{-1} fed to rainbow trout did not affect feed utilisation efficiency but it was noted that in this study the phytase was top-sprayed onto the soy-protein based diets before use (Forster *et al.*, 1999). The use of a dephytinised canola protein concentrate in a rainbow trout diet likewise had no significant effect on feed intake (Teskeredzíć *et al.,* 1995; Thiessen *et al*., 2004). Similarly, in shrimp, 500 FTU kg^{-1} top sprayed onto soybean based diets fed to *P. monodon* did not significantly improve the FCRs (Biswas *et al.,* 2007) nor did a supplement of 2000 FTU kg^{-1} added to the diets given to *L. vannamei* (Chen *et al.,* 2005). In line with the latter study, the current experiment showed *M. rosenbergii* fed diets supplemented with up to 2000 FTU kg^{-1} did not significantly improve feed intake and FCR.

Fox *et al.* (2006) reported that researchers at the Fisheries and Mariculture Laboratory - Marine Science Institute at the University of Texas concluded that shrimp fed soybean meal based practical basal diets deficient in P that were supplemented with 600 FTU kg^{-1} showed moderate improvements in their feed performances. It is important to note that a variety of methods to supplement diets with phytase have been used in these past studies and the method used may explain the diversified results that are reported. In the present study, microbial phytase was added to the diet formulation in a manner similar to that in a number of earlier studies, *e.g.* Oliva-Teles *et al*. (1998), Forster *et al*. (1999), Sugiura *et al*. (2001), Cheng & Hardy (2003, 2005), Liebert & Portz (2004, 2007) and in Chen *et al*. (2005). In addition to incorporating the

phytase directly into the feed, phytase has been applied directly as a top spraying or coating as used in the studies conducted by Jackson *et al.* (1996), Li & Robinson, (1997), Vielma *et al*. (1998, 2000, 2004), Papatryphon *et al*. (1999), Debnath *et al.* (2005) and Biswas *et al*. (2007). Other methods for adding phytase include the pre-treatment of ingredients prior to formulation (Van Weerd *et al*., 1999; Sugiura *et al*., 2001; Yoo *et al.,* 2005; Cao *et al*., 2008) and using dephytinised ingredients (Teskeredźić *et al.,* 1995; Thiessen *et al.,* 2004). Clearly each method has its pros and cons and further investigation is needed before deciding which may be the best method.

A study conduct by Debnath *et al.* (2005) found that a supplement of 500 FTU kg^{-1} fed to *P. pangasius* resulted in an optimum PER. Higher PER values were also reported for Nile tilapia fed phytase pre-treated diets, indicating that the phytase treatment improved the nutritional quality of the plant protein meal (Cao *et al.,* 2008). This, however, is a marked contrast to shrimp, where the *P. monodon* fed diets supplemented with 500 FTU kg^{-1} showed no significant effect on PER when compared to those in the control group (Biswas *et al*., 2007). The results of the present trial are in agreement with the latter study, where PER was not significantly affected by the inclusion of microbial phytase in the diets. This is probably due to the high levels of plant protein source used as the main ingredient and the short duration of the trial. The effectiveness and limitations of food and feed supplementation with phytate-degrading enzymes may also depend on their sensitivity to protease digestion. It has been shown that the phytate degrading enzyme from *A. niger* was more stable in the presence of pepsin or pancreatin than the corresponding enzyme from wheat (Phillippy, 1999). Other issues that may have also possibly influenced PER and feed efficiency in the phytase supplemented diets are the liquid or dry phytase form used to prepare the test feeds, the actual activity of the supplemented phytase, and the half-life of the phytase source.

4.4.3 Apparent nutrient utilisation

In the previous PA trial (*i.e.* that detailed in **Chapter 3** and **Section 3.3.4**), the apparent nutrient (protein, lipid and energy) utilisations were evidently affected by the inclusions of dietary PA. Increasing dietary PA from 0.26 g PA kg^{-1} to 26.16 g PA kg^{-1} in the diet significantly decreased the nutrient utilisation of protein, lipid and energy. Interestingly, the current study found an improvement in protein utilisation as a consequence of phytase added to the diet. In the current trial, the ANPU was significantly higher than that seen in the control group when doses of 500–2000 FTU kg^{-1} microbial phytase were added to the diet. The improved protein digestibility due to the addition of phytase may explain the enhanced protein utilisation. Furthermore, an increase was also seen in lipid digestibility. The increased protein availability, from the action of phytase on PA releasing PA–protein complexes, also positively affected the protein and lipid utilisation and consequently enhanced the growth of the prawns. These findings are in agreement with a number of earlier studies (see for example Storebakken *et al*., 1998; Vielma *et al.,* 1998; Sugiura *et al.,* 2001; Liebert & Portz, 2005). Nonetheless, the energy utilisation in *M. rosenbergii* remained unaffected by the phytase supplement in the current study.

4.4.4 Apparent digestibility coefficient

One of the challenges in working with juvenile *M. rosenbergii* in the present experiment was their small size and hence the delicate faecal samples they produce. The inadequate faecal samples that were collected did not permit replications, however, despite the limited material available it is hoped that the data gathered from analysing the faecal samples that were collected may help provide some insight regarding the effects of microbial phytase on the digestion abilities of *M. rosenbergii*.

Phytic acid forms complexes with proteins and chemically purified PA decreases protein

digestibility (Spinelli *et al*., 1983). As reported in **Chapter 3** and **Section 3.3.5**, increasing dietary PA from 0.26 g PA kg^{-1} to 26.16 g PA kg^{-1} in the diet was found to decrease protein and lipid digestibility. Phytase is known to release the phosphate groups of phytate as well as those on proteins (Agranoff, 2009) therefore, possibly increasing protein digestibility - this was demonstrated in the present study. The dry matter, protein and lipid digestibility coefficients increased with an increasing supplement of phytase in the diets which contained approximately 15 g PA kg^{-1}. This is most certainly due to the positive effects of the enzyme activity on PA by releasing the bindings of PA–protein, hence, increasing protein availability. In *Macrobrachium*, lipid is a component of circulatory or membrane lipoproteins and serves as a substrate for the synthesis of various compounds (Guillaume *et al.,* 2001). The increasing protein and lipid digestibility may, therefore, lead to the improved growth performances observed in this current study. These results are in agreement with earlier studies reported for Nile tilapia (Furuya *et al.,* 2001; Liebert & Portz (2007), *P. pangasius* (see Debnath *et al*., 2005) and rainbow trout (Suguira *et al*., 2001; Vielma *et al*., 2004). Conversely, phytase pre-treated soy protein concentrate had no significant effects on the apparent digestibility of protein in Atlantic salmon (Storebakken *et al.,* 1998). The lack of an effect on protein digestibility in fish fed soy protein concentrate diets where phytase was added by top spraying agrees with earlier observations with Atlantic salmon (Sajjadi & Carter, 2004; Denstadli *et al*., 2007), rainbow trout (Vielma *et al*., 2000; Cheng & Hardy, 2002) and striped bass (Papatryphon *et al*., 1999). The lack of an effect on protein digestibility in these studies may be due to the manner in which the phytase was supplemented, differences in the quality of the protein based feed ingredients used or the gastrointestinal pH of the species in question.

4.4.5 Whole body proximate composition

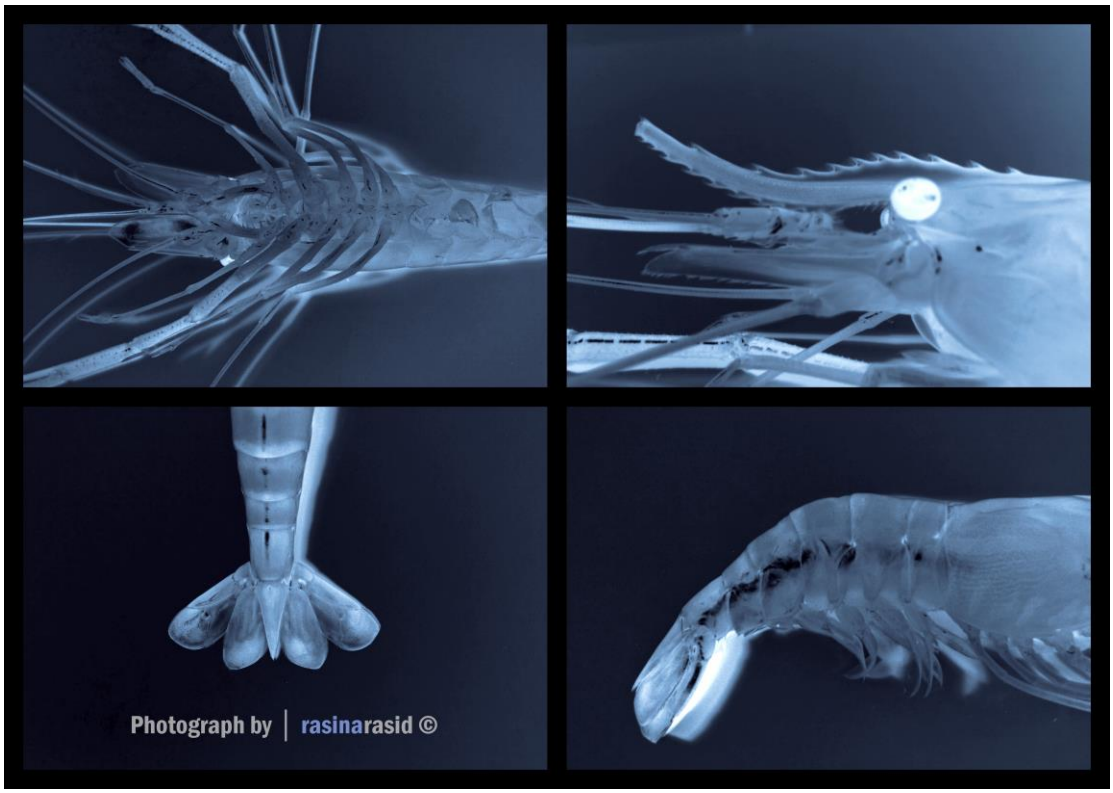
In **Chapter 3** and **Section 3.3.6**, it was shown that inclusion of PA in the diet significantly affected the whole body chemical composition. It was also shown that increasing amounts of PA significantly decreased the protein and lipid content while the ash content increased. The present study, however, demonstrated that a supplement of microbial phytase into diets containing 15 g PA kg^{-1} did not significantly influence the chemical composition of the carcass. This is in agreement with the findings of Chen *et al.* (2005) and Biswas *et al*. (2007) who reported that the whole body chemical composition of juvenile *L. vannamei* and *P. monodon* were not influenced by dietary phytase supplements. Nevertheless, increasing amounts of microbial phytase added to the diets were observed to gradually increase the protein and lipid content although not significantly. The ash content, however, was found to gradually decrease as the amount of phytase added to the diet increased. This suggests that there is better protein availability from microbial phytase supplemented diets, which may reduce the adverse consequences of dietary PA. The reduction of PA–protein complexes and increased nutrient availability may also explain this observation that is indirectly supported by the increasing protein content in the carcass (Liebert & Portz, 2005). The higher lipid composition in the carcass of *M. rosenbergii* fed microbial phytase diets compared to the control group is possibly associated to the increased lipid availability and storage. The results also suggest that microbial phytase could have a positive effect on the use of protein and lipid for growth when the prawns are fed plant protein rich diets containing 15 g PA kg^{-1}.

4.5 Conclusion

In conclusion, the results from this study suggest that firstly, the addition of phytase to diets of *M. rosenbergii* containing approximately 15 g kg^{-1} PA significantly improve nutrient utilisation

and digestibility. However, graded levels of phytase supplementation did not significantly improve growth performances and feed efficiency of *M. rosenbergii*. In addition, the inclusion of phytase did not significantly affect tissue compositions. Lastly, high levels of protein derived from plant sources can be used in the diets with no negative effects on the growth, feed utilisation, digestibility, nutrient utilisation and chemical composition of *M. rosenbergii* when their diet is supplemented with microbial phytase. Despite these promising indications, further research is needed to fully determine the economic efficiency of this approach before the incorporation of microbial phytase into diets rich in plant proteins can be considered in commercial scale *M. rosenbergii* culture.

Chapter 5 - The effects of mineral concentration in *Macrobrachium rosenbergii* fed diets with graded levels of phytic acid



The effects of mineral concentration in *Macrobrachium rosenbergii* fed diets with graded levels of phytic acid

5.1 Introduction

Phytic acid (PA) is present in most plant protein and is a major concern when plant protein ingredients are used in aquatic diets as it may impair the availability of essential minerals (Denstadli *et al*., 2006). Phytate is the primary form of phosphorus (P) in grains and plant seeds where a major portion (65–85%) of the total amount of P is bound as PA (Lall, 1991; NRC, 1993; Raboy, 1997; Pallauf & Rimbach, 1997).

Phytic acid is known to be a very strong mineral-binding agent that can inhibit mineral availability by chelating divalent and trivalent cations such as calcium (Ca^{2+}), magnesium (Mg^{2+}), zinc (Zn^{2+}), iron (Fe^{2+}), copper (Cu^{2+}) and phosphorus (P) (Pallauf & Rimbach,1997; Papatryphon *et al*., 1999; Francis *et al*., 2001). The anti-nutritional effect of PA is based on its molecule structure of six phosphate groups carrying twelve negative charges and, depending on the local pH conditions, can bind different di- and trivalent cations into stable complexes (Pallauf *et al*., 1998).

The utilisation of minerals is particularly important in crustaceans because of their role in strengthening the exoskeleton (Conklin *et al*., 1975). One of the unique physiological requirements of crustaceans that differ from vertebrates is the moulting cycle, which is necessary and forms part of the growth cycle (Aitken, 1980; Greenaway, 1985). Prior to moulting, minerals must be removed from the old exoskeleton to soften it and to permit the animal to release it. Although some minerals are temporarily stored in tissues such as the hepatopancreas and within the haemolymph for the subsequent remineralisation of the new exoskeleton, a significant proportion are lost during moulting (Davis & Kurmaly, 1993; Wang *et*

al., 2003). In nature, the cast exuviae is consumed, presumably as a mechanism to recover the resources they contain including large quantities of minerals (Huner *et al*., 1979). Minerals that are not conserved in the exuviae must be obtained externally (Davis & Kurmaly, 1993; Wang *et al*., 2003).

Most crustaceans can assimilate some minerals from the external aquatic environment that are used to satisfy part of the nutritional requirements of the animal other than those derived from dietary sources (Guillaume *et al*., 2001; NRC, 2011). Uptake is closely linked to absorption capacity via the gills, integument or mouth (Deshimaru *et al*., 1978; Davis & Kurmaly, 1993). The evaluation of the dietary requirements for particular minerals, therefore, can be particularly difficult to determine and is complicated by the ability of crustaceans (and other aquatic animals) to absorb these directly from the aquatic environment (Gilles & Piquex, 1983).

Phosphorus, for example, is an essential element of the exoskeleton of crustaceans and is required for growth. Commercial shrimp diets typically contain 1.5–2.5% phosphorus (P), most of which is from the fishmeal component (Peñaflorida, 1999). Phosphorus from PA is poorly bioavailable (Lásztity & Lásztity, 1990) as this organic form of P must first be hydrolysed by phytase to inositol and to phosphoric acid before it can be utilised and absorbed by the animal. Deficiency in phosphorus leads to poor growth, poor feed efficiency, excess lipid deposition and an increase in the activity of certain enzymes (NRC, 1993; Guillaume *et al*., 2001). A number of studies have investigated the effect of including PA in shrimp diets on the availability of P in *Marsupenaeus* [syn*. Penaeus*] *japonicus* and *Litopenaeus vannamei* (see Civera & Guillaume, 1989; Davis *et al*., 1993) and also in the diets presented to several fish species such as Atlantic salmon, *Salmo salar* (see Storebakken *et al*., 2000; Helland *et al*., 2006), Chinook salmon, *Oncorhynchus tshawytscha* (see Richardson *et al*., 1985), cobia,

Rachycentron canadum (see Lin, 2005), and rainbow trout, *Oncorhynchus mykiss* (see Overturf *et al*., 2003).

Calcium (Ca) is reported to be directly involved with P where it affects P availability (Davis *et.al,* 1993; Boonyaratpalin, 1996). Phytate (also known as phytin) is a mixed calcium, magnesium and potassium salt of PA that is present as a chelate and storage form for P in cereals, oilseeds and legume (Pallauf & Rimbach, 1997). Ca is crucial in the biological processes of aquatic animals where it is essential in the moulting process of prawn and can affect the hardening of the newly formed shell moulting (Brown *et al*., 1991). The nutritional interaction and effects between Ca and PA on mineral metabolism has not, however, been directly studied in freshwater prawns, including *M. rosenbergii*. Studies investigating the effects of Ca and PA have been conducted on *M. japonicus* and *L. vannamei* (see Civera & Guillaume, 1989) and on several fish species including Atlantic salmon, *S. salar* (see Storebakken *et al*., 2000; Helland *et al*., 2006), channel catfish, *Ictalurus punctatus* (see Gatlin & Philips, 1989), common carp, *Cyprinus carpio* (see Hossain & Jauncey, 1991) and rainbow trout, *O. mykiss* (see Spinelli *et al*., 1983; Overturf *et al*., 2003).

