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Digestive profile and capacity of the mud crab (*Scylla serrata*)

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ABSTRACT

Commercial farming of mud crab *Scylla serrata* is a significant industry throughout South East Asia. The limited scientific knowledge of mud crab nutritional requirements and digestive processes, however, is recognised as a major constraint to the future growth of this industry. To better understand the mechanisms of digestion in the mud crab we have analysed the diversity of digestive enzymes from the hepatopancreas. Significant protease, amylase, cellulase and xylanase activities were detected in soluble extracts from this organ.

Temperature profiles for all enzymes were basically similar with optimal activities observed at 50^0C. Examination of pH tolerance revealed optimal activities for protease and amylase at pH 7.0 while optimal activities for cellulase and xylanase were observed at pH 5.5. Under optimum conditions, protease and amylase activities were approximately two orders of magnitude greater than those seen for either cellulase or xylanase. Interestingly, hepatopancreatic extracts were able to liberate glucose from either starch or carboxymethyl (CM)-cellulose suggesting that a range of carbohydrates may be utilised as energy sources. The effects of dietary carbohydrates on feed digestibility, digestive enzyme levels and growth were also studied by inclusion of additional starch or CM-cellulose at the expense of casein in formulated diets. It was shown that amylase, cellulase and xylanase activities in extracts from the hepatopancreas were highest in mud crabs fed diets containing 47% carbohydrate. Based on the findings, we suggest that the ability of the mud crab to modulate digestive enzyme activities may represent

a mechanism to maximise access to essential nutrients when the dietary profile changes.

STATEMENT OF ORIGINAL AUTHORSHIP

I, Marko Pavasovic, hereby declare that this thesis is based on my own work and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made.

Marko Pavasovic

Signed……

Date……

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1. INTRODUCTION

Crustacean aquaculture is a rapidly growing industry in Australia and South East Asia. It is now recognised as an industry of high value contributing 16% of the total value of aquaculture production in 2000, despite a much smaller contribution by weight 3.6% (Tacon, 2003). In recent times, aquatic crustaceans such as marine and freshwater prawns, lobsters, crabs and crayfish have become very important aquaculture commodities. For example, the global production of crabs from aquaculture increased over ten fold from 1987 to 1996, resulting in the second highest aquaculture expansion ratio in the world (New, 1999). In Australia crustacean production reached 4,098 t in 2001/02, with prawns accounting for the bulk of production (88.7%) and freshwater crayfish, crabs and soft-shell crabs accounting for the remainder (Love and Langenkamp, 2003).

The success of crustacean aquaculture production depends on providing the culture species with adequate and appropriate nutrition. Unfortunately, specific nutritional data on most cultured crustacean species is relatively scarce, especially when compared with the nutritional data available for terrestrial farmed animals (D'Abramo, 1997). In many instances, formulated diets used in crustacean aquaculture are designed primarily for the penaeid prawn species and typically contain high levels of protein rich (>50%) ingredients such as fishmeal. This strategy has often proven expensive or has resulted in low feed conversion efficiencies which severely limit the viability of aquaculture ventures for non-penaeid crustaceans (Evans, 1992). For example, diets given to mud crabs during the fattening periods of production typically include products such as trash fish that are prone to significant fluctuations in price and availability and can contribute to poor water quality (Hutabarat, 1999). This problem is particularly evident in some

grow-out systems for mud crabs, where wet-weight feeding of 5% of trash fish per day leads to costs which are about half the income generated from production (Keenan, 1999a).

One strategy for reducing the costs associated with crustacean aquaculture is to explore the possibility of utilising relatively cheap carbohydrate based ingredients to supply energy thereby sparing the use of protein in formulated diets. Interestingly, research has shown that many crustacean species of economic importance possess the necessary enzymes for hydrolysis of carbohydrates (D'Abramo, 1997). This has led to the hypothesis that diets may be formulated for crustaceans which replace a significant proportion of the expensive protein component with less expensive carbohydrates obtained from plant material, agricultural products or by-products (Xue *et al*., 1999).