Zinc (Zn) appears to be the trace element whose bioavailability is most influenced by PA (Pallauf & Rimbach, 1997; Pallauf *et al*., 1998). Phytic acid as well as Ca and P levels, have been reported to influence Zn absorption and retention (Hardy & Shearer, 1985; McClain & Gatlin, 1988; Satoh *et al*., 1987, 1989; Denstadli *et al.*, 2006). High levels of Ca may exacerbate the inhibitory effect of PA on Zn absorption by forming Ca–PA–Zn complexes (Lönnerdal, 2000). Zinc is crucial for development, functioning and normal growth (NRC, 1980), although the main role is as a co-factor in many enzymatic systems involved in the utilisation of nutrients (Guillaume *et al.*, 2001) and is indispensable in the diet (Yamaguchi, 1998).

A deficiency in Zn has been reported to result in reduced growth rates in channel catfish and rainbow trout (Gatlin & Wilson, 1983; Satoh *et al*., 1983). Adverse effects of dietary Ca and PA on Zn availability have been observed in studies on Chinook salmon (see Richardson *et al*., 1985) and on channel catfish, *I. punctatus* (see Gatlin & Philips, 1989; Satoh *et al*., 1989). In addition to these, there have also been studies investigating the effect of PA on Zn availability in *L. vannamei* (see Davis *et al*., 1992, 1993) and in Atlantic salmon (Storebakken *et al*., 2000; Helland *et al*., 2006), cobia (see Lin, 2005), common carp (see Hossain & Jauncey, 1991), rainbow trout (Spinelli *et al*., 1983; Apines *et al*., 2003; Overturf *et al*., 2003) and in blue tilapia, *Oreochromis aureus* (see McClain & Gatlin, 1988).

Phytic acid is also reported to influence the concentration and bioavailability of magnesium (Mg) in common carp (Hossain & Jauncey, 1991) and in Atlantic salmon (Storebakken *et al*., 1998; Helland *et al*., 2006). Magnesium plays an essential role as a co-factor in numerous enzymatic reactions related to P, intermediary metabolism functions involved in growth, protein synthesis and in osmoregulation (NRC, 1993; Davis & Kurmaly, 1993; Guillaume *et al*., 2001). Magnesium requirements, however, can be met by absorption from the aquatic environment or extracted from dietary sources (Shearer & Asgard, 1990; Ali, 1999). The availability of Mg from plant ingredients is also reported to be quite high (Guillaume *et al*., 2001). Magnesium deficiency, however, does not suppress growth in shrimp species like *L. vannamei* (see Davis *et al*., 1992), but instead results in decreased growth, lethargy, muscle degeneration, vertebrae deformity and cataracts in fish (NRC, 1993).

One unique physiological difference in the physiology of the freshwater prawn, *Macrobrachium rosenbergii* compared to fish species is the use of a copper (Cu) based respiratory pigment, haemocyanin (Icely & Nott, 1980). A study undertaken by Depledge (1989) estimated that the

metabolic requirement of Cu in decapod crustaceans was such that 40–60% of the total body burden of Cu is required for and associated with the haemolymph. Copper constitutes the main element of proteins such as haemocyanin, which contains 0.17% Cu (Mangum, 1983) in crustaceans, as a respiratory pigment and therefore, its requirement is vital and markedly higher when compared to that found and needed by various fish species (Davis *et al.,* 1992; Davis & Kurmaly, 1993; Guillaume *et al*., 2001). Although Cu can be absorbed from the aquatic environment, its uptake is insufficient and supplementation from dietary sources, mainly from sources of animal origin, is required (Davis *et al*., 1993; Guillaume *et al*., 2001). Copper deficiency in the shrimp *L. vannamei* resulted in poor growth, enlargement of the heart with reduced Cu levels in the carapace, hepatopancreas and haemolymph (Davis *et al*., 1992). The effects of PA and Cu have also been the basis of study in experiments involving rainbow trout and common carp (Spinelli *et al*., 1983; Hossain & Jauncey, 1993; Apines *et al*., 2003), however, no similar studies conducted with *M. rosenbergii* are known.

Crustaceans are reported to have lower iron (Fe) requirements than fish (Guillaume *et al*., 2001). Research undertaken by Deshimaru *et al*. (1978) and by Kanazawa *et al.* (1984) found that excessive dietary supplementation of Fe appeared to have potentially adverse effects on the growth of *M. japonicus.* Iron can also be absorbed from the crustacean's aquatic surroundings or from the diet containing sources of animal origin (NRC, 1993; Guillaume *et al*., 2001). Iron is one of the primary metals involved in lipid oxidation and is a potent catalyst of lipid peroxidation, in addition to its ability to initiate the breakdown of PUFA (poly-unsaturated fatty acids; Davis & Kurmaly, 1993). Phytic acid is known to chelate Fe and its effects in diets given to rainbow trout (Spinelli *et al*., 1983) and common carp (Hossain & Jauncey, 1993) have been investigated.

Manganese (Mn) can form complexes with PA *in vitro* (Vohra *et al*., 1965); even though its binding affinity is reported to be lower than that for copper and zinc. Manganese functions either as an integral part of metalloenzymes in protein, lipid and carbohydrate metabolism or as a co-factor that activates metal-enzymes complexes (NRC, 1993; Guillaume *et al.,* 2001). Manganese can be absorbed from the aquatic surroundings (Miller *et al*., 1980), however, there is also an essential requirement for additional Mn from the diet (Davis & Kurmaly, 1993). Deficiency in Mn has been reported to result in poor growth rate, deformities and decreased enzyme activity in rainbow trout, carp, tilapia and shrimp (see Ishak & Dollar, 1968; Ogino & Yang, 1980; Yamamoto *et al*., 1983; Kanazawa *et al*., 1984; Guillaume *et al*., 2001). The crucial question remains on its binding affinity to PA and sensitivity to the freshwater prawn, *M. rosenbergii*, which is yet to be established.

Potassium (K) and sodium (Na) are often overlooked as these elements are readily absorbed from the animal's surrounding aquatic medium and can be found in ingredients in diets containing materials of plant origin (Davis & Kurmaly, 1993; Guillaume *et al*., 2001). Freshwater species show a wide range in their requirements for K (*i.e*. 0.3–1.2%) and this may be linked to the K concentration in the environment (Guillaume *et al*., 2001). Freshwater contains lower levels of K and Na and there is the possibility of chelation between K/Na with PA. Dietary input of K is essential, particularly in purified diets, as a deficiency can lead to anorexia, convulsions and massive mortalities in fish species (Guillaume *et al.,* 2001).

Most of the published accounts regarding the effects of PA on minerals have been focused in fish species and on selected / limited minerals. At the time that the research presented in this thesis was conducted, there were no known studies of this nature conducted with *M. rosenbergii*. The aim of the present study, therefore, was to elucidate the effects of PA on these

macro minerals and trace elements based on material from a dose-response growth trial in terms of moulting, whole body, muscle tissue and carapace mineral compositions.

5.2 Materials and methodology

5.2.1 Experimental system

The experiment was carried out as a growth trial within the Tropical Aquarium facilities in the Institute of Aquaculture, University of Stirling, UK and was conducted over a period of 140 days. Twenty four rectangular tanks (26.4 L capacity) were utilised. Each dietary treatment comprised four replicate tanks with each tank containing five individually housed experimental animals that were randomly assigned (see **Section 2.1** for details). A recirculating water system was used such that there was a controlled water flow of 1 L min^{-1} to each tank; all tanks were maintained at 28.7 ± 0.4°C. Supplemental aeration in each tank was provided using air stones. Water quality was monitored weekly and parameters (*i.e*. pH, dissolved oxygen, general hardness, calcium hardness, ammonia, nitrite and nitrate levels) were maintained within levels acceptable for the culture of *M. rosenbergii* (see **Table 2.1**). Prawns were subjected to a constant photoperiod of 12 h light: 12 h darkness.

5.2.2 Experimental animals

One hundred and twenty juvenile *M. rosenbergii* (mean initial weight 0.20 ± 0.01 g and mean initial carapace length of 6.22 ± 0.52 mm) obtained from a single batch of fertilised eggs were randomly allocated to the individual cylindrical pots in each tank. The prawns were hand fed the experimental diets twice daily (09:00, 16:00) to apparent satiation.

5.2.3 Sampling procedures

Prior to the start of the experiment, 120 juvenile *M. rosenbergii* were killed to determine the initial proximate composition. At the end of the experiment, the prawns from each treatment were killed by submergence in iced water for 1 min and then frozen at -20ºC until their proximate composition could be analysed. Only animals with a hard exoskeleton were killed and used for analysis. Prawns were starved for 24 h prior to sacrifice after which they were individually weighed and the length of their carapace measured before they were frozen. Throughout the 140-day trial, the prawns were individually weighed and measured (*i.e*. carapace length) every 20 days (details are provided in **Section 2.3.1**). Mortality and moulting events were observed twice daily as an index of diet performance and were recorded as they occurred. Survival is expressed as a percentage.

5.2.4 Experimental diets

Six experimental diets were formulated, prepared and produced at the Institute of Aquaculture, University of Stirling. The diets were formulated to meet the known optimal nutrient requirements of *M. rosenbergii* (New & Valenti, 2000; Mitra *et al.,* 2005). Fish meal, soy protein concentrate and wheat meal were selected as the main ingredients. Diets were formulated to provide approximately 420.0 g kg^{-1} of protein, 70.0 g kg^{-1} of lipid and 20 kJ g^{-1} total energy. The formulated diets were prepared such that they also included an increasing supplement of PA at the nominal inclusion rates of 0, 5, 10, 15, 20 and 25 g kg^{-1} PA. Yttrium oxide (Y_2O_3) was used as an inert marker at 2.0 g kg^{-1} in the diets and trimethylaminehydrochloride (TMAH) was utilised as an attractant (see **Section 2.5.8** and **Section 2.4.2** for details). The compositions of the vitamin and mineral pre-mixes added to the experimental diets are shown in **Table 2.2** and **Table 2.3**, respectively.

The dry ingredients were ground to a powder before the PA, fish oil (BioMar Ltd) and water components were added. A California Pellet Mill (Model CL2, San Francisco, California) was used to pellet the diets using a die size of 1.0 mm. Pellets were dried for 24 h at 40°C. Once cooled, they were packed in labelled, polythene bags and frozen at -20°C until required (complete details in **Section 2.4.1**). The chemo-attractant trimethylaminehydrochloride (TMAH) was sprayed onto a week's ration of pellets at a time (details provided in **Section 2.4.2**). The formulations, proximate chemical analysis and mineral composition of the experimental diets are shown in **Table 5.1**.

5.2.5 Chemical analysis

The nutrient compositions (*i.e.* moisture, crude protein, crude lipid, ash and gross energy content) of the six experimental diets and the whole body samples were determined by proximate analysis based on methods described in AOAC (1995) as described in the **General Materials and Methods** section of this thesis. The PA contents of each of the experimental diets were analysed according to the methods described in **Section 2.6**. For the analysis of minerals, samples of the whole body, muscle tissue and carapace were homogenised prior to analysis. Samples were first digested in 5 ml of 69% nitric acid for 1 h using an MarsXpress microwave (CEM Corporation, USA). Once cooled, the samples were diluted to 10 ml with distilled water before running them inductively on the coupled plasma mass spectrophotometer (ICP-MS) (Thermo XSeries 2) (**Section 2.5.9**). The concentration of P in each sample, however, was determined using the methods described in **Section 2.5.10**.

Table 5.1. Feed formulations and proximate composition (g kg^{-1}, as fed) of the experimental diets presented to *Macrobrachium rosenbergii*.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Ingredients	PAM0	PAM5	PAM10	PAM15	PAM20	PAM25
Fishmeal[1]	320.0	320.0	320.0	320.0	320.0	320.0
Soy prot. conc.[2]	285.0	285.0	285.0	285.0	285.0	285.0
Wheat meal	210.0	210.0	210.0	210.0	210.0	210.0
Corn starch	88.0	80.2	72.4	64.6	56.8	50.0
Fish oil[3]	40.0	40.0	40.0	40.0	40.0	40.0
Vit. premix[4]	15.0	15.0	15.0	15.0	15.0	15.0
Min. premix[5]	15.0	15.0	15.0	15.0	15.0	15.0
Yttrium oxide[6]	2.0	2.0	2.0	2.0	2.0	2.0
Carboxymethylcellulose[7]	25.0	25.0	25.0	25.0	25.0	25.0
Phytic acid[8]	0.26	6.48	11.28	16.53	21.45	26.16
Proximate composition (g kg^{-1}, as fed)						
Dry matter	908.5	908.3	908.1	908.4	907.5	907.4
Crude protein	429.4	426.7	425.9	428.8	427.3	428.5
Crude lipid	69.8	70.3	70.4	70.9	71.8	72.0
Crude fibre	20.6	20.4	19.5	20.1	18.4	18.8
Ash	68.2	69.4	69.9	70.0	70.8	71.7
NFE[9]	320.2	320.2	319.1	312.9	311.4	307.2
Gross energy[10]	19.7	19.6	19.6	19.6	19.5	19.0
Phytic acid	0.26	6.49	11.28	16.53	21.44	26.16
Mineral composition (g kg^{-1}, as fed)						
Phosphorus (P)	8.7	10.8	11.2	12.3	13.8	15.3
Calcium (Ca)	8.04	8.16	8.71	8.75	8.83	9.48
Zinc (Zn)	0.20	0.22	0.21	0.18	0.19	0.19
Magnesium (Mg)	2.43	2.24	2.42	2.42	2.45	2.48
Copper (Cu)	0.05	0.05	0.06	0.04	0.04	0.04
Iron (Fe)	0.55	0.52	0.52	0.52	0.55	0.49
Manganese (Mn)	0.07	0.08	0.08	0.08	0.09	0.09
Potassium (K)	11.47	11.59	12.67	12.47	12.48	12.61
Sodium (Na)	4.66	5.24	5.36	5.46	5.92	6.71

[1]Ewos Ltd; [2]Soy protein concentrate, BioMar UK Ltd; [3]Herring oil; [4]Vitamin premix (**Table 2.2**); [5]Mineral premix (**Table 2.3**); [6]Sigma Aldrich, 205168; [7]Sigma Aldrich, C5013; [8]Phytic acid sodium salt hydrate from rice, Sigma Aldrich, P8810; [9]Nitrogen free extract; [10](kJ g^{-1}).

5.2.6 Moults

Moult events were checked for twice daily and when found were recorded and then the exuviae were left for the prawns to consume. This is done to allow the animal to feed on as they would do in their natural environment. Total number of moults, the average number of moults and, moulting frequency per treatment (details described in **Section 2.8.2**) were calculated according to Briggs *et al.* (1991).

5.2.7 Calculations and statistical analyses

Statistical analyses were performed using SPSS 18.0 (SPSS Inc, 2010) as described in detail in **Section 2.10**. Non-homogeneous data (Levene's test) were subjected to either a square root, log or arcsin transformation before analysis. All the data are presented as the mean ± SD and were analysed by a one-way ANOVA and when appropriate, Tukey's Honest Significant Difference test was applied in order to rank significant different means. Differences were regarded as significant when $p<0.05$ (Zar, 1999). If there appears to be a relationship between two variables, significant differences of mineral concentration were estimated by the linear regression analyses method based on the mineral concentration of the samples per treatment versus the dietary PA levels.

5.3 Results

5.3.1 Chemical composition of diets

The analysed proximate composition of the six experimental diets are shown in **Table 5.1**. The protein content of the experimental diets, regardless of the PA inclusion level, varied little between them (425.9–429.4 g kg^{-1}) as did the lipid content (69.8–72.0 g kg^{-1}) and the fibre content (18.8–20.6 g kg^{-1}). Addition of PA resulted in gradually elevated amounts of ash in the

diets. All diets were similar with a gross energy value of approximately 19.6 kJ g^{-1}. The nominal inclusions of PA added into the diet were 0, 5, 10, 15, 20 and 25 g PA kg^{-1} but following analysis, they were determined to be slightly higher values at 0.26, 6.48, 11.28, 16.53, 21.45 and 26.16 g PA kg^{-1} due to the natural PA content of some of the ingredients used in the formulation of the diets.

The essential mineral content of all the experimental diets, except for Ca, were sufficient to satisfy the recommended requirements for the culture of shrimp (see **Table 5.2**; Akiyama *et al*., 1991; Davis & Kurmaly, 1993). The inclusions of PA resulted in a linear increased concentration of P (r^2=0.908), which ranged from 8.7 to 15.3 g P kg^{-1} in reflection of the increased inclusion of PA. The increased dietary P, however, might not necessarily be bioavailable. The Ca composition of the diets also increased slightly from 8.04 to 9.48 g kg^{-1} Ca. Calcium was added at a lower level than is typically added because excessive Ca levels may reduce the bioavailability of other nutrients (Davis & Kurmaly, 1993). The ratio of Ca:P is an important attribute for normal growth, thus, the dietary Ca:P ratio of all the experimental diets were between 1.2–1.3: 1 as recommended by Davis & Kurmaly, 1993 .

Table 5.2. Recommended mineral requirements for shrimp (Source: Akiyama *et al*., 1991; Davis & Kurmaly, 1993).

Mineral	Requirement (g kg^{-1})
Phosphorus (P)*	10.0 - 20.0
Calcium (Ca)	12.5 - 20.0
Zinc (Zn)	0.11 - 0.15
Magnesium (Mg)	2.0
Copper (Cu)	0.035 - 0.05
Iron (Fe)	0.15 - 0.30
Manganese (Mn)	0.02
Potassium (K)	9.0 - 10.0
Sodium (Na)	5.0 - 6.0

* Includes references from Deshimaru & Kuroki (1974); Kanazawa *et al.* (1984); Cheng *et al.* (2006).