It is widely believed that a more scientific approach to aquaculture nutrition is required before significant improvements in feed technologies can occur (Houser and Akiyama, 1997). A key focus of the current study will be to use the mud crab, *Scylla serrata*, as a model system to determine the capacity of cultured crabs to digest protein and various classes of carbohydrates incorporated into formulated diets. Another goal is to determine if mud crab digestive processes or feed digestibility are altered by changes in the protein or carbohydrate profiles in the diet.

1.2 Biological characteristics of mud crab

1.2.1 Basic biology

The mud crab (*Scylla serrata)* belongs to the phylum Arthropoda, sub-phylum Crustacea. Like most cultured crustaceans (prawns, shrimp, lobsters, crayfish and crab) mud crab belongs to the Order Decapoda. A full classification of the mud crab is presented in (Table 1.1) (King, 1995). The mud crab (Fig. 1.1) is a relatively large crab, has a very smooth broad carapace and can grow to in excess of 3 kilograms (Kaliola *et al.,* 1993). The colour of the mud crab can vary from dark brown to mottled green, depending on the environment. Located on each side of the carapace are nine spines, all of them very similar in size and the hind legs are flattened in order to aid the animal in swimming (Kaliola *et al.,* 1993). Male mud crabs are normally larger in size than female mud crabs (Keenan, 1999).

Table 1.1 Classification of mud crab, *Scylla serrata*, (adapted from King, 1995 and Keenan, 1999).

Phylum	***Arthropoda***
Subphylum	***Crustacea***
Class	***Malacostraca***
Subclass	***Eucarida***
Order	***Decapoda***
Suborder	***Reptantia***
Infraorder	***Brachyura***
Family	***Portinuidae***
Genus	***Scylla***
Species	***serrata,*** *olivica, paramamosain, tranquebarica*

Figure 1.1 Mud crab - *Scylla serrata*.

1.2.2 Distribution

Mud crabs are distributed widely throughout the Indo-west Pacific region, mainly through the warmer regions. Their natural distribution stretches from the East coast of Africa all the way to Tahiti. In Australia, mud crabs are found in the warmer areas ranging from Exmouth Gulf in Western Australia to Bega River in New South Wales (Kaliola *et al*., 1993).

Mud crabs are marine, mostly estuarine animals. This means that they inhabit sheltered intertidal mudflats normally on river mouths and areas covered in mangroves. Although crabs inhabit mainly shallow areas, the females in berry (i.e. carrying eggs) are known to venture up to 50 km offshore to produce their eggs.

1.2.3 Current status of mud crab fisheries

In recent decades overfishing of many marine fisheries has seriously depleted the global stocks (New, 1999). One of the main sectors of marine fisheries that has been affected by over harvesting is the crustacean sector. This has serious economic implications since several crustacean fisheries are of particularly high value. For example, the mud crab has a very high market value and throughout the Western world is viewed as a delicacy, largely due to its low fat, high protein, vitamin and mineral content as well as fine taste (Baliao, 2000). The mud crab is also an important source of food for coastal inhabitants in the Indo-Pacific region where it is widely distributed (Baliao, 2000).

In Australia a substantial mud crab fishery stretches all the way from Western Australia, along the north coast and down to New South Wales with locations centred mostly on estuaries and lower reaches of rivers. The smallest portion of the industry is located in Western Australia in the north-eastern corner of the state. Mud crab fisheries in the Northern Territory have expanded only recently to an area between the Roper and McArthur rivers, located in the South West corner of the Gulf of Carpentaria. Prior to this the bulk of the Northern Territory mud crab industry was concentrated around Darwin (Kaliola *et al*, 1993). In Queensland, the fishery is concentrated south of Rockhampton with the most intensive sector located between Gladstone and Maryborough and for New South Wales between Port Macquarie and Newcastle. Although the New South Wales fishery is centred on this small area, mud crabs are caught all the way from the Queensland border down to Sydney (Kaliola *et al.,* 1993).