5.3.2 Moult frequency

The total, average and moult frequency of the juvenile *M. rosenbergii* fed the graded PA diets throughout the 140 day experimental period are shown in **Table 5.3**. Moult frequency ranged from 2.64 to 3.39% but was not significantly affected by the level of PA included in the diet. Increasing PA content resulted in a higher moult frequency than those receiving lower levels. The experimental diet containing 16.53 g PA kg^{-1} showed the highest moulting frequency. There was relatively high survival throughout the experimental groups and this ranged from 85.0–95.0%.

Table 5.3. Moulting events in the *Macrobrachium rosenbergii* fed diets containing graded levels of phytic acid for a period of 140 days. The numbers given represent the observations made from the 20 animals in each test group (*i.e.* four replicates of 5 animals each).

	Dietary phytic acid, g kg^{-1}					
	0.26	6.48	11.28	16.53	21.45	26.16
∑ number of moults	74	84	88	95	91	80
Av. number of moults	3.7	4.2	4.4	4.8	4.6	4.0
Moult frequency (%)	2.64	3.00	3.14	3.39	3.25	2.86
Survival (%)	85.0 ± 0.50	95.0 ± 0.50	90.0 ± 0.60	95.0 ± 0.50	95.0 ± 0.50	90.0 ± 0.60

Av. = average

5.3.3 Whole body proximate mineral composition

The initial and final whole body mineral compositions are presented in **Table 5.4**. At the end of the experimental period the inclusions of PA significantly affect the P content where a consistent decrease in the P content with increasing PA inclusion was seen. Specifically, the P content in the whole body of groups fed 21.45 to 26.16 g PA kg^{-1} were significantly lower (p<0.05) when compared to the control and to the other experimental groups. Linear regression analyses were conducted to examine the relationship between Ca concentration in the whole

body and increasing levels of dietary PA. The results of the linear regression model indicated Ca content was evidently elevated and linked to the increasing PA inclusion (r^2= .61, F(1, 9)=14.1 , p<0.005). There were no significant differences between the groups for the Zn (0.13%–0.19%), Mg (3.15%–5.35%), K (16.77%–19.95%) and Na (5.00%–6.56%) content of the whole body that varied little. The Cu content varied from 0.06 to 0.10% and the Fe content from 0.03 to 0.08% with the highest observed levels for both trace elements being seen in the *M. rosenbergii* receiving the diet containing 11.28 g PA kg^{-1}. There were no significant differences between the groups for either of these elements when tested for using a one-way ANOVA. For the trace element Mn, there were no differences between experimental groups and levels did not appear to be influenced by the level of PA within the diet. Graded inclusion of PA resulted in significant increase (p<0.05) in the ash concentration in the whole body of *M. rosenbergii*, from 2.18% to 5.95%.

5.3.4 Proximate mineral composition of muscle tissue

The mineral composition of the muscle tissues from the *M. rosenbergii* fed the six experimental diets for 140 days are shown in **Table 5.5**. The P levels ranging between 8.04 g kg^{-1} to 10.11 g kg^{-1} within the muscle tissues were not significantly affected by the inclusion of PA within the diet. A consistent decrease from 2.74 to 1.57 g kg^{-1} in the Ca content with increasing PA inclusion was seen and linear regression analyses were conducted. The results of the linear regression model indicated Ca content evidently decrease and linked to the increasing PA inclusion (r^2= .49, F(1, 9)=8.85, p<0.01). The analysis of the other minerals showed insignificant interactions and changes in the muscle tissues. The Zn content in treatment group 0.26 g PA kg^{-1} to 26.16 g PA kg^{-1} ranged between 0.06–0.08 g kg^{-1}, the level of Mg between 1.59–1.88 g kg^{-1}, while the content of K varied between 8.89–10.98 g kg^{-1}, respectively. The Cu

(0.03–0.06 g kg^{-1}), Fe (0.03–0.05 g kg^{-1}) and Mn (0.002–0.004 g kg^{-1}) contents of the muscle tissues also varied little. The Na content decreased slightly from 4.17 to 3.61 g kg^{-1} with increasing PA inclusion, however, there were no significant interactions when tested for using a one-way ANOVA.

5.3.5 Proximate mineral composition of the carapace

The mineral composition of the carapace from *M. rosenbergii* is presented in **Table 5.6**. The main effects seen on the tissue was the increase in the concentration of Ca levels and decreased P levels, with increasing PA levels. Linear regression analyses were conducted. The results of the linear regression model indicated higher levels of PA tended to increase the amount of Ca in the carapace, *i.e.* in the control group, without an additional inclusion of PA, increased from 8.94 g Ca kg^{-1} to 11.59 g Ca kg^{-1} whereas the amount found in the *M. rosenbergii* receiving the diet with 26.16 g PA kg^{-1}, had 12.06 g Ca kg^{-1} (r^2= .63, F(1, 9)=15.2, p<0.004). In contrast, the graded inclusion of PA in the experimental diets resulted in a significant reduced P content in the carapace from 8.81 to 7.20 g P kg^{-1} (r^2= .28, F(1, 15)=5.86, p<0.03). Notably, the Zn and Cu content were significantly affected (p<0.05) by the PA in the diets. Specifically, treatment group fed 16.53 g PA kg^{-1} had higher Zn and Cu content, *i.e*. 0.12 g Zn kg^{-1} and 0.09 g Cu kg^{-1}, than the other groups which ranged from 0.03–0.06 g Zn kg^{-1} and 0.05–0.06 g Cu kg^{-1}, respectively. Magnesium levels ranged from 2.41–2.69 g Mg kg^{-1} but these were also not statistically significantly affected by the dietary inclusion of PA. In addition, there were no significant interactions or changes in the trace elements Fe and Mn in the carapace with PA inclusion and in most cases the overall mineral level effects was not significant. The inclusion of 16.53 g PA kg^{-1} in the diet, however, did result in significant changes to the K and Na content in the carapace, *i.e*. approximately 4.82 g K kg^{-1} and 11.88 g

Na kg^{-1}, when compared to the levels found in the other experimental groups, *i.e*. 2.51–3.47 g K kg^{-1} and 4.52–5.73 g Na kg^{-1}, respectively.

Table 5.4. Whole body proximate mineral compositions (g kg^{-1} dry weight) and ash content (% wet weight) of the *Macrobrachium rosenbergii* fed experimental diets with increasing amounts of phytic acid (0.26 to 26.16 g PA kg^{-1}) for a period of 140 days.

		Dietary phytic acid, g PA kg^{-1}					
	Initial[1]	0.26	6.48	11.28	16.53	21.45	26.16
Phosphorus (P)	12.05 ± 0.53	12.82 ± 0.76[a]	12.56 ± 0.26[a]	12.18 ± 0.49[a]	12.06 ± 0.70[a]	11.68 ± 1.24[b]	11.51 ± 0.48[b]
Calcium (Ca)*	14.37 ± 0.23	19.47 ± 1.74	19.93 ± 0.25	20.51 ± 1.07	21.08 ± 0.17	21.28 ± 1.49	23.95 ± 0.50
Zinc (Zn)	0.14 ± 0.01	0.13 ± 0.02	0.19 ± 0.01	0.16 ± 0.01	0.18 ± 0.02	0.19 ± 0.02	0.19 ± 0.01
Magnesium (Mg)	2.99 ± 0.06	3.87 ± 0.17	3.38 ± 0.08	4.11 ± 0.41	5.35 ± 0.40	3.15 ± 0.50	3.65 ± 0.69
Copper (Cu)	0.11 ± 0.01	0.06 ± 0.03	0.08 ± 0.01	0.07 ± 0.10	0.10 ± 0.00	0.08 ± 0.00	0.09 ± 0.00
Iron (Fe)	0.03 ± 0.01	0.04 ± 0.00	0.03 ± 0.02	0.04 ± 0.04	0.04 ± 0.01	0.05 ± 0.04	0.04 ± 0.01
Manganese (Mn)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.03
Potassium (K)	15.69 ± 0.39	19.15 ± 0.08	19.03 ± 1.24	18.22 ± 0.22	19.95 ± 0.15	17.14 ± 0.91	16.77 ± 2.19
Sodium (Na)	7.20 ± 0.79	5.00 ± 1.38	5.44 ± 0.95	6.32 ± 0.60	6.43 ± 0.35	5.69 ± 0.89	6.56 ± 0.73
Ash (%, wet weight)	2.10 ± 0.15	2.18 ± 0.47[a]	2.32 ± 0.16[a]	4.02 ± 0.41[b]	5.01 ± 0.23[bc]	5.36 ± 0.18[c]	5.95 ± 0.15[c]

Values presented represent the mean ± SD of three replicates.
Values within the same row with different letters are significantly different (p<0.05). For parameters where there were no significant differences no superscript values are provided.
[1]Values not included in the one-way Anova.
*Significant differences using linear regression analyses.

Table 5.5. Muscle tissue proximate mineral compositions (g kg^{-1} dry weight) of the *Macrobrachium rosenbergii* fed experimental diets with increasing amounts of phytic acid (0.26 to 26.16 g PA kg^{-1}) for a period of 140 days.

		Dietary phytic acid, g PA kg^{-1}					
	Initial[1]	0.26	6.48	11.28	16.53	21.45	26.16
Phosphorus (P)	9.42 ± 0.64	10.11 ± 0.29	8.82 ± 1.24	8.56 ± 0.82	8.74 ± 0.54	8.03 ± 2.21	8.04 ± 0.45
Calcium (Ca)*	7.25 ± 0.20	2.74 ± 0.73	2.65 ± 0.49	2.18 ± 0.39	1.94 ± 0.26	1.79 ± 0.13	1.57 ± 0.20
Zinc (Zn)	0.09 ± 0.01	0.07 ± 0.00	0.08 ± 0.02	0.07 ± 0.00	0.07 ± 0.01	0.06 ± 0.00	0.07 ± 0.00
Magnesium (Mg)	1.86 ± 0.07	1.88 ± 0.03	1.85 ± 0.16	1.69 ± 0.10	1.70 ± 0.25	1.59 ± 0.00	1.82 ± 0.01
Copper (Cu)	0.08 ± 0.02	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.05 ± 0.01
Iron (Fe)	0.07 ± 0.03	0.05 ± 0.03	0.05 ± 0.02	0.04 ± 0.00	0.06 ± 0.05	0.03 ± 0.01	0.05 ± 0.02
Manganese (Mn)	0.004 ± 0.00	0.002 ± 0.00	0.004 ± 0.00	0.002 ± 0.00	0.003 ± 0.00	0.002 ± 0.00	0.002 ± 0.00
Potassium (K)	11.61 ± 0.60	10.98 ± 0.84	9.71 ± 1.70	10.23 ± 0.03	10.85 ± 0.01	8.89 ± 0.89	9.95 ± 1.31
Sodium (Na)	5.98 ± 0.36	4.09 ± 0.16	4.17 ± 0.64	3.83 ± 0.02	3.85 ± 0.65	3.77 ± 0.06	3.61 ± 0.80

Values presented represent the mean ± SD of three replicates.
Statistical analysis found no significant differences between the different diets for any of the measured parameters.
[1]Values not included in the one-way Anova.
*Significant differences using linear regression analyses.

Table 5.6. Carapace proximate mineral composition (g kg^{-1}dry weight) of the *Macrobrachium rosenbergii* fed experimental diets with increasing amounts of phytic acid (0.26 to 26.16 g PA kg^{-1}) for a period of 140 days.

		Dietary phytic acid, g PA kg^{-1}					
	Initial[1]	0.26	6.48	11.28	16.53	21.45	26.16
Phosphorus (P)*	10.37 ± 3.53	8.81 ± 0.80	8.40 ± 1.11	8.57 ± 0.51	7.88 ± 0.35	7.82 ± 1.10	7.20 ± 1.28
Calcium (Ca)*	8.63 ± 0.26	8.94 ± 0.62	9.04 ± 1.54	9.55 ± 0.40	10.83 ± 0.59	11.59 ± 1.45	12.06 ± 1.33
Zinc (Zn)	0.03 ± 0.00	0.04 ± 0.00^{a}	0.03 ± 0.00^{a}	0.06 ± 0.02^{ab}	0.12 ± 0.03^{ab}	0.03 ± 0.01^{a}	0.03 ± 0.01^{a}
Magnesium (Mg)	2.29 ± 0.12	2.41 ± 0.34	2.48 ± 0.29	2.65 ± 0.19	2.69 ± 0.18	2.61 ± 0.36	2.51 ± 0.27
Copper (Cu)	0.16 ± 0.00	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.06 ± 0.00^{ab}	0.09 ± 0.02^{b}	0.05 ± 0.00^{a}	0.05 ± 0.01^{a}
Iron (Fe)	0.06 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.07 ± 0.02	0.06 ± 0.06	0.03 ± 0.01	0.02 ± 0.03
Manganese (Mn)	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Potassium (K)	9.54 ± 0.17	3.35 ± 0.12^{a}	3.41 ± 0.06^{a}	3.47 ± 0.13^{a}	4.82 ± 0.12^{b}	2.51 ± 0.40^{a}	2.81 ± 0.45^{a}
Sodium (Na)	6.43 ± 0.19	5.35 ± 0.18^{a}	4.72 ± 0.16^{a}	5.73 ± 0.99^{a}	11.88 ± 0.90^{b}	4.52 ± 0.77^{a}	4.80 ± 0.87^{a}

Values presented represent the mean ± SD of three replicates.
Values within the same row with different letters are significantly different (p<0.05). For parameters where there were no significant differences no superscript values are provided.
[1]Values not included in the one-way Anova.
*Significant differences using linear regression analyses.

5.4 Discussion

The presence of PA in a number of potentially useful plant ingredients, *i.e*. soybean meal, wheat meal, rapeseed meal and cotton seed meal (**Table 1.3**) has been a major impediment to their use as replacements for fishmeal in aquafeeds as PA effectively binds different di- and trivalent cations to form insoluble complexes (Reddy *et al.,* 1989). The aim of the present study was, therefore, to elucidate the effects of dietary PA with minerals on the growth (somatic tissue) of *M. rosenbergii*. Specifically, this study also set out to determine the changes in whole body mineral composition and that in the muscle tissues and in the carapace, when different levels of PA were included within the diet. The hypothesis under test was that PA, as a mineral-binding agent by chelating di- and trivalent cations, inhibits mineral availability.

5.4.1 Growth performance: Moult frequency

The frequency of moulting is a function of growth in crustaceans as they periodically shed their old exoskeleton resulting in a slight increase in animal external dimensions described as somatic tissue growth (Ismael & New, 2000). The findings from the present study demonstrate that the inclusion of PA in the diet of *M. rosenbergii* did not reduce the frequency of moulting. In fact, moult frequencies were higher in all PA enriched diets compared to the control group (without additional PA) although this was not significant. Similar findings of a previous study undertaken by Cheng & Guillaume in 1984 who studied mineral metabolism of *M. japonicus*, particularly the role of P source and reported sodium phytate, as disodium phosphate is the best inorganic P tested which allowed normal exoskeleton mineralisation. This was also support by Civera & Guillaume (1989) where juvenile shrimp, *M. japonicus,* fed diets enriched with 15 to 20 g kg^{-1} of sodium phytate had no detrimental effects on the number of moults; they did, however, show a tendency to increase the frequency of moulting events. Future

investigation is needed to validate further the role and the mechanism of PA as an inorganic P in mineralisation of exoskeleton.

The experimental diets of the present trial were formulated to meet the recommended nutritional and mineral requirements of *M. rosenbergii (*Mitra *et al*., 2005*)*. The optimal nutrient and satisfactory mineral formulation should have provided the nutrients necessary for normal moulting to occur. A previous study by Floreto *et al.* (2000) reported that a balanced amino acid and other nutrients were necessary for normal moulting to occur in the American lobster, *Homarus americanus*. Ambasankar *et al.* (2006) also suggested that the Ca: P ratio was an important consideration in normal growth and therefore, findings from the present trial suggest that a ratio of 1:1.2–1.3 was ideal for *M. rosenbergii*.

The moult cycle and mineral status are essentially correlated as the latter plays a crucial role in *M. rosenbergii*. Changes in the biochemistry and physiology of various tissues occur during the moult cycle including the reabsorption of Ca and other minerals during the pre-moult stage; the deposition of Ca and other materials as the exoskeleton hardens during the post-moult; and the storage of organic and mineral reserves during the inter-moult (Skinner, 1985). At the end of the current experimental trial, only hard exoskeleton animals were euthansed and processed; most of the experimental animals were either in the pre-moult or intermoult stages. Other factor that may have also influenced moult frequency in *M. rosenbergii* includes environmental conditions (*i.e*. Ca levels in the surrounding water, dissolved oxygen and temperature), food availability, hormone level and space (Chang, 1985; Cheng & Chang, 1994; Wilder *et al.,* 2009). The potential role of PA in the moult cycle is largely unknown, however, the results from the present study suggest the prospect of interactions between PA and minerals. In addition, the findings allude to *M. rosenbergii* being able to tolerate relatively high levels of PA without

severely compromising somatic tissue growth.

5.4.2 Whole body mineral composition

A number of earlier studies indicate that whole body element concentrations are homeostatically controlled (Shearer, 1984; Satoh *et al*., 1987) and therefore less than normal whole body levels of a particular element is indicative of a sub-clinical element deficiency (Storebakken *et al*., 1998). High levels of dietary PA indicate to have lower chemical composition of *M. rosenbergii*, as presented in the previous PA growth trial (see **Chapter 3**;

Table 3.5). An accompanying effect on whole body mineral composition and deposition due to PA interference with dietary mineral digestibility was expected. These effects were, however, less severe than anticipated. In the present study, the inclusion of PA in the diets presented to *M. rosenbergii* did not appear to cause detrimental effects to their other whole body mineral composition other than P. No severe pathology or prominent signs of disturbances in the mineral homeostasis of the experimental animals were observed, but there were several indications of a sub-optimal mineral availability in the prawns.

Phosphorus is expected to be a challenge in diets containing high levels of PA. Previous studies performed on *L. vannamei* reported that the presence of 15 g kg^{-1} PA in their diets resulted in a depressed P bioavailability (Davis *et al.*, 1993). At the end of the present experimental period, the concentration of the important mineral P in the whole body analysis of the carcass showed a declining trend with an increasing inclusion of dietary PA. This was most probably due to PA binding strongly with P making it unavailable. Considering that P is an essential macro minerals for the hardening of the exoskeleton, a decrease in the amount of P as consequence of dietary PA appears to influence moult frequency and to some extent the somatic tissue growth of *M. rosenbergii*. The nature of P salt, reported to influence growth and

moult frequencies, may have been an essential element overlooked. Cheng and Guillaume (1984) found that disodium phosphate increased feed consumption whereas sodium phytate increased both feed consumption and growth rate of *M. japonicus*. The mechanisms involved are complex; it may involve calcification, absorption, and acid-base balance. In addition, *M. rosenbergii* are able to uptake minerals from its aquatic surroundings; an imbalance of these elements, therefore, could be compensated for immediately by an uptake by active transport via the gills, skin or mouth (NRC, 1983; Davis & Kurmaly, 1993; Guillaume *et al*., 2001).

In the present trial, the diet formulated contained approximately 12.0 g P kg^{-1} and 8.7 g Ca kg^{-1}. Pan *et al*. (2005) recommended total dietary P level 13.3 g P kg^{-1} and Ca supplementation of 17.0 g Ca kg^{-1} for *L. vannamei*. Davis and Arnold (1998) reported that the maximum growth was obtained when *L. vannamei* was fed practical diet with original P of 9.8 g P kg^{-1} and supplemental P of 1.4 g P kg^{-1} as primarily calcium phosphorus monobasic. In Indian white shrimp, the best performance was recorded in shrimp fed diet with 10 g kg^{-1} total dietary P. Higher P and Ca supplementation rather suppressed growth (Ambasankar & Ali, 2002). It is important, however, to consider that a significant P deficiency could take a long time to appear depending on the P reserves of the animals (Civera & Guillaume, 1989) and that the P requirements of *M. rosenbergii* may have been overestimated and are lower than those cited for various shrimp species, *e.g. L. vannamei* and *M. japonicus* (see Davis *et al.*, 1992; Davis & Kurmaly, 1993).

In the present trial, significant differences in the ash content of the whole body were observed. The results of the present study are clearly in agreement with earlier studies conducted on shrimp species, *i.e. M. japonicus and L. vannamei*. Civera & Guillaume (1989) reported similar findings where the ash (P) content of the exoskeleton of *M. japonicus,* and for *L. vannamei*

although but less clearly, was significantly increased while there was a simultaneous elevation in the Ca content. The increase of Ca concentration in *M. rosenbergii* (r^2= .61, F(1, 9)=14.1, p<0.005) receiving increasing doses of dietary PA may be explained, at least partly, to the relative levels of Ca storage in the carcass. Decreases in Ca availability occur as a result of deficiencies in Ca dietary levels, which are caused by the formation of PA–Ca complexes. Conventionally, when the availability of Ca decreases, *e.g.* as a result of PA–Ca chelation, *M. rosenbergii* typically attempts to conserve Ca as a means of compensating for the diminishing levels. This exceptional regulatory mechanism was demonstrated in an earlier study by Brown *et al*. (1991) on *M. rosenbergii*, who found that there was a greater deposition of Ca when the hardness of the water was lower. It is interesting that in a contrast to the crustacean species, *i.e. M. japonicus*, *L. vannamei* and *M. rosenbergii*, the inclusion of PA in the diets presented to most fish species appeared to reduce the Ca composition within the whole body. Atlantic salmon, for example, fed diets of untreated soybean concentrate containing 18 g PA kg^{-1} had a lower whole body ash, P and Ca composition (Storebakken *et al.*, 1998). These observations are supported by the study of Helland *et al.* (2006) who concluded that an inclusion of 20.7 g PA kg^{-1} significantly reduced the P and Ca concentration of the whole body of Atlantic salmon thereby indicating deficiencies. This in general, supports the notion that the effects of PA varies from species to species and therefore, justifies the importance of the current study.

Zinc, however, may not be as important in *M. rosenbergii* as it is in fish species where it is required for the mineralisation of bones. In a contrast to the findings of the present study, a level of 15 g PA kg^{-1} in the purified diets presented to *L. vannamei* appeared to depress Zn bioavailability (Davis *et al.*, 1990). High levels of Ca were reported to excerbate the inhibitory effects of PA on Zn which are attributable to the formation of insoluble Ca–PA–Zn complexes (McClain & Gatlin; 1988; Pallauf & Rimbach, 1997; Lönnerdal, 2000). It could be explained that

phytate *per se* does not appear to reduce Zn availability but an excess of Ca or Mg alone could have decreased the availability of Zn. This is in agreement with a previous study undertaken by Spinelli *et al*. (1983) working on rainbow trout. Equally, Gatlin & Philips (1989) reported a reduced bioavailability of Zn in the bones of channel catfish that were exacerbated by the presence of high concentrations of dietary Ca and of 15 g kg^{-1} dietary phytate. The presence of high PA levels coupled with excess Ca present also appeared to decrease Zn bioavailability resulting in cataract formation in Chinook salmon (Richardson *et al*., 1985). No apparent adverse effects though were observed in the present study. It is probable that the source of protein used, *i.e*. purified diets and casein, instead of fishmeal in the previous studies abovementioned may have influenced the significant decrease in Zn bioavailability. Fishmeal was used as the main protein source in the present study and diet formulations were carefully formulated to prevent excessive Ca levels, which may have otherwise reduced the bioavailability of other nutrients (Davis & Kurmaly, 1993). The use of fishmeal may explain why only minor effects on Zn content in the whole body were seen in this trial.

The inclusion of PA in this trial did not result in negative effects of Mg concentration in the whole body of *M. rosenbergii*. A deficiency of Mg in the present study is unlikely, as the concentration of Mg derived from plant protein ingredients is reported to be moderately high (Guillaume *et al*., 2001). In the present trial, the feed formulation contained approximately 49.5% plant protein, SPC and wheat meal and dietary Mg was approximately 2.4 g Mg kg^{-1}. The requirement level of dietary available Mg for *M. rosenbergii* is similar to that of *L. vannamei*, 2.60–3.46 g Mg kg^{-1} (Cheng *et al.*, 2005). The Mg content in the whole body showed no significant differences between the graded levels of PA treatments, indicating that *M. rosenbergii* has a very strong regulatory mechanism of Mg in secretion and reabsorption to make the ion in tissues stable. *Macrobrachium rosenbergii, P. monodon* and most crustaceans

are able to regulate Mg between hemolymph and aquatic environment by effector organs, including gills, integument and antennal glands (Lin *et al*., 2000).

Copper is an important trace element for freshwater prawns as it forms an important component (*i.e.* about 0.17%) of haemocyanin (Mangum, 1983). In the present trial, Cu concentrations in the whole body of *M. rosenbergii* were not affected by graded inclusions of PA in the diet. The reason for the lack of an observed effect on Cu in *M. rosenbergii* fed a PA supplemented diet is not clear. It is possible though that there were lower levels of insoluble PA–Cu complexes formed and that the capacity of other chelators, protein in particular, to replace PA from PA–Cu complexes, may have helped in making Cu available.

Phytic acid can also form a Fe chelate (Graf *et al*., 1987). In the present study, the differences in the Fe compositions across all treatment groups were minor and varied from 0.03 to 0.05 g kg^{-1} and were not statistically significant from one another. The coordination chemistry of Fe and PA chelation is largely unknown, however, the similar measured concentrations determined in the present study are also most probably due to the low solubility of these polyferric PA chelates (Graf *et al*., 1987).

No reports on the effects of dietary PA on Mn are available for shrimp species, *i.e*. *L. vannamei* or *M. japonicus*, although Mn has been reported to form complexes with PA *in vitro* (Vohra *et al*., 1965). It is, however, worthy to note that the trace element Mn, in the current trial, was not entirely influenced by the inclusion of dietary PA. This is most likely due to the weaker binding affinity of Mn when compared to Zn or Cu (Lönnerdal, 2002). Moreover, the Mn requirements for freshwater prawns such as *M. rosenbergii* are likely to be lower and could be acquired from its aquatic surroundings or from dietary sources.

The macro mineral potassium (K) in the current trial was not affected by dietary inclusion of PA. The K content of the whole bodies of *M. rosenbergii* fed the PA supplemented diets were found to be slightly lower than those animals fed the control diet and there were no correlations to the graded inclusion of PA in the diet. This is presumably because dietary supplementation of K is not necessary and dietary K levels does not influence the whole body K concentration, similar to those reported in *L. vannamei* by Zhu *et al*. (2006).

The specificity of PA and its binding to minerals is pH dependent and is a crucial factor that could explain the results observed in the current trials with *M. rosenbergii*. When the concentration of di-/trivalent cations exceeds the concentration of PA, insoluble chelates of PA and the minerals can form at neutral and basic pH and precipitate out (Adeola & Sands, 2003). In most crustaceans, the foregut fluid has a pH of between 5 and 7 (Dall & Moriarty, 1983), and so the absence of acid digestion in *M. rosenbergii* (Guillaume, 1997) is probably an advantageous factor in this context.

The concentrations of the macro minerals and trace elements determined from the diets that were prepared for the current trial were within the published recommended requirements (see **Table 5.2**). The trial found that the inclusion of dietary PA did have several major dietary effects on whole body element compositions. Generally, the adverse effects of dietary PA on the macro minerals and trace elements probably depends on a number of factors that includes the concentration of PA within the diet, the duration over which “the diet” is fed, the strength of its binding with different minerals, and, pH levels of acid digestion. There are also other factors which may also have influenced the whole body mineral concentrations of *M. rosenbergii* receiving diets high in PA and these include: species-specific responses; the presence of other mineral binding agents such as dietary fibre, oxalic acid and tannins all of which may compete

with PA for binding with minerals (Thompson, 1993); the source of PA used and whether this is derived from dietary protein, intestinal or bacterial phytase; and, the metabolic adaptation of the specific species to high dietary PA in the diet.

5.4.3 Muscle tissue mineral composition

The interactions were between Ca and dietary PA were apparent where the increase in dietary PA levels resulted in consistent decreased of Ca concentrations in the muscle tissues (r^2= .49, F(1, 9)=8.85, p<0.01). Research findings in contradiction to this though have been reported for *M. japonicus*, where Ca levels in the muscle tissues were apparently unaffected by the presence of PA (Civera & Guillaume, 1989). The fall in the Ca concentrations in the muscle tissues is presumably due to the formation of insoluble PA–Ca complexes. The P composition in the muscle tissues of *M. rosenbergii,* however, was not significantly affected by the inclusion of dietary PA. The extraordinary ability of *M. rosenbergii* to compensate for low mineral concentrations either through intake via their diet or from the aquatic environment may explain the lower impact of PA chelations on P in the muscle tissues that were seen in the present trial. More studies, therefore, are needed to have a better understanding of the relationship of dietary PA and P sources and to determine the ability of *M. rosenbergii* to hydrolyse the PA and take advantage of its inositol content.

The differences in the trace element compositions (*i.e*. Zn, Cu and Fe) in the muscle tissues of *M. rosenbergii* were subtle among the dietary treatment groups and control. There was, consequentially, no apparent correlation in the observations and the increasing inclusions of PA. Within the compositions, the concentration of Mn in the muscle tissues, notably, appeared uninfluenced by dietary PA. It is presumed, therefore, that the muscle tissue of *M. rosenbergii* is not a major site for storage of these trace elements.

Likewise, changes in the macro mineral compositions (*i.e*. Mg, K and Na) in the muscle tissues were not significantly evident. The concentration of Mg in the animals fed the control diet was only slightly higher than the levels determined in the treatment groups. A decrease in the Na concentration from 4.09 to 3.61 g kg^{-1} with an increase in the inclusion rate of PA (*i.e.* between the control and the treatment group receiving the highest inclusion of PA) in the muscles was seen. These results suggest the prospect of PA chelations with Mg and Na does occur in the muscle tissues but at a lower level. In addition, some of the PA binding functions could be saturated by the present of these minerals within the diets and/or the aquatic environment. An imbalance of these cations due to the insoluble PA formation could, therefore, be compensated for by active transport. On the other hand, no consistency in the K concentration across all diet groups was seen.

The reasons for these variable responses are not readily apparent but may reflect differences in the digestive, metabolic and storage systems of each crustacean species. In addition, a number of other factors may have influenced the effect of these macro minerals and trace elements, including: the intake levels of the nutrient; interactions with other nutrients; chelators; inhibitors; physiological and pathological states of the animal; and, water chemistry.

5.4.4 Mineral composition of the carapace

Growth is a less sensitive indicator of mineral status than is the mineral content of a specific tissue, as animals can often sacrifice mineral stores to compensate for deficiencies (Baker, 1986). The carapace, therefore, was selected for analysis because it is a distinct structural tissue (Dall & Moriarty, 1983).

The increasing inclusions of PA in the diets were found to decrease the P composition in the

carapace of *M. rosenbergii* (r^2= .28, F(1, 15)=5.86, p<0.03). Contrast observations were recorded for the Ca compositions where graded inclusions of PA increased Ca levels in carapace (r^2= .63, F(1, 9)=15.2, p<0.004). A similar finding has been reported in a previous study undertaken by Civera and Guillaume (1989) where 17.5–20 g kg^{-1} phytic P tend to increase the Ca content of the hepatopancreas in *M. japonicus*. It is presumable that *M. rosenbergii* was not able to compensate the decreasing mineral availabilities through the uptake from its aquatic surroundings due to the the insoluble formation of PA–P and PA–Ca complexes. The high concentrations of Ca in the carapace highlight the importance of the carapace as the main area for storage in *M. rosenbergii*.

In the present study, the inclusion of PA in the diets presented to *M. rosenbergii* result in significant differences in the concentrations of Zn, Cu, K and Na found in the carapace. At the end of the trial, there were perceptible trends in the concentrations of Zn, Cu, K and Na. The increasing Zn, Cu, K and Na concentration levels correlate with increasing levels of PA in the diet reaching a peak at 11.28 g PA kg^{-1} before decreasing at higher levels of PA, *i.e*. 21.45–26.16 g PA kg^{-1}. The reason of this may be due to the ionoregulation ability of *M. rosenbergii*. For example, in some euryhaline shrimp, haemolymph K levels can be regulated to some extent by means of Na/K-ATPase when environmental K concentrations vary greatly (Dall & Smith, 1981). Zinc, Cu, K and Na plays essential role in aiding the exuviation process (Wilder *et al*., 2009). It is, therefore, possible that PA at this inclusion level of 11.28 g PA kg^{-1} may facilitate in accumulating these minerals, retained in preparation for edysis process before moulting as most samples collected were at pre-moult and inter-moult stage. Not very much is known on the metabolism and the response of Zn, Cu, K and Na concentration in *M. rosenbergii* when fed graded PA levels; hence, further research is needed.

The inclusions of dietary PA are unlikely to cause deficiencies in the trace elements of Fe, Mg and Mn in *M. rosenbergii* as the concentrations of these elements in the carapace did not differ significantly between treatment groups.

5.5 Conclusion

The results of this study suggest that the inclusion of high doses of PA in the diets presented to *M. rosenbergii* showed no negative effects on somatic tissue growth. Evidently, P and Ca content in the whole body were significantly decreased by the increasing inclusion of PA in the diets fed to *M. rosenbergii*. It was also determined that Pas are mineral binding agents, binding Ca in particular and reducing their availability. Moderate changes in the composition of Zn, Mg, Fe, Cu, K and Na in the presence of dietary PA inclusions were also seen. In conclusion, the results confirm that the chelation of PA with minerals is a concern and that considerations should be taken when formulating diets for *M. rosenbergii* and using plant-based ingredients that may result in a high PA content in the diet and subsequently an impact on mineral availability.