Mud crab catches are generally higher during summer, particularly for areas in Queensland and Northern Territory. Unfortunately, the climate in the far north makes many areas inaccessible for crabbing due to the heavy rains in the wet season (Kaliola *et al.,* 1993).

1.2.4 Mud crabs in aquaculture

Mud crab, (*S. serrata*) is an abundant marine / brackish water species that is considered to have excellent potential for culture in Australia. The natural distribution of mud crab, *Scylla* sp., ranges from tropical to temperate zones (Keenan, 1999a). Australia has healthy wild mud crab stocks and also large areas of coastal land, which are well suited to development of mud crab farming systems.

Elsewhere in the Asia / South-east Asian region there is evidence of mud crab farming extending back for over 100 years (Yalin and Quingsheng, 1994). Farming of mud crab has received much attention as the species is generally regarded as a high quality food source (Fortes, 1999). Demand for this species has been on the increase because of the qualities it possesses, including large size, high meat yield (Rattanachote and Dangwatanakul, 1992), high meat quality and rapid growth during culture (Millamena and Quinitio, 1999).

Modern production technologies for mud crabs are very similar to those used for prawn farming which utilise large growout ponds, with or without mangroves and intertidal pens (Keenan, 1999; Fortes, 1999). The current stocking density for mangrove pen culture is 5-7 crabs/m^2, while non-mangrove density is

significantly lower at 1-3 crabs/m^2, both achieved with supplemental diets (Keenan 1999).

Although there has been significant interest in mud crab aquaculture in Australia and South-east Asia, only in recent years has the future of the industry looked promising with many opportunities available for further expansion. Despite this, there are several issues which need to be addressed in order to secure further progress (Keenan, 1999). These include; improving hatchery production and stock selection, controlling cannibalism, improving pond management and water quality, controlling diseases and improving nutrition (Keenan, 1999).

1.3 Anatomy and physiology of digestive tract of the mud crab

1.3.1 Anatomy of digestive tract

As in other crustaceans, the digestive tract of the crab (Fig. 1.2) is divided into three parts; foregut, midgut and hindgut. The foregut is comprised of a mouth, oesophagus and stomach. The midgut is a tube with an anterior and posterior caecum and midgut gland (hepatopancreas). The hindgut is a simple straight tube, which finishes at the anus (Barker and Gibson, 1978; Ceccaldi, 1997). Barker and Gibson (1978) described mud crab digestive tract morphology and histology, which is briefly outlined in Table 1.2.

Table 1.2 Anatomy of digestive tract of mud crab (Barker and Gibson, 1978)

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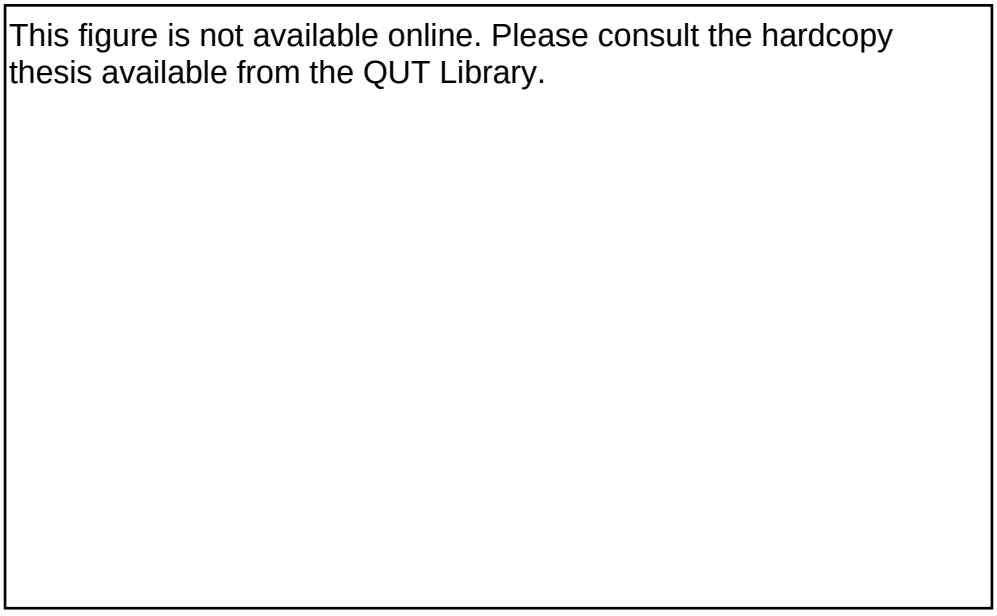