Chapter 6 - Microbial phytase and its effects on the mineral concentrations in *Macrobrachium rosenbergii*

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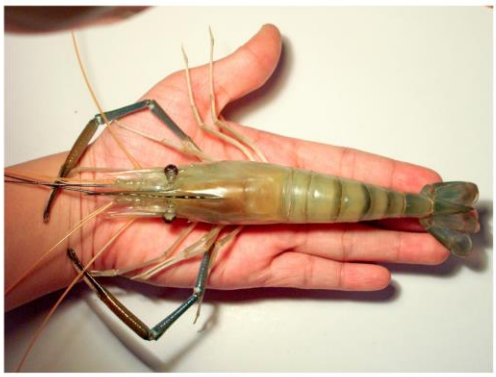

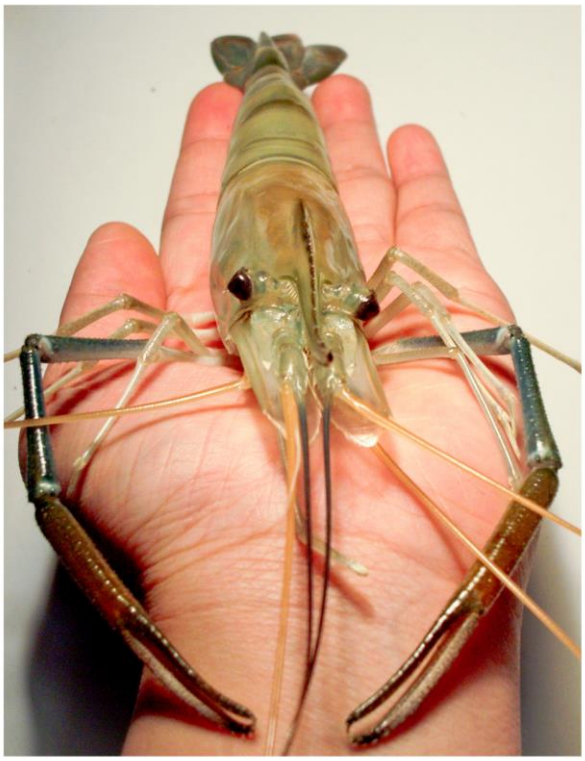

Microbial phytase and its effects on the mineral concentrations in

Macrobrachium rosenbergii

6.1 Introduction

Inorganic phosphorus is commonly supplemented to meet the low available phosphorus (P) in plant protein diets (Cao *et al.,* 2008). Since dietary phytic acid (PA) has been found to interfere with the availability of minerals, it seems more rational to degrade PA and consequently liberate P and other minerals binding with phytase than to add inorganic P (Denstadli *et al*., 2007). In addition, the beneficial effect of phytase of releasing macro minerals and trace elements from complexes with PA may also enhance growth performances (Selle *et al*., 2000; Brenes *et al.*, 2003).

The effects of phytase supplementation on mineral availability in fish species have been studied more extensively. In general, the most extensive mineral investigated by the supplementation of phytase is P. The availability of P was found to be markedly enhanced by the supplementation of phytase in plant protein based diets fed to Atlantic salmon, *Salmo salar* (see Storebakken *et al.,* 1998; Denstadli *et al.,* 2007), common carp, *Cyprinus carpio* (see Schäfer *et al*., 1995), channel catfish, *Ictalurus punctatus* (see Jackson *et al*., 1996; Li & Robinson, 1997), Korean rockfish, *Sebastes schlegeli* (see Yoo *et al*., 2005), rohu, *Labeo rohita* (see Baruah *et al*., 2005), Pangas catfish, *Pangasius pangasius* (see Debnath *et al*., 2004), rainbow trout, *Oncorhynchus mykiss* (see Cain & Garling, 1995; Rodehutscord & Pfeffer, 1995; Forster *et al*., 1999; Suguira *et al*., 2001; Cheng & Hardy, 2002; Vielma *et al*., 2004), striped bass, *Morone saxatilis* (see Hughes & Soares, 1998; Papatryphon *et al.*, 1999; Papatryphon & Soares, 2001), European sea bass, *Dicentrarchus labrax* (see Oliva-Teles *et al*., 1998) and Nile tilapia, *Oreochromis niloticus* (see Liebert & Portz, 2005; Cao *et al.,* 2008). In Nile tilapia, phytase supplementation of between 500 and 1500 FTU kg^{-1} improved P

availability (Cao *et al*., 2008) and bone mineralisation (Furuya *et al.,* 2001), whereas the addition of 750 FTU kg^{-1} resulted in the same performance as including inorganic P (Liebert & Portz, 2005). In contrast to these studies, Chen *et al*. (2005) reported that phytase supplementation of the diets presented to *Litopenaeus vannamei* did not affect the P content of the whole body.

Calcium (Ca) availability has been reported to improve with the supplementation of phytase of between 500 and 1500 FTU kg^{-1} when given to Nile tilapia (Furuya *et al*., 2001). Portz & Liebert (2005) reported that a phytase supplementation of 1000 FTU kg^{-1} in the diet given to Nile tilapia resulted in growth rates and mineral utilisation that were similar to a plant based diet that was supplemented with inorganic P. Storebakken *et al.* (1998) also demonstrated that the pre-treatment of a soy concentrate-based diet with a supplement of phytase presented to Atlantic salmon resulted in the complete hydrolysis of PA and improved the Ca availability and utilisation equivalent to a FM based diet. In contrast to this, Ca availability in *L. vannamei* was reported to be unaffected by phytase supplementation in the diets given to them (Chen *et al*., 2005).

A number of previous studies have also demonstrated an improved zinc (Zn) availability and utilisation in Atlantic salmon (Storebakken *et al.,* 1998), *P. pangasius* (see Debnath *et al*., 2005) and rainbow trout (Suguira *et al*., 2001) when their diets were supplemented with phytase. Additionally, Zn absorption was marginally improved when at least 2000 FTU kg^{-1} was supplemented in the diet fed to striped bass (Papatryphon *et al.,* 1999). Storebakken *et al*. (1998) reported improved magnesium (Mg) availability in Atlantic salmon, while Vielma *et al.* (1998) reported that Mg deposition was enhanced in rainbow trout when diets were supplemented with phytase. Although copper (Cu) availability was not significantly affected by dietary PA, as seen in the previous PA trial detailed in **Chapter 5**, it is an essential trace

element as it constitutes a principal component of haemocyanin, a protein that plays a role in crustaceans similar to that of haemoglobin in oxygen transportation (Guillaume *et al*., 2001). A number of earlier studies have demonstrated positive effects regarding Cu when the experimental diets were supplemented with phytase, and these studies include those conducted with *P. pangasius* (see Debnath *et al*., 2005), rainbow trout (Suguira *et al*., 2001) and striped bass (Papatryphon *et al*., 1999). In addition to these, there was an apparent improvement in the absorption of iron (Fe) in the studies performed with *P. pangasius* (see Debnath *et al*., 2005) and striped bass (Papatryphon *et al*., 1999) when the diets were supplemented with phytase. Vielma *et al*. (1998) reported that a phytase treatment of 1000 FTU kg^{-1} enhanced the deposition of manganese (Mn) in the tissues of rainbow trout, while Debnath *et al.* (2005) found that Mn absorption in *P. pangasius* was better in the experimental groups receiving the phytase supplements than those in the control group. There are, however, no known studies on the effects of phytase on Cu, Fe and Mn in *M. rosenbergii*.

Very little is known regarding the effects of phytase supplementation on mineral availability in shrimp. In 2004, researchers at the Universidad Autónoma de Nuevo León, Mexico, reported that a supplement of 1000 FTU g^{-1} in diets containing 40% pea protein flour fed to Pacific white shrimp resulted in higher dry matter and protein compared to those receiving diets without (reported in Fox *et al*., 2006). A study by Chen *et al.* (2005) suggested that phytase supplementation had significant effects on the compositions of the carapace and serum but not on the growth performance of juvenile *L. vannamei.* To date, however, no known report has yet to be found on the effects of phytase supplementation on the minerals in the freshwater prawn, *M. rosenbergii*.

The aim of the current study, therefore, was to investigate the effects, interactions and efficiency of microbial phytase on the mineral availability of P, Ca, Zn, Mg, Cu, Fe, Mn, K and

Na in *M. rosenbergii* when added to plant protein based diets containing high level of PA presented to them.

6.2 Materials and methodology

6.2.1 Experimental system

The experiment was conducted within the Prawn Unit in the Tropical Aquarium of the Institute of Aquaculture University of Stirling over a period of 80 days. Twelve tanks (26.4 L) (three replicates per treatment) containing five nylon mesh pots per tank each pot housing an individual animal were used (for precise details, see **Section 2.1**). A recirculating water system supplied water to each tank at a flow rate of 1 L min^{-1}; the water depth in each tank was 17 cm. Air stones were used to provide additional aeration to each tank. The average water temperature throughout the experimental period was 28.4 ± 0.13°C. Water quality parameters, *i.e*. dissolved oxygen, pH, general hardness, calcium hardness, ammonia, nitrite and nitrate, were determined and monitored weekly and maintained within acceptable levels (see **Table 2.1**). A consistent photoperiod regime of 12 h daylight and 12 h darkness was maintained throughout the trial.

6.2.2 Experimental animals

Sixty juvenile Malaysian freshwater prawns, *M. rosenbergii,* obtained from a single batch of eggs, with an average initial weight of 0.61 ± 0.26 g $juvenile^{-1}$ and an average initial carapace length of 9.62 ± 1.59 mm $juvenile^{-1}$ were used for this experiment. Each animal (5 animals per tank; 3 replicates per treatment) was randomly assigned to the nylon mesh pots, where each mesh pot held a single animal so as to prevent cannibalism (**Figure 2.2**). Experimental animals were fed experimental diets to apparent satiation twice daily at 08:30 and 16:30 by hand.

6.2.3 Sampling procedures

The weight and carapace length of each experimental animal was measured each 20 days throughout the 80 day experiment (details in **Section 2.3.1**). Prior to the start of the experiment, a total of 104 juvenile *M. rosenbergii* were sampled, for the purposes of determining the initial proximate composition, by immersion in ice water for 1 minute and then frozen until they could be analysed. On completion of the 80-day experiment, only those animals with a hard exoskeleton within each test group were euthanized and processed. Faecal samples were collected by siphon, pooled for each treatment and then immediately centrifuged (Centaur 2 Sanyo) for 10 min at 2268 × g to remove excess water before they were frozen and stored until they could be analysed. Moulting and mortality events were recorded daily. Exuviae were noted and left to allow the prawn to naturally feed on throughout the experiment.

6.2.4 Experimental diets

Four experimental diets containing high levels of plant protein ingredients were formulated and prepared in the processing room situated within the Institute of Aquaculture, University of Stirling. The diets were formulated to meet the known, optimal nutrient requirements of *M. rosenbergii* (New & Valenti, 2000; Mitra *et al.,* 2005). High protein soybean meal, wheat gluten and wheat meal were used as the primary protein sources and fish soluble (see precise details in **Section 2.4.1**) were added as a flavour to make the pellets more palatable to the prawns. The diets were formulated to produce approximately 440.0 g kg^{-1} of protein, 92.0 g kg^{-1} of lipid and 20 kJ g^{-1} total energy.

The experimental diets consisted of three diets formulated to provide different levels of microbial phytase supplement (*i.e.* 0 FTU kg^{-1}, 1000 FTU kg^{-1} and 2000 FTU kg^{-1}) and a control. The control diet, unlike the three test group diets (*i.e.* 0 FTU kg^{-1}, 1000 FTU kg^{-1} and

2000 FTU kg^{-1}), included a mineral premix (35 g kg^{-1}) and monosodium phosphate content (8 g kg^{-1}) (**Table 6.1**). High levels of dietary PA of up to 15 g kg^{-1} (Sigma Aldrich) were added to augment the responses of phytase supplementation and retain consistency. Each dose of microbial phytase was prepared by adding it to 50 mL of distilled water and then adding this to 1 kg of the formulated diet. Distilled water was added to the control and 0 FTU kg^{-1} diet, to maintain equal levels of moisture. The preparation of the experimental diets is described in detail in **Section 2.4**. The formulations and the proximate compositions of the experimental diets are shown in **Table 6.1**.

6.2.5 Chemical analysis

Moisture, crude protein, crude lipid, ash and the crude fibre contents of the experimental diets and from whole body samples were determined as described in the "General Materials and Methods" section of this thesis (see **Section 2.5**). The methods used are based on those described in AOAC (1995). Minerals were analysed by the analytical methods described in **Sections 2.5.9** and **2.5.10**. Phytic acid analyses were determined using the methodology described in **Section 2.6**. Phytase concentrations in the dietary treatments were analysed according to the procedures described by Engelen *et al.* (1994) and are provided in detail in **Section 2.7**.

6.2.6 Moults

The total number of moult events, the average number of moults and the moulting frequency per treatment (details described in **Section 2.8.2**) were calculated according to the method in Briggs *et al.* (1991). The experimental animals were assessed twice per day and any moults were recorded; the exuviae were left to allow the animal to feed on these as they would in their natural environment.

Table 6.1. Feed formulations, proximate composition (g kg^{-1}, as fed) and mineral composition of the experimental diets presented to the juvenile *Macrobrachium rosenbergii* over a period of 80 days. The experimental diets are identified as 0–2000 FTU kg^{-1} of microbial phytase.

Ingredients	Diet 1 Control	Diet 2 0 FTU kg^{-1}	Diet 3 1000 FTU kg^{-1}	Diet 4 2000 FTU kg^{-1}
High protein soybean[1]	355.0	355.0	355.0	355.0
Wheat gluten[1]	300.0	300.0	300.0	300.0
Wheat meal	115.0	158.0	158.0	158.0
Fish soluble[1]	50.0	50.0	50.0	50.0
Fish oil[2]	80.0	80.0	80.0	80.0
Vitamin pre-mix[3]	10.0	10.0	10.0	10.0
Mineral pre-mix[4]	35.0	0.0	0.0	0.0
Yttrium oxide[5]	2.0	2.0	2.0	2.0
Monosodium phosphate[6]	8.0	0.0	0.0	0.0
Carboxymethylcellulose[7]	30.0	30.0	29.0	28.0
Phytic acid[8]	15.0	15.0	15.0	15.0
Phytase[9] (FTU kg^{-1})	0.0	0.0	1000	2000
Proximate composition (g kg^{-1}, as fed)				
Dry matter	921.0	924.3	925.6	925.5
Crude protein	445.0	440.1	441.9	438.6
Crude lipid	91.9	93.5	92.6	93.7
Crude fibre	12.5	12.7	12.9	12.8
Ash	80.7	42.7	42.5	44.6
Nitrogen free extract	290.9	335.3	335.7	335.8
Gross energy (kJ g^{-1})	20.30	20.89	20.79	20.83
Phytic acid	15.37	14.67	13.96	12.73
Phytase (FTU kg^{-1})	59.0	61.0	1127.0	2119.0
Mineral composition (g kg^{-1}, as fed)				
Phosphorus (P)	9.91	8.01	7.85	7.93
Calcium (Ca)	7.76	2.63	2.51	2.25
Zinc (Zn)	0.06	0.04	0.04	0.04
Magnesium (Mg)	1.60	1.39	1.32	1.21
Copper (Cu)	<0.01	<0.01	<0.01	<0.01
Iron (Fe)	0.2	0.1	0.1	0.1
Manganese (Mn)	0.04	0.02	0.02	0.02
Potassium (K)	8.39	8.31	8.15	7.53
Sodium (Na)	4.44	2.92	2.81	2.51

[1]BioMar UK Ltd; [2]Herring oil; [3,4]As listed in **Table 2.2** and **Table 2.3**, according to Jauncey & Ross (1982); [5]Sigma Aldrich, 205168; [6]Sodium phosphate monobasic, Sigma Aldrich, S8282; [7]Sigma Aldrich, C5013; [8]Phytic acid sodium salt hydrate from rice, Sigma Aldrich, P8810; [9]Microbial phytase, Sigma Aldrich, P1259.

6.2.7 Calculation and statistical analysis

Growth performances, *i.e.* weight gain, carapace length increment, specific growth rate (SGR) and hepatosomatic index (HSI), were calculated using the formulae provided in **Section 2.8**. The animals were weighed and measured at the start of the trial and every 20 days thereafter until the end of 80 day trial producing four sets of data in total.

Statistical analyses were performed using SPSS 18 (SPSS Inc, 2010) and the data are presented as the means ± SD. Data that were identified as non-homogeneous by using Levene's test, were subjected to square root or arcsin transformation before analysis. Significant differences between the dietary treatments were determined by one-way ANOVA. Where appropriate, Tukey's Honest Significant Difference test was applied in order to rank the significantly different means. Differences were regarded as significant when $p<0.05$ (Zar, 1999).

6.3 Results

6.3.1 Chemical composition of diets

Proximate analysis of the four experimental diets showed that the dietary protein, lipid and fibre levels were approximately 441.4 g kg^{-1}, 92.9 g kg^{-1} and 12.7 g kg^{-1}, respectively, and were consistent among the four dietary treatments (**Table 6.1**). The ash content, however, were two-fold in the control diet, *i.e.* 80.7 g kg^{-1}, due to the inclusion of a mineral premix and monosodium phosphate compared to diets containing microbial phytase and lacking mineral premix (*i.e.* 0 FTU kg^{-1}, 1000 FTU kg^{-1} and 2000 FTU kg^{-1}). The gross energy values were consistent at approximately 20 kJ g^{-1} regardless of the diet. Phytase activity levels in the dietary treatments were close to the intended levels. Some phytase activity, however, was also observed in the 0 FTU kg^{-1} diet and control treatment and will be commented upon. The diets

were formulated to meet all the known nutrient requirements of *M. rosenbergii*, although they were designed to contain high levels of PA in order to determine the responses of phytase supplementation. The increasing inclusion of microbial phytase had a direct effect on the PA composition of the diets. Specifically, the PA content of the 0 FTU kg^{-1} to 2000 FTU kg^{-1} diet decreased slightly from 14.67 g PA kg^{-1} to 12.73 g PA kg^{-1}, which is likely due to the enzymatic activity of phytase.

The mineral compositions of the four experimental diets are presented in **Table 6.1**. The mineral premix was eliminated from the formulations except for the control treatment. The absence of the mineral premixes resulted in minimal P and Ca concentrations, which resulted from the animals or from inorganic sources and were found to range between 7.85–9.91 g P kg^{-1} and 2.25–2.63 g Ca kg^{-1} when fed 0–2000 FTU kg^{-1} diets. Plant protein based ingredients are reported to be moderately high in Mg (Guillaume *et al*., 1999), hence, it was not surprising that the Mg and K levels that were found in the 0–2000 FTU kg^{-1} diets and ranged between 1.21–1.39 g Mg kg^{-1} and 7.53–8.31 g K kg^{-1}. Traces of the other mineral elements, *i.e.* Zn, Cu, Fe, Mn and Na, were also detected in the formulations and were obtained completely from the ingredients.

6.3.2 Growth performances

All groups of *M. rosenbergii* displayed adequate growth and feed utilisation, *i.e*. increase in the carapace length, weight gain, SGR, feed intake (FI), feed conversion ratio (FCR) and protein efficiency ratio (PER) after 80 days (**Table 6.2**). After the 80 day period, increases in the carapace length ranged from 3.34 to 4.48 mm, although the differences between the groups were not statistically significant. The terminal weight of *M. rosenbergii* fed the control diet was significantly lower than those in the other treatment groups. Weight gain ranged from 0.98 to

1.36 g and *M. rosenbergii* fed the control diet had significant lower weight gain compared to all other groups. The SGRs ranged between 1.30–1.53 among the treatment groups but they were not statistically significant. The HSIs were determined to range from 2.97% to 3.93%, with the control having a value of 3.23%. Survival between the treatment varied from 46.7 to 80.0%; significant higher (p<0.05) levels of mortality were seen in the *M. rosenbergii* fed the diet containing 1000 FTU kg^{-1} phytase.

The feed intake ranged from 0.015–0.021 over the experimental period. FCR was determined to vary from 1.02 to 1.25. No significant differences were shown by ANOVA between the four treatment groups either on feed intake or FCR. Similarly, PER ranged between 1.84 and 2.30 and no significant differences among the groups were seen.

6.3.3 Whole body proximate composition

At the start of the feeding trial, the proximate composition of the *M. rosenbergii* whole body was 75.23% moisture, 15.98 g kg^{-1} crude protein, 1.39 g kg^{-1} crude lipid, 2.12 g kg^{-1} ash and 4.50 kJ g^{-1} gross energy (**Table 6.3**). After the 80 day feed trial, the moisture content ranged from 75.00% to 76.54% while the lipid content ranged from 1.08 g kg^{-1} to 1.56 g kg^{-1}. The total protein content in the control was 16.35 g kg^{-1}, while in the phytase supplemented group it varied from 15.47 g kg^{-1} to 16.49 g kg^{-1}. There were, however, no significant differences among the dietary treatments. The ash content was observed to increase slightly from 1.56 g kg^{-1} to 1.74 g kg^{-1}. The energy content of the final carcass ranged between 4.76 kJ g^{-1} to 5.08 kJ g^{-1} between treatment groups. Statistical analysis using ANOVA, however, did not reveal any significant differences between the four treatments.

6.3.4 Moults

The total, average and moult frequency of the juvenile *M. rosenbergii* fed the diets containing microbial phytase supplementations are presented in **Table 6.4**. The frequency of moulting appeared to be largely unaffected by the graded supplementations of microbial phytase and this ranged from 3.33–4.08%. The freshwater prawns fed diets supplemented with 2000 FTU kg^{-1} phytase were recorded as having the highest moulting frequency, although the result was not statistically significant from the other diet groups. The moult frequency in the control treatment group was 3.75%.

6.3.5 Whole body proximate mineral compositions

The initial and final whole body mineral compositions for each dietary treatment and the effects and interactions of the mineral level and microbial phytase inclusions are shown in **Table 6.5**. In general, the increasing inclusions of microbial phytase in the diets significantly increased the Ca content in the carcass. For instance, a dietary treatment with either a 1000 or 2000 FTU g kg^{-1} inclusion resulted in the prawns having significantly higher (p<0.05) levels of Ca when compared to the prawns in the control group and in the group not receiving an inclusion of phytase, *i.e.* 0 FTU kg^{-1}. Equally, similar significant interactions (p<0.05) were observed for other macro minerals including Mg, K and Na with the graded inclusion of microbial phytase in the diet. Specifically, the prawns receiving the diets either 1000 or 2000 FTU g kg^{-1} had significantly higher (p<0.05) concentrations of Mg, K and Na that varied from 0.22 g kg^{-1} to 0.33 g kg^{-1}, 1.02 g kg^{-1} to 1.37 g kg^{-1} and 0.50 g kg^{-1} to 0.68 g kg^{-1}, respectively. In contrast to this though, the mineral P and the trace elements Zn, Cu, Fe and Mn were not influenced by the graded inclusion levels of microbial phytase in the diet. No significant differences in the P and trace element content of the whole body of the prawn in the four different phytase treatments were seen.

6.3.6 Muscle tissue proximate mineral composition

The initial and final mineral composition of the muscle tissues sampled from the experimental prawns are shown in **Table 6.6**. Of note are the significant interactions (p<0.05) of the Ca and Zn levels in the muscle with the increasing inclusion of microbial phytase. Specifically, Ca and Zn were found in higher concentrations in the muscles in the 2000 FTU kg^{-1} phytase treatment. On the other hand, no significant differences were seen for K even though the K content in the muscles increased slightly from 1.57 to 1.73 g kg^{-1} with an increasing inclusion of microbial phytase. The muscle P composition in treatments 0–2000 FTU kg^{-1} varied from 6.76 g kg^{-1} to 8.54 g kg^{-1}, while the P content in the control treatment was 7.83 g kg^{-1}. Graded inclusions of phytase did not affect the P composition in the muscle tissues of the experimental prawns and no significant interactions were shown (ANOVA). There were no significant interactions with the other minerals. The levels of Mg, Cu, Fe and Na in the muscles varied little and were determined to be between 0.14–0.17 g kg^{-1}, 0.03–0.05 g kg^{-1}, 0.02–0.03 g kg^{-1} and 0.40–0.48 g kg^{-1}, respectively. Likewise, the Mn content of the muscle tissue appeared to be unaffected by the levels of microbial phytase included in the experimental diets.

6.3.7 Proximate mineral composition of the carapace

The initial and final mineral composition of the carapace from the experimental prawns fed diets containing graded amounts of microbial phytase are presented in **Table 6.7**. The main effect observed was the significant increase (p<0.05) in the concentration of Ca from 12.17 to 13.58 g kg^{-1} in the carapace in the groups fed diets containing 0–2000 FTU kg^{-1} compared to those in the control group which were 11.17 g kg^{-1}. Equally, the treatment group fed diets containing between 1000–2000 FTU kg^{-1} demonstrated significantly higher (p<0.05) Mg levels (*i.e*. 0.32–0.34 g Mg kg^{-1}) in the carapace when compared to the prawns sampled from the 0 FTU kg^{-1} and the control groups who had levels of between 0.26–0.27 g Mg kg^{-1}. Conversely, the

inclusion of 1000–2000 FTU kg^{-1} resulted in a slight but significant decrease (p<0.05), *i.e*. a value of 0.10–0.11 g Zn kg^{-1}, in the concentration of Zn composition within the carapace when compared to the levels found in the prawn in the control and 0 FTU kg^{-1} groups which had Zn concentrations of 0.14 g Zn kg^{-1}. There were no significant interactions or changes in the minerals P, K, Na nor in the trace elements Cu, Fe and Mn and each appeared to be unaffected by the inclusion of microbial phytase in the prawn diets.

Table 6.2. Growth performance of the *Macrobrachium rosenbergii* fed diets supplemented with microbial phytase for a period of 80 days.

		Dietary microbial phytase, FTU kg^{-1}		
	Control	0	1000	2000
Initial carapace length (mm)	8.31 ± 0.54	10.84 ± 1.14	8.88 ± 1.30	10.47 ± 1.79
Final carapace length (mm)	12.36 ± 0.73	14.18 ± 0.26	13.36 ± 0.91	14.20 ± 0.56
Carapace length increment (mm)	4.05 ± 0.98	3.34 ± 1.19	4.48 ± 1.85	3.73 ± 2.23
Initial weight (g)	0.43 ± 0.10	0.79 ± 0.27	0.55 ± 0.29	0.70 ± 0.04
Final weight (g)	1.42 ± 0.03^{a}	2.15 ± 0.09^{b}	1.90 ± 0.20^{b}	2.05 ± 0.09^{b}
Weight gain (g)	0.98 ± 0.09^{a}	1.36 ± 0.21^{b}	1.30 ± 0.32^{b}	1.35 ± 0.17^{b}
Av. daily weight gain (g prawn^{-1})	0.013 ± 0.00	0.017 ± 0.00	0.016 ± 0.01	0.017 ± 0.00
Specific growth rate (% day^{-1})	1.47 ± 0.16	1.30 ± 0.38	1.53 ± 0.36	1.36 ± 0.30
Feed intake (g prawn^{-1} day^{-1})	0.015 ± 0.00	0.021 ± 0.00	0.016 ± 0.00	0.020 ± 0.00
FCR	1.21 ± 0.35	1.25 ± 0.02	1.02 ± 0.16	1.19 ± 0.13
PER	1.98 ± 0.17	1.84 ± 0.01	2.30 ± 0.25	1.92 ± 0.23
Hepatosomatic index (HSI), (%)	3.23 ± 0.23	2.97 ± 0.53	3.93 ± 0.01	3.58 ± 0.34
Survival (%)	80.0 ± 0.00^{a}	80.0 ± 0.00^{a}	46.70 ± 0.60^{b}	66.70 ± 0.60^{ab}

Values are means ± SD of three replicates.
Values within the same row with different letters are significantly different (p<0.05). For parameters where there were no significant differences no superscript values are provided.

Table 6.3. Whole body proximate composition (g kg^{-1} wet weight) of the *Macrobrachium rosenbergii* fed diets supplemented with microbial phytase for 80 days.

			Dietary microbial phytase, FTU kg^{-1}		
	Initial[1]	Control	0	1000	2000
Moisture (%)	75.23 ± 0.72	75.30 ± 1.61	76.54 ± 2.79	75.00 ± 1.01	75.43 ± 3.65
Crude protein	15.98 ± 0.00	16.35 ± 1.16	15.47 ± 1.83	16.49 ± 0.55	16.22 ± 2.37
Crude lipid	1.39 ± 0.01	1.56 ± 0.13	1.08 ± 0.04	1.21 ± 0.20	1.21 ± 0.31
Ash*	2.12 ± 0.09	1.71	1.56	1.74	1.61
Gross energy(kJ g^{-1})*	4.50	5.01	4.76	5.08	4.98

Values are the mean ± SD of three replicates.
*One replicate due to limited sample.
Statistical analysis found no significant differences between the different diets for any of the measured parameters.
[1]Values not included in the one-way Anova.

Table 6.4. Moulting of the *Macrobrachium rosenbergii* fed diets supplemented with microbial phytase diets for 80 days.

		Dietary microbial phytase, FTU kg^{-1}		
	Control	0	1000	2000
∑ number of moults	45	40	40	49
Av. number of moults	3.00	2.67	2.67	3.27
Moults frequency (%)	3.75	3.33	3.33	4.08

Av. = average

Table 6.5. Whole body proximate mineral compositions (g kg^{-1} dry weight) of the *Macrobrachium rosenbergii* fed graded levels of microbial phytase diets for 80 days.

			Dietary microbial phytase, FTU kg^{-1}		
	Initial[1]	Control	0	1000	2000
Phosphorus (P)	12.05 ± 0.53	13.15 ± 0.44	12.35 ± 0.62	12.55 ± 1.28	12.52 ± 1.71
Calcium (Ca)	5.47 ± 0.44	5.71 ± 1.00^{a}	5.76 ± 0.46^{a}	7.48 ± 1.10^{ab}	8.45 ± 1.04^{b}
Zinc (Zn)	0.12 ± 0.02	0.14 ± 0.04	0.13 ± 0.06	0.09 ± 0.08	0.15 ± 0.02
Magnesium (Mg)	0.20 ± 0.02	0.22 ± 0.03^{a}	0.22 ± 0.01^{a}	0.26 ± 0.03^{ab}	0.33 ± 0.04^{b}
Copper (Cu)	0.07 ± 0.01	0.08 ± 0.01	0.05 ± 0.00	0.07 ± 0.01	0.08 ± 0.01
Iron (Fe)	0.03 ± 0.01	0.06 ± 0.03	0.03 ± 0.01	0.03 ± 0.04	0.02 ± 0.00
Manganese (Mn)	0.01 ± 0.00	0.01 ± 0.00	<0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Potassium (K)	0.98 ± 0.09	1.05 ± 0.16^{a}	1.02 ± 0.04^{a}	1.33 ± 0.13^{ab}	1.37 ± 0.12^{b}
Sodium (Na)	0.44 ± 0.03	0.50 ± 0.06^{a}	0.51 ± 0.03^{a}	0.65 ± 0.06^{b}	0.68 ± 0.07^{b}

Values are the mean ± SD of three replicates.
Values within the same row with different letters are significantly different (p<0.05). For parameters where there were no significant differences no superscript values are provided.
[1]Values not included in the one-way Anova.

Table 6.6. Muscle tissue proximate mineral compositions (g kg^{-1} dry weight) of the experimental *Macrobrachium rosenbergii* fed graded levels of microbial phytase diets for 80 days.

			Dietary microbial phytase, FTU kg^{-1}		
	Initial[1]	Control	0	1000	2000
Phosphorus (P)	9.42 ± 0.64	7.83 ± 0.85	7.41 ± 0.95	8.54 ± 1.86	6.76 ± 0.65
Calcium (Ca)	0.29 ± 0.05	0.61 ± 0.08^{b}	0.13 ± 0.01^{a}	0.17 ± 0.03^{a}	0.66 ± 0.06^{b}
Zinc (Zn)	0.12 ± 0.02	0.11 ± 0.01^{a}	0.08 ± 0.02^{ab}	0.07 ± 0.01^{b}	0.15 ± 0.02^{c}
Magnesium (Mg)	0.15 ± 0.01	0.16 ± 0.01	0.14 ± 0.01	0.14 ± 0.02	0.17 ± 0.01
Copper (Cu)	0.04 ± 0.00	0.05 ± 0.02	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Iron (Fe)	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.02	0.02 ± 0.01
Manganese (Mn)	<0.01 ± 0.00	<0.01 ± 0.00	<0.01 ± 0.00	<0.01 ± 0.00	<0.01 ± 0.00
Potassium (K)	1.56 ± 0.04	1.57 ± 0.06	1.58 ± 0.06	1.59 ± 0.20	1.73 ± 0.09
Sodium (Na)	0.36 ± 0.01	0.48 ± 0.04	0.42 ± 0.02	0.42 ± 0.05	0.40 ± 0.03

Values are the mean ± SD of three replicates.
Values within the same row with different letters are significantly different (p<0.05). For parameters where there were no significant differences no superscript values are provided.
[1]Values not included in the one-way Anova.

Table 6.7. Carapace proximate mineral composition (g kg^{-1} dry weight) of the *Macrobrachium rosenbergii* fed graded levels of microbial phytase diets for 80 days.

			Dietary microbial phytase, FTU kg^{-1}		
	Initial[1]	Control	0	1000	2000
Phosphorus (P)	10.37 ± 3.53	12.55 ± 0.08	11.57 ± 1.01	11.54 ± 0.77	11.19 ± 2.19
Calcium (Ca)	10.82 ± 0.66	11.17 ± 0.16^{a}	12.17 ± 0.57^{b}	12.66 ± 0.29^{bc}	13.58 ± 0.30^{c}
Zinc (Zn)	0.15 ± 0.01	0.14 ± 0.01^{a}	0.14 ± 0.01^{a}	0.11 ± 0.01^{b}	0.10 ± 0.01^{b}
Magnesium (Mg)	0.33 ± 0.02	0.27 ± 0.00^{a}	0.26 ± 0.01^{a}	0.32 ± 0.00^{b}	0.34 ± 0.01^{b}
Copper (Cu)	0.07 ± 0.00	0.08 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.08 ± 0.00
Iron (Fe)	0.03 ± 0.02	0.01 ± 0.02	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.01
Manganese (Mn)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	<0.01 ± 0.00	0.01 ± 0.00
Potassium (K)	0.56 ± 0.04	0.55 ± 0.01	0.51 ± 0.01	0.52 ± 0.03	0.54 ± 0.03
Sodium (Na)	0.51 ± 0.03	0.49 ± 0.01	0.50 ± 0.02	0.46 ± 0.02	0.50 ± 0.02

Values are the mean ± SD of three replicates.
Values within the same row with different letters are significantly different (p<0.05). For parameters where there were no significant differences no superscript values are provided.
[1]Values not included in the one-way Anova.