Figure 1.2 Internal anatomy of portunid crab without gills and musculature (McLaughlin, 1983).

1.3.2 Physiology of digestive tract

Food taken by the mouth is passed through the oesophagus to the anterior (cardiac) part of the stomach. Digestive liquid (fluid) from the hepatopancreas flows forward through the posterior (pyloric) part into the anterior part of the stomach, where food is mixed with digestive liquid and ground (tritutation) in the gastric mill (Dall and Moriarty, 1983; Ceccaldi, 1997).

The mass of digestive liquid and food passes through the ventral section of the anterior part of the stomach and goes backward into the posterior part of the stomach where it passes through a filter which excludes all particles larger than 1µm. The filtered mass of digestive liquid and food then continues into the hepatopancreas (Dall and Moriarty, 1983; Ceccaldi, 1997).

In the process of food digestion, the hepatopancreas has the following functions; secretion and synthesis of digestive enzymes, absorption of digested food, metabolism of proteins, carbohydrates, lipids and vitamins, mineral reserve maintenance and distribution of stored reserves (Ceccaldi, 1997).

To clearly understand the physiological significance of the hepatopancreas, it is important to understand its histological structure. The hepatopancreas has four cell types; E-cells or Embryonalzellen, R-cells or Restzellen, F-cells or Fibrezelen and B-cells or Blasenzellen, (Barker and Gibson, 1978; Dall and Moriarty, 1983; Anderson, 1989; Dall, 1992; Ceccaldi, 1997). Al-Mohann and Nott (1987) described a new type of cell, the M-cell or midget cell.

E-cells (embryonic) are apical cells of each hepatopancreas tubule and give

arise to R-cells and F-cells, however it is F-cells, which differentiate into B-cells (Barker and Gibson, 1978; Dall and Moriarty, 1983; Ceccaldi, 1997).

The primarily function of R-cells (absorptive cells) is storage. These cells have microvilli, which are densely arranged allowing for efficient absorption. Mature cells contain lipid droplets (accumulation), glycogen particles (accumulation) and elements (Cu, P, etc.) (Barker and Gibson, 1978; Dall and Moriarty, 1983; Dall, 1992; Ceccaldi, 1997).

F-cells (fibrillar cells) have microvilli similar to R-cells which are also believed to contribute to absorption. These cells secrete and synthesise digestive enzymes and they differentiate into B-cells (Barker and Gibson, 1978; Dall and Moriarty, 1983; Dall, 1992; Ceccaldi, 1997).

B-cells (vacuolar cells) have two main functions; intra-cellular digestion of absorbed nutrients and removal of insoluble waste (Dall, 1992).

M-cells (midget cells – not in diagram) are involved in nutrient absorption, (Guillaume and Ceccaldi, 2001) and have a storage function (Ceccaldi, 1997). The process of digestion and differentiation of hepatopancreas tubules is presented diagrammatically (Fig. 1. 3) (Dall, 1992).