6.4 Discussion

6.4.1 Growth performance and moult frequency

Previous studies have reported that phytase supplemented in the diets presented to *L. vannamei* (see Chen *et al*., 2005) and *P. monodon* (see Biswas *et al*., 2007) at 500, 1000 and 2000 FTU kg^{-1} did not result in significant weight gains. The results of the present study are in agreement with the studies mentioned above where no significant differences among phytase supplemented treatments (0–2000 FTU kg^{-1}) were observed. These earlier studies, however, mentioned that they did not eliminate the mineral premix in the diets and so could not positively isolate the effects of phytase exclusively on the mineral availabilities in regard to weight gain. In general, the growth performances and the FCR of *M. rosenbergii* of this stage and size, with regard to the increase carapace length, weight gain, SGR and HSI for the duration of the experimental period, in all experimental groups, were satisfactory and in agreement with other studies (Chen *et al*., 2005; Biswas *et al*., 2007). This was predicted as the diets were formulated to meet the established nutritional requirements for *M. rosenbergii*. The hepatosomatic index was well within the 2–6% of total body wet weight (Ceccaldi, 1997) that was expected for this organ in decapod crustaceans. The SGR was not statistically significant regardless of the dietary treatment given. Furthermore, these groups of prawns and those maintained on the control diet were equivalent in performance, comparable to inorganic P supplementation.

In agreement with the findings of various studies conducted with penaeid shrimp, the addition of between 500–2000 FTU kg^{-1} phytase in the diets presented to *L. vannamei* (see Chen *et al*., 2005) and *P. monodon* (see Biswas *et al*., 2007) did not affect their whole body composition. The changes made to the experimental diets in the present trial, *i.e* the omission of a mineral

premix but the inclusion of microbial phytase, had some small effects on the final chemical composition of the whole *M. rosenbergii* carcass. The ash content, for example, was noticeable higher in those receiving diets supplemented with phytase which indicates better mineralisation. Phytases have the capacity to dephosphorylate phytate to a series of lower inositol phosphate esters (*i.e. myo*-inositol pentaphosphate to *myo*-inositol monophosphate) and ultimately, to inositol and inorganic P (Selle *et al*., 2000). This is an encouraging indication for an alternative ingredient used in prawn feeds being converted to inorganic P.

Somatic tissue growth is a more discriminative parameter than weight gain when correlated to the growth rate of *M. rosenbergii*. This is particularly true in prawns because the initial weight by moulting results from the absorption of water whereas real growth is achieved by increment of somatic tissue growth (Ismael & New, 2000). The moult frequency did not appear to have been significantly affected by the absence of the mineral premix and the microbial phytase supplements. In the present study, the survival of *M. rosenbergii* ranged from 46.7% to 80%. A survival rate of below 80% is not unusual in an environmentally controlled shrimp nutrition study where the test animals are subjected to repeated sampling (weighing and measuring) and water changes over the 80 d trial. A small number of mortalities, however, were observed as prawns moulted (**Figure 6.1**). Such mortality events have been referred to as “moult death syndrome” (D’Abramo & New, 2000) and, although the cause of this syndrome is not known, it is thought to be primarily dietary (Johnson & Bueno, 2000). The moulting process also creates severe physiological stress, such as changes in the body chemistry and feeding habits, aggressive behaviour and higher risk to infection. The composition of the experimental diets, which derived approximately 77–81% of the protein from plant ingredients, could also be a contributing factor. Tidwell *et al.* (1993) found that replacement of FM with SBM caused significant decreases in concentrations of some amino acids, *i.e.* lysine and methionine, and

several fatty acids of the diet. Mineral physiology in Crustacea is highly dependent on the moult cycle and uptake across the gills is a key source of minerals. Hence, indirect effects of a disrupted moult cycle on mineral composition might be expected and possibly that are unrelated to the physiological effects of dietary PA.

Figure 6.1. Experimental animal believed to be affected by the so-called "moult death syndrome".

6.4.2 Mineral composition of the whole body

Metal ions have been shown to modulate with phytase activity. Previous studies with various fish species have shown that a supplementation of phytase results in suggested improvements in their mineral compositions (Cain & Garling, 1995; Rodehutscord & Pfeffer, 1995; Schäfer *et al*., 1995; Jackson *et al*., 1996; Li & Robinson, 1997; Hughes & Soares, 1998; Oliva-Teles *et al*., 1998; Storebakken *et al.,* 1998; Forster *et al*., 1999; Papatryphon *et al.*, 1999; Papatryphon & Soares, 2001; Suguira *et al*., 2001; Cheng & Hardy, 2002; Vielma *et al*., 2004; Debnath *et al*., 2004; Baruah *et al*., 2005; Liebert & Portz, 2005; Yoo *et al*., 2005; Denstadli *et al.,* 2007; Cao *et al.,* 2008). Interestingly, in the present study, the supplementation of microbial phytase in the diets presented to *M. rosenbergii* did improve the macro mineral and trace element compositions of the whole body. The Ca, Mg, K, Na and Cu concentrations in the whole body

of *M. rosenbergii* were significantly higher in the phytase supplemented groups, *i.e* those receiving between 1000–2000 FTU kg^{-1} compared to those without, *i.e*. those receiving 0 FTU kg^{-1} and those in the control group (**Table 5.4**). The microbial phytase may have hydrolysed the bonds between the minerals and PA, thus, facilitating and improving mineral availability.

The Ca availability in Nile tilapia was reported to improve with the supplementation of phytase in their diets (Furuya *et al*., 2001; Portz & Liebert, 2004). In contrast to this, the Ca composition in the whole body of *L. vannamei* fed diets containing up to 2000 FTU kg^{-1} was unaffected by the supplementation (Chen *et al.*, 2005). Mineral pre-mixes, however, were not excluded in the diets of the latter study. Microbial phytase supplementation, however, must have played an important role in the availability of the trace element Cu, K and Na. The results indicate that the positive effect was unrelated to the inclusion levels but was most likely due to the general effect of the phytase supplementation in particular.

Phosphorus and the trace element, *e.g*. Zn, Fe and Mn, content of the whole bodies of *M. rosenbergii* were less affected by the phytase supplementation in their diets. Similar findings were reported in penaeids where no significant differences in the P content of the whole body of *L. vannamei* fed diets supplemented with up to 2000 FTU kg^{-1} were found (Chen *et al*., 2005). It is, however, worthy to note that the P reserves of each group of animals should be considered, as a P deficiency or enhancement could take a long time to appear (Civera & Guillaume, 1989). The current findings for *M. rosenbergii*, however, are in contrast to those determined for fish species where the P availability was found to be greatly enhanced by the supplementation of phytase in plant protein based diets (Cain & Garling, 1995; Rodehutscord & Pfeffer, 1995; Schäfer *et al*., 1995; Jackson *et al*., 1996; Li & Robinson, 1997; Hughes & Soares, 1998; Oliva-Teles *et al*., 1998; Storebakken *et al.,* 1998; Forster *et al*., 1999;

Papatryphon *et al*., 1999; Furuya *et al.,* 2001; Papatryphon & Soares, 2001; Suguira *et al*., 2001; Cheng & Hardy, 2002; Debnath *et al*., 2004; Vielma *et al*., 2004; Baruah *et al*., 2005; Liebert & Portz, 2005; Yoo *et al*., 2005; Denstadli *et al.,* 2007; Cao *et al.,* 2008). This is likely due to the higher requirement of P for bone mineralisation in fish species, and a deficiency, therefore, would result in a more severe impact than it might be in prawn or shrimp species.

6.4.3 Mineral composition of muscle tissue

In the previous dietary PA mineral trial (**Section 5.4.3**), Ca compositions in the muscle tissues of *M. rosenbergii* were found to be affected by the high dietary PA levels. The results of the present study ascertained that microbial phytase resulted in the complete hydrolysis of PA and improved Ca availability. Specifically, a supplementation of 2000 FTU kg^{-1} resulted in a significantly higher (p<0.05) Ca composition when compared to the other treatment groups, *i.e.* those in the 0–1000 FTU kg^{-1} diet groups, and were equivalent to the prawns in the control. The Zn composition of the *M. rosenbergii* muscles showed a similar trend. In line with previous studies, several fish species demonstrably had improved Zn availabilities and utilisations due to phytase supplementation in their diets (Storebakken *et al.,* 1998; Papatryphon *et al.,* 1999; Suguira *et al*., 2001; Debnath *et al*., 2005).

Nevertheless, the high levels of dietary PA and the low availability of minerals from the diet did not appear to cause mobilisation of P, Mg, Cu, Fe, Mn, K and Na from the muscle tissues of the prawns. The results of the present study indicate that a phytase supplementation of up to 2000 FTU kg^{-1} did not influence the availabilities of these minerals in the muscle tissues although they were absent from their diets. This confirms the ability of *M. rosenbergii* to compensate for any the deficiencies by acquiring these from their aquatic surroundings without compromising their growth performance.

6.4.4 Mineral composition of the carapace

The hepatopancreas situated within the carapace region is a principal storage organ for minerals in *M. rosenbergii* (Fieber & Lutz, 1982). Water taken up during ecdysis at the intermoult stage is gradually replaced by tissue growth, the enrichment of organic matter and mineral reserves resulting in the growth of the prawn (Saravanan *et al*., 2008).

An earlier study on the penaeid, *L. vannamei,* reported that the Ca content of the exoskeleton in animals fed diets supplemented with 500–1000 FTU kg^{-1} were significantly higher than those fed a control diet or one containing 2000 FTU kg^{-1} (Chen *et. al*., 2005). In the present study, the Ca concentration in the carapace of *M. rosenbergii* was significantly higher (p<0.05) in the groups presented with diets that included a phytase supplement compared to those that did not, *i.e*. those given a 0 FTU kg^{-1} diet or those in the control group. Similar results were observed for Mg. The results of the present study, therefore, indicate that phytase has a strong positive effect on the Ca and Mg composition of the carapace. Other than the direct uptake from the aquatic environment (Steffens, 1989; NRC, 1993; Guillaume *et al.,* 2001), it is presumed that the enhancement in the Ca and Mg concentration in the carapace was as a result of the increasing availability of these minerals from the hydrolyses of PA by microbial phytase which releases the PA–CA and PA–Mg complexes. It is important to note that the results of the present trial suggest that the inclusion of 1000–2000 FTU kg^{-1}, when in the presence of 15 g PA kg^{-1}, in the diets presented to *M. rosenbergii* has resulted in a significant increase (p<0.05) in the Ca and Mg levels in the carapace. This approach, which could lead to moderate increases in growth performance and higher moult frequencies, is promising for the use of microbial phytase in commercial diets. The present trial was, however, conducted over a relatively short time period, and it is possible that this could have masked the real extent of the positive changes occurring over the whole production cycle of *M. rosenbergii*.

In the present study, the differences in the Zn compositions of the carapace of the experimental *M. rosenbergii* receiving various inclusions of phytase were significantly affected. Specifically, the Zn content in the groups receiving phytase supplements in their diets were significantly lower, *i.e*. 0.10–0.11 g Zn kg^{-1}, compared to those who did not receive a phytase supplement or were in the control group, *i.e*. 0.14 g Zn kg^{-1}. The differences were minimal which indicates that the concentration of Zn were not influenced by the level of phytase but was possibly due to the lack of Zn storage in the carapace. This is likely due, at least in part, to the primary ingredients used in the diet, *i.e*. plant protein, which contains lower Zn compared to dietary sources of animal origin. In addition, it is likely that the *M. rosenbergii* were not able to compensate the shortfall, as absorption of Zn from the aquatic environment is reported to be low (Guillaume *et al*., 2001). This discovery also suggests that Zn plays an important role during moulting as a majority of the mortalities observed were due to moult death syndrome. The mechanism by which Zn is responsible for moulting in *M. rosenbergii* is still unclear. Zinc is known to have antibacterial properties (Dupont *et al*., 1994), but there is still a lack of scientific evidence to understand the exact mode of action, particularly during moulting events.

In the present trial P, Cu, Fe, Mn, K and Na in the carapace tissue were not affected by the amount of phytase included in the experimental diets. This agrees with the earlier findings in this thesis in that the minerals were found to be unaffected by the inclusion of PA in the diet (see **Section 5.4.4**). This suggests that P is not a limiting factor in plant based diets, *e.g*. soybean and wheat meal, with the supplement of phytase, as no negative effects such as poor growth was observed. These results are appealing, as a mineral premix was excluded from the experimental diets and the level of minerals in the test diets were below the recommended requirements, the *M. rosenbergii* were still able to offset the shortfall. Besides the positive role of microbial phytase in releasing the minerals from PA complexes, these results may also be

associated with changes in other regulatory mechanisms of mineral balance such as branchial uptake from the surrounding environment. Studies by Corbin *et al*. (1983) and Tidwell *et al*. (1995) suggested that the major portion of macro-nutrients, *i.e.* protein, for freshwater prawns needs to be supplied in the formulated diets, but required levels of micro-nutrients, *i.e*. vitamins and minerals, could be derived from natural productivity or stimulated productivity of natural foods in culture ponds. A better understanding of the role of natural productivity in prawn nutrition and development of methods for selective management of desirable components are needed (MacLean *et al*., 1989). This could result in decreased production costs based upon providing a combination of pelleted rations and pond organisms to satisfy nutrient requirements (Corbin *et al*., 1983).

6.5 Conclusion

In conclusion, the results of this study suggest that the dietary phytase supplements had no significant effects on the growth performances and whole body chemical compositions of the *M. rosenbergii* under the present experimental conditions. Microbial phytase supplement, however, significantly improves the mineral concentrations of Ca, Mg, K, Na and Cu in the whole body, Ca and Zn concentrations in the muscle tissues and Ca concentrations in the carapace of of *M. rosenbergii.* The trial findings confirm that microbial phytase does play an important and positive role in releasing macro minerals and trace elements from complexes with PA making them more readily available for *M. rosenbergii*.

Chapter 7 - General discussion, conclusions and future perspectives

In many aquaculture operations today, feed accounts for over half of the variable operating cost, and protein constitutes a major cost of formulated diets. In view of the fact of the rising cost of aqua feeds, alternative protein sources such as plant proteins have been studied intensively due to the limited, unpredictable supply and high cost of fishmeal (FM). Ongoing research is essential particularly in freshwater prawn production where the cost of feed is one of the major expenditures. Importantly, an increase in using plant protein ingredients as an alternative protein source does not appear as a viable option for the future unless the feed formulation is addressed. One of the major problems limiting the use of plant protein is the presence of anti-nutritional factors, such as phytic acid (PA), commonly found in most plant protein ingredients (Storebakken *et al.*, 2000; Francis *et al*., 2001). The overall objective of this thesis was, therefore, to determine the effects of PA when included at various inclusion rates in diets presented to *Macrobrachium rosenbergii* with the aim of establishing a clearer understanding of its role and utility. This body of research sets out to promote sustainability by focusing on the substitution of fishmeal with sustainable ingredients, *i.e*. plant proteins. This thesis presents novel research investigating the effect of PA, an anti-nutritional factor that is found largely in plant protein feed ingredients in aqua feed and its neutralising enzyme, microbial phytase. Phytic acid is a serious concern because of its ability to form complexes with proteins rendering them unavailable with the consequential impacts on growth performances. Furthermore, the stable structure and high chelation capacity of PA with multivalent cations and, in particular, minerals, *i.e.* P, Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, K^{2+} and Na^{2+}, is a major concern. This is seen as a limiting factor that needs to be investigated in *M. rosenbergii*. The effect of phytase enzyme in catalysing the hydrolysis of PA was also investigated. By working efficiently on the substrate in the prevailing conditions, the supplementation of phytase could diminish the anti-nutritive effects of PA and reduce the cost of diets by removing and / or

reducing the need for supplements of inorganic phosphate.

A targeted approach in this thesis was adopted whereby graded levels of PA and phytase were added to a series of experimental diets given to *M. rosenbergii* in order to establish their effects and tolerance levels. Specifically, in the first key experiment (see **Chapter 3**), graded levels of dietary PA were added to experimental diets to investigate their effects on growth performance. In the second major experiment (see **Chapter 4**), a growth trial was conducted which explored the supplementation of the diets with graded levels of microbial phytase set out to evaluate the potential use of microbial phytase. The third experiment (see **Chapter 5**) went on to elucidate the effects of graded levels of PA on mineral compositions in *M. rosenbergii*. Lastly, the effects of microbial phytase in hydrolysing PA and their effects on mineral compositions were investigated in the fourth major experiment which is detailed in **Chapter 6**. This research was conducted fundamentally to provide valuable information for future optimisation and implementation of plant proteins in the diets of *M. rosenbergii*.

7.1 Main findings

The main research findings from each experimental trial can be briefly summarised as below.

The results of the first experiment (see **Chapter 3**), suggest that the inclusion of between 0.26 to 26.16 g PA kg^{-1} within the diet fed to *M. rosenbergii* resulted in:

- No significant effect on the growth performances and survival between the groups of test animals.
- No negative impact on feed utilisation.
- An impact resulting in a significant decrease ($p<0.05$) with increasing PA inclusion on the apparent protein, lipid and energy utilisations, particularly within the groups of

prawns fed the diet with the highest inclusions of PA, *i.e*. the 21.45–26.16 g PA kg^{-1} diets.

- A decrease in apparent protein and lipid digestibility with increasing PA inclusion.
- Histological examination of the digestive gland found no apparent deviations between the treatment and the control groups.
- Significant changes in the proximate composition of the whole body of *M. rosenbergi*, especially in the group fed the highest inclusion level of PA, *i.e*. 26.16 g PA kg^{-1}. The protein (p<0.04), lipid (p<0.01) and gross energy (p<0.05) in the final carcass significantly deceased with increasing PA inclusion whereas a reverse trend was seen for the ash content (p<0.01).