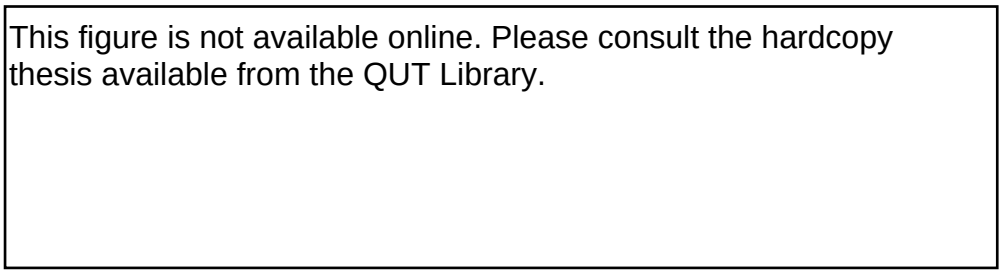

Figure 1.3 Scheme of differentiation and function of digestive gland tubule, however M-cells are not shown in this scheme, because their function currently, is not clear (Dall, 1992).

After the digestive gland, food is directed into the midgut. In the midgut some absorption of nutrients occurs, but the main role of this structure is water and electrolytic regulation. Anterior and posterior caeca are involved in the process of nutrient absorption. Following the midgut, the food passes into the hindgut where water absorption and formation of faeces takes place (Anderson, 1989).

Although the anatomy of the mud crab digestive system is well documented, there are many aspects of the digestive physiology of this species which are poorly understood. For example, data is lacking about the capacity of mud crabs to digest many of the ingredients commonly included in aquaculture diets. Future development of the mud crab industry will require a thorough understanding of the key nutrient requirements of this species and the digestive strategies employed to access these nutrients.

1.4 Feed in natural environments and in aquaculture

Traditionally, mud crabs have been described as carnivores or predators (Hill, 1979), however, when their stomach contents have been examined they have often been found to contain plant material (Hill, 1976; Tacon and Akiyama, 1997). Observations of natural feed ingredients can be used as a basis for targeting potential ingredients for formulated feeds for mud crabs in intensive farming systems.

1.4.1 Natural feed ingredients

In natural habitats larval mud crabs usually eat plankton while the main diet of adults and subadults are molluscs (shellfish). Traditionally, mud crabs have been viewed as carnivores that show a preference for natural diets containing molluscs, crustaceans and dead fish (Hill, 1979), while they will also cannibalise smaller mud crabs. Mud crabs are known to prefer live food and locate prey by sight, touch and chemical means (Kailola *et al*., 1993; Baliao, 2000). Feeds provided as complete or supplementary diets in mud crab (*S. serrata*) culture are summarised below in (Table 1. 3) (Tacon and Akiyama, 1997).

Table 1.3. Feeds of mud crab, adapted from (Tacon and Akiyama, 1997).

FOOD TYPE	DESCRIPTION
CRUSTACEANS	Penaeid prawns – general
MOLLUSCS	Clam (*Mercenaria mercenaria*) Horse mussel (*Modiolus sp*.)
FISH	Trash fish – general
VERTEBRATES	Slaughterhouse waste – general
PLANTS	Aquatic macrophytes (sea grass *Halophila ovalis*, filamentous algae (*Chaetomorpha sp*.,and *Enteromorpha sp*.)

1.4.2 Processed feed ingredients

A number of successful diets have been specifically formulated for crustacean species and used in aquaculture (Tarshis, 1978). At present, however, relatively little specific data exists on the suitability of processed feed ingredients for mud crab culture. Potentially, a wide range of processed feed ingredients are readily available for inclusion in formulated mud crab diets based on natural mud crab diets. For example, New (1987) recognised several key categories of feed ingredients that have significant potential in formulated aquaculture diets;

- *grasses* - grasses (dried) are limited in feed value and used as minor feed ingredients for prawns and fish (except herbivorous fishes).

- *fruits and vegetables* - they have not been used commonly in prawn nutrition as feed ingredients, but are available as seasonal waste and products from processing or harvesting of fruits and vegetables. They are a source of carbohydrates.

- *root crops* - are excellent sources of carbohydrates; their value as feed ingredients for prawns is limited because of poor utilisation but they appear to have potential for crabs.

- *cereals* - cereals and cereal products also have a very high level of carbohydrates. They can be important feed ingredients, because their starch content increases water stability of the feed and they can contribute significant amounts of proteins, lipids and are an excellent source of B-group vitamins.

- *legumes* - a few legumes have been used successfully as feed ingredients in aquaculture and they are rich in proteins and minerals.

- *oil-bearing seeds and oil cakes* - are important ingredients in feed formulations because they have a high level of protein (oil seeds are high in protein

content (usually from 20 to 50 %) and a low level of carbohydrate.

- *miscellaneous fodder plants* - the leaves and other parts of many plants, other than those specifically grown for fodder can be used as ingredients for food in aquaculture (plants in this category may have local significance as feed ingredients but can have low digestibility).

- *animal products* - animal by-products are an excellent source of proteins, vitamins and poly-unsaturated fatty acids.

- *additives* - these are feed ingredients which are added as binders, antioxidants, vitamins, synthetic amino acids, prophylactic medicines, hormones or growth promoters.

Each category has unique characteristics which should be considered in the process of feed preparation. For example, feed ingredients for prawns are chosen on the basis of cost, nutrient content and availability (New, 1987). Processed feed ingredients commonly utilised in crustacean aquaculture diets are listed inTable1.4. Processed feed ingredients include all animal and plant food items which have first been subjected to drying, fermenting, compositing, grinding, pelleting or mixing with other food items into a compounded diet (Tacon and Akiyama, 1997).

Table 1.4 Observed dietary inclusion level in % of the selected processed feed ingredients used within practical complete diets for marine penaeid shrimp and fresh water crustaceans (Tacon and Akiyama, 1997).

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1.4.3 Specific feed ingredients

The nutritional value of any feed ingredient depends on its physical characteristics, including particle size, density, water solubility, pelletability and its biochemical composition. The best feed ingredients for prawns are ingredients which have a biochemical structure which is very similar to the biochemical structure of the organism consuming them (Tacon and Akiyama, 1997).

Any natural or processed feed ingredient used in manufactured feeds must contain the essential nutrients required to support the candidate species. All nutrients in manufactured feeds are grouped into classes according to chemical composition as presented in Fig. 1.4 and discussed in the following sections.