The results from the second experiment (see **Chapter 4**) which set out to investigate the potential and optimal use of microbial phytase, across the range 0–2000 FTU kg^{-1}, when included within the diets fed to *M. rosenbergii*, found that:

- Low fish meal diets / high plant protein ingredients can be used with no negative effects on growth and feed utilisation with the supplement of microbial phytase.
- Protein utilisation in the treatment groups fed diets supplemented with 500–2000 FTU kg^{-1} were significantly higher (p<0.05) when compared to the control group. A significant difference (p<0.05) in the lipid utilisation was also observed in the treatment group fed 2000 FTU kg^{-1} when compared with the animals in the control group.
- An increasing supplement of phytase in the diet resulted in an increase in dry matter and in the digestibility's of protein and lipid.

- There were no significant differences in the proximate composition of the whole body of *M. rosenbergii* due to supplements of phytase. There were moderate increases in the protein, lipid and gross energy of the final carcass with an increasing supplement of phytase, while a reverse trend was seen in the ash content.

The results of the third experiment which are detailed in **Chapter 5**, which investigated the effects of a dietary inclusion of PA (*i.e.* between 0.26 to 26.16 g PA kg^{-1}) on mineral availability found that:

- There were no major detrimental effects on moult frequency.
- There were significant increases (p<0.05) in the ash composition of the whole body with an increase in the inclusion of PA and this was most noticeable in the groups with the highest levels of PA, *i.e.* 16.53–26.16 g PA kg^{-1}.
- Higher PA inclusion, *i.e.* 21.45–26.16 g PA kg^{-1}, significantly reduced (p<0.05) the P content in the whole body. Interestingly, a reverse trend (r^2= .61, F(1, 9)=14.1, p<0.005) was recorded for the Ca content which resulted from an increasing inclusion of PA.
- The Ca composition in the muscle tissues of *M. rosenbergii* significantly decreases (r^2= .49, F(1, 9)=8.85, p<0.01) with increasing dietary PA levels.
- The graded inclusion of PA in the experimental diets resulted in a significant reduced (r^2= .28, F(1, 15)=5.86, p<0.03) P content in the carapace.
- There were significant changes (p<0.05) in the carapace Zn, Cu, K and Na compositions, particularly in the prawns fed the diet containing 11.28 g PA kg^{-1}, which

suggests that the specific minerals were either selectively utilised or retained in the carapace.

The fourth experimental trial which is detailed in **Chapter 6**, set out to elucidate the effects and efficiency of microbial phytase, when added to plant based diets from which a mineral premix had been eliminated, on the mineral availability in *M. rosenbergii*. The key findings from the experiment, suggest that:

- There were no significant negative effects on growth and feed efficiency. Treatment group without a phytase supplement (0 FTU kg^{-1}) recorded the least growth.
- Survival of the experimental prawns was significantly affected (p<0.05), particularly in the treatment group fed the 1000 FTU kg^{-1} diet.
- The elimination of the mineral premix and the supplementation of microbial phytase did not significantly affect the moult frequency of the experimental prawns.
- There were no significant changes in the proximate composition of the whole body of *M. rosenbergii* between treatment groups.
- There were significant changes (p<0.05) in the Ca and macro mineral content, *i.e.* Mg, K and Na, in the whole body of *M. rosenbergii* when the diets were supplemented with microbial phytase. The Ca content of the whole body was significantly higher (p<0.05) in treatment groups fed microbial phytase compared to those prawns receiving diets either the control diet or the diet lacking a supplement.
- The Ca and Zn compositions in the muscle tissues were significantly higher (p<0.05) in the treatment group fed the highest microbial phytase supplement, *i.e*. 2000 FTU kg^{-1}.

- Microbial phytase inclusions significantly increase (p<0.05) the Ca and Mg content of the carapace.
- An inclusion of microbial phytase and the elimination of a mineral premix results in a significant reduction (p<0.05) in the Zn content of the carapace.

7.2 General discussion

7.2.1 Impact of phytic acid on growth

Research into the potential adverse impacts of PA within the diets of *M. rosenbergii* is lacking despite reports of its detrimental effects on growth in fish, poultry, swine and rats (Davies & Nightingale, 1975; Pallauf & Rimbach, 1997; Francis *et al*., 2001; Rapp *et al*., 2001; Yonekura & Suzuki, 2003). Findings from the present work suggest that PA does not compromise the development of juvenile *M. rosenbergii* when the following parameters are considered: the increase of carapace length, weight gained, specific growth rate (SGR), feed conversion ratio (FCR), protein conversion ratio (PER), hepatosomatic index (HSI), and, the survival. Although the results were not significant, the growth performance and feed efficiency of the prawns fed the diets containing 26.16 g PA kg^{-1} were evidently the slowest and lowest (see **Figure 3.1**). It is, however, acknowledged that the experiment was conducted under laboratory conditions and that the prawns were held separately, in isolation, to prevent cannibalism and territorialism which restricted their social interactions necessary for normal growth. Further, although this experiment used 120 animals (26.4 L tanks), it is still considered a small-scale trial which ran for a shorter duration than the complete production cycle.

Interestingly, studies on the nutrient digestibility and utilisation suggest that protein apparent digestibility coefficient (ADCP) and lipid digestibility (ADCL) are evidently reduced with

increasing inclusions of PA, and although this could potentially have an effect on the performance of *M. rosenbergii*, no significant effects on growth were seen. The scale of the current trial also meant that there were limits to the volume of faeces that could be collected, and as such the amount collected was not sufficient to permit a detailed study.

Equally, there was a clear inverse relationship between the protein, lipid and energy composition in the whole body of *M. rosenbergii* with increasing levels of PA in the diets. A possible explanation of these could be that the interactions of PA with various proteins resulted in PA–protein complexes which may have changed the protein structure or shape, thus, reducing their enzymatic activity, protein solubility and proteolytic digestibility. Furthermore, the decrease in the lipid content may be an indication of the higher energetic costs of overcoming the shortcomings of protein availability which results in the lipid and energy being exploited and utilised in order to compensate for the loss. This is supported by the significant decrease (p<0.05) in the nutrient utilisation of protein, lipid and energy (ANPU, ANLU and ANEU) with increasing PA inclusions which clearly indicates a reduction in the ingested nutrient retained by deposition in the whole body composition of *M. rosenbergii*. It is therefore possible that growth performances could have been significantly different had the protein level in the diets of the present trial were lower comparable to the minimum levels in commercial feed.

7.2.2 Beneficial of microbial phytase supplement on growth

The motivation to use phytase in aqua feed is due to the emphasis placed on reducing the current dependency on fish meal by substituting it with plant protein sources for efficient animal production with minimal environmental damage. The study conducted in **Chapter 4** exploited this by investigating the potential of microbial phytase from *Aspergillus niger* and its activity specifically in hydrolysing PA when added as a supplement in plant based diets with the aim of

improving nutritional efficiency. The results of this trial suggest that the growth performance of juvenile *M. rosenbergii*, including increase in the carapace length, weight gain, SGR, and the feed utilisation (feed intake, FCR and PER), did not differ significantly between the treatment groups. It is acknowledged though that this experiment was of a relatively short duration, *i.e.* 80 days, and that it was conducted within a laboratory environment which means that the prawns may not perform as well as they might in a farm pond environment where there is less disturbance from the experimentor. The highest growth performances were recorded for the prawns in the treatment group fed the 2000 FTU kg^{-1} diet, and part of this might be explained by the better utilisation of their dietary protein and lipid. The inclusion of 1000–2000 FTU kg^{-1} significantly increased (p<0.05) protein utilisation in *M. rosenbergii* whereas the treatment group fed the 2000 FTU kg^{-1} had a significantly increased (p<0.05) lipid utilisation when compared to the control group. There were no significant differences in moisture, protein, lipid, ash and gross energy contents in the whole body of *M. rosenbergii* between the treatment groups, although gradual increases were observed in the protein and lipid content in the whole body with an increasing phytase inclusion whereas a reverse relationship was observed with the ash content. A vital element of the study that may have been overlooked is the stomach pH of the juvenile *M. rosenbergii* which may not been within the optimal range for phytase activity, *i.e*. pH 4.5–5.6 (Kerovuo, 2000). The basic digestive system of juvenile *M. rosenbergii* is probably not sufficiently acidic, therefore, a more comprehensive study looking into adding 3% citric acid into the diets as suggested by Radcliffe *et al.* (1998) to provide an optimum environment for microbial phytase activity in *M. rosenbergii* should be considered.

In summary, this study provides interesting information on the possibility of reducing FM in the diets of *M. rosenbergii*. In comparison to an earlier study conducted by Du & Niu (2003) which investigated the substitution of FM with soybean meal in the diet presented to *M. rosenbergii*,

the findings from the current research suggest that the efficiency of plant based diets can be improved through the use of microbial phytase. It must be remembered, however, that these results must be considered as preliminary and for a better understanding on the effects and to realize the full benefits of adding phytase to plant based diets, further investigation to find the best method of phytase usage in the diets whether by pre-treatment, top spraying, coating or by adding phytase over a wide range of temperatures during the diet preparation should be conducted. As the engineering of feed enzymes is in constant development, further research looking at the wide variety of appropriate enzymes available, *e.g*. bacteria (*Bacillus subtilis*) and yeast (*Saccharomyces cerevisiae*), is also necessary.

7.2.3 Chelation of PA with minerals

Despite the effects of PA on mineral availability having been extensively studied in fish (Spinelli *et al*., 1983; Sajjadi & Carter, 2004; Helland *et al*., 2006), the roles of PA in reducing the availability of minerals in crustaceans is still limited. Minerals are known to play a crucial role in the nutritional balance, during edysis and in the growth processes of crustaceans. Phytic acid is a principal chelating agent and is capable of impairing divalent mineral bioavailability through binding and thus making them unavailable for absorption (Pallauf & Rimbach, 1997). The key findings from **Chapter 5** in this thesis, indicate that PA did not cause any major detrimental effects on moult frequency in the juvenile population of *M. rosenbergii* but instead found that there were higher frequencies with an increasing inclusion of PA in the diet when compared to the control group. This observation has also been seen in the marine shrimp, *M. japonicus*, in earlier studies conducted by Cheng & Guillaume (1984) and Civera & Guillaume (1989). It is possible that decrease bioavailability of P due to PA is not a major concern as described in other aquatic species, *i.e*. mostly fish.

Although there were no clear signs of mineral deficiencies in this trial, there were several indications of sub-optimal mineral availability in the juvenile *M. rosenbergii*. The significant reduction (p<0.05) in the P content of the whole body samples taken from the groups of prawns fed the higher PA inclusions, *i.e*. 21.45 g PA kg^{-1} and 26.16 g PA kg^{-1}, is likely correlated to their low growth as seen in the previous PA growth trial (see **Chapter 3**). A significant increase (p<0.05) in the ash composition of the whole body with increasing PA inclusions was seen while there was a concurrent elevation in the Ca content. These findings are in agreement with earlier studies performed with marine shrimp species such as *M. japonicus* and *L. vannamei,* by Civera & Guillaume (1989). From their findings, these latter authors suggested that the negatively charged PA chelating with the positively charged Ca forms PA–Ca complexes that decrease the Ca availability but the exceptional regulatory mechanisms exhibited by *M. rosenbergii* typically attempt to conserve diminishing minerals as a means of compensating for shortages. This demonstrates the ability of *M. rosenbergii* to cope and adapt to acute deficiencies. Whether these results were indicative of the dietary influences of the minerals due only to the presence of PA in the experimental diets though remains uncertain, as unfortunately, this could not be validated in this trial due to limitations in the small size of the experimental animals and the limited number available meant that there were difficulties in collecting sufficient amounts of hepatopancreas from the animals in each treatment group to permit a robust analysis.

It is important to acknowledge, however, that mineral deficiency studies in crustaceans, and as here in this trial with *M. rosenbergii*, are complex and this may be reflected in their physiological requirements, ability and behavioural traits. Although a number of different indicators have been assessed here to provide a broad assessment of the changes to the mineral status or content within *M. rosenbergii* when fed diets containing PA, there are a variety of other

parameters to consider including the mineral content of the culture water, the elimination of the dietary mineral premix, and the mineral requirements of the culture species is necessary to provide an even more comprehensive understanding of the impacts of PA chelation with di- and tri-valent minerals.

The highest concentrations of Zn, Cu, K and Na retained within the carapace, were seen in the prawns fed on the diet containing 11.28 g PA kg^{-1}, suggesting that there is possibly a beneficial role of PA in retaining these minerals in preparation for the ecdysis process. These specific minerals are known to play essential roles in aiding the exuviation process and therefore, it would be of interest to elucidate the role of PA in the accumulation of these selected minerals at different time points throughout the moult cycle.

7.2.4 Efficiency of microbial phytase and mineral availability

Research into the potential use of microbial phytase in plant based diets is still lacking despite the clear importance of this enzyme, particularly in releasing macro minerals and trace elements from complexes with PA that consequentially can enhance growth performance (Selle *et al*., 2000; Brenes *et al*., 2003). The research findings from the experimental trial detailed in **Chapter 6** are promising and suggest that a supplement of phytase in plant protein based diets fed to *M. rosenbergii* does enhance mineral availability which is confirmed by the elimination of the mineral premix from the diet formulation. It is also acknowledged, however, that survival was impaired and therefore further work is required before any firm conclusions can be made and a balance assessment of the pros and cons to the use of phytase can be made. There was no evidence of a clear pattern in the P composition in the presence of phytase supplement and this may have been due to the relatively short duration of the experimental study and therefore, longer trials would need to be conducted to fully appraise the benefits of using phytase as a

mean of releasing phytate bound P. Nonetheless, the significant changes in the mineral compositions that were observed are attributed to the inclusion of phytase in the diets that resulted in higher concentrations of: 1) Ca, Mg, K and Na being found in the whole body; 2) Ca and Zn in the muscle tissue; and, 3) Ca and Mg in the carapace of *M. rosenbergii*. In addition to these observations, the deficiency of Zn in the carapace has led to an important discovery. The trace element Zn plays a vital role during the moulting process and a deficiency can result in mortality (Wilder *et al*., 2009). In the present trial, a number of the mortality events that were seen were due to an incomplete moult – a condition known as moult death syndrome. This situation also necessitates further investigation to look into the role of Zn during ecdysis with a view to preventing moult death syndrome events.

Most of what is known about the mineral requirements of *M. rosenbergii* have been largely derived from studies conducted with the two marine shrimp species, *M. japonicus* and *P. monodon*. These species are carnivorous and so it is likely that there will be differences in their mineral requirements and concentrations. In conclusion, an understanding of the mineral roles is of key importance in order to improve and enhance the nutritional characteristics, particularly the mineral availability in the feed for growth as well as for health and so further fundamental research is needed to fully exploit this prospective enzyme in releasing mineral binding complexes with PA. Moreover, an indepth study on the roles of minerals is needed for a better understanding of the causes and prevention of moult death syndrome, with an emphasis of such studies being conducted with *M. rosenbergii*.

7.3 General conclusions

This thesis presents novel data resulting from a series of investigations that have looked at the effects of dietary PA on *M. rosenbergii.* In conclusion, not only has this thesis assessed some

of the serious concerns regarding the impact of high levels of PA in the diets on growth performance but it has also looked at the changes in nutrient utilisation, nutrient digestibility and, the proximate composition of the whole body of *M. rosenbergii*. These changes may have a serious impact on commercial prawn production and so caution should be exercised to ensure that the anti-nutritive effects of PA are minimised. Mineral deficiencies can result in numerous physiological problems that could be detrimental to large-scale production. Even though nutrient deficiencies are serious, mineral imbalances or the lack of particular minerals can also result in significant reduction in production efficiency and result in unnecessary increases in feed cost. The supplementation of microbial phytase in *M. rosenbergii* feed can be expected to provide both economic and environmental benefits by: 1) increasing the amount of plant protein currently used in aqua feeds and by doing so; 2) decrease the current dependency of FM without compromising nutritional consistency and growth. The valuable information generated from this PhD project can now be used in a species targeted approach to reduce the levels of FM used in aqua feeds and optimise the plant protein ingredients used in maintaining a sustainable future for the aquaculture industry and simultaneously facilitate the efficient culture of *M. rosenbergii*.

7.4 Future perspectives

Some of the areas identified as research priorities for future development in these fields of investigation can be summarised as follows:

- A detailed in depth study in to the interactions between various ANFs in plant protein based diets as many of the plant ingredients contain more than one ANF, *e.g*. phytic acid – trypsin inhibitors, phytic acid – tannins, saponin – gossypol *etc*. Studies are also

needed to expose the effects of mixtures of ANFs in proportions similar to plant derived nutritional sources.

- The potential positive effects of microbial phytase on the growth of *M. rosenbergii* should be examined over longer periods of the production cycle, ensuring that the quality, duration and costs of production of the final product is not compromised. Lastly, the economic benefits of using various local plant protein ingredients and the costs of using phytase supplements require proper evaluation.

- It is recognised that there are limitations to and consequences of running experiments in controlled experimental environments such as research aquaria. Further dietary trials conducted over a longer part of the production cycle, therefore, should be carried out to fully explore their effects and the mechanisms involved. It would be interesting, therefore, if the laboratory conditions can be extrapolated to a larger scale and in common practice field conditions, *i.e*. a semi-intensive system.

- Information concerning the mineral requirements of freshwater prawns is limited and sometimes are in contradiction to those required by species of marine shrimp. A comprehensive study on the mineral requirements, including their bioavailability, dietary interactions and general recommendations for dietary supplementation for *M. rosenbergii* is therefore necessary to provide a robust foundation and understanding of the mineral nutrition of freshwater prawns. Such studies should include a comprehensive evaluation of each mineral and should define what concentrations are essential in the formulation of each species-specific diet.

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