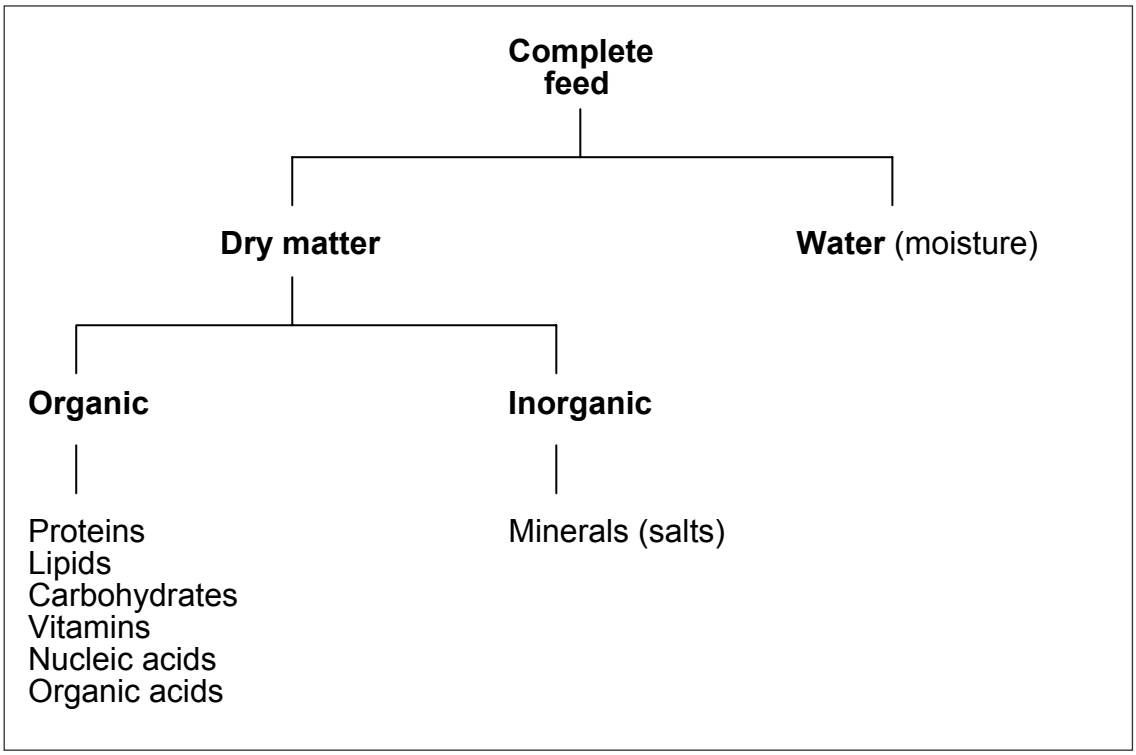

Figure 1.4 Scheme of hierarchical subdivision of a feed showing the major chemical components, adapted from (Jobling, 2001).

1.4.3.1 Protein

Proteins are major nutrients present in many feed ingredients of both plant and animal origin (Jobling, 2001) and are normally the most expensive ingredients in artificial diets for both aquatic and terrestrial animals. Proteins are large organic molecules consisting of amino acid monomers. Although over 200 amino acids have been isolated from biological materials, only 20 are commonly found in most proteins (McDonald *et al*., 2002) and therefore need to be considered in nutritional

studies (DeSilva and Anderson, 1995). Amino acids are classified as essential (animals cannot synthesise or may not synthesise in sufficient quantity), conditionally essential (can be synthesised from other essential amino acids, - i.e. cystine and tyrosine can be synthesised from methionine and phenylalanine) and non-essential (can be synthesised from other compounds). This classification is presented in Table 1. 5.

Table 1.5 Classification of essential, conditionally essential and non-essential amino acids, adapted from (Jobling, 2001).

ESSENTIAL	CONDITIONALLY ESSENTIAL	NON-ESSENTIAL
Arginine	Cystine	Alanine
Histidine	Tyrosine	Asparagine
Isoleucine		Aspartic acid
Leucine		Glutamine
Lysine		Glutamic acid
Methionine		Glycine
Phenylalanine		Proline
Threonine		Serine
Tryptphan		
Valine		

To facilitate maximum growth and reproduction animals require an optimal level of protein in their diet (New, 1987; Guillamue, 1997). The optimal level of dietary protein will vary with the dietary characteristics (digestibility and biological value of protein), animal size, age and species, as well as temperature and salinity (Guillamue, 1997). Crustaceans and other aquatic animals generally require higher levels of proteins than do most terrestrial animals (DeSilva and Anderson, 1995).

1.4.3.2 Carbohydrates

Carbohydrates are one of the major energy sources in diets besides proteins and lipids and are an important energy store in plants (Webster and Lim, 2002). Carbohydrates are typically the cheapest of the major feed ingredients used in artificial diets for aquatic or terrestrial animals. They can be found in abundance in many plant-based ingredients and have a molecular structure based upon carbon, hydrogen and oxygen (Shiau, 1997), although some contain small proportions of nitrogen (chitin) (Jobling, 2001), and sulphur (heparin) (Campbell and Smith, 1988). As shown in (Fig. 1.5), carbohydrates are classified as monosaccharides (simple sugars such as glucose), oligosaccharides (sugars made up of 2-10 monomer units) or polysaccharides (polymers made up of a large number of monomer units). Furthermore, polysaccharides can be classified as homopolysaccharides (single monomer monosaccharide) or heteropolysaccharides (made up of two or more types of monomer units and derived products).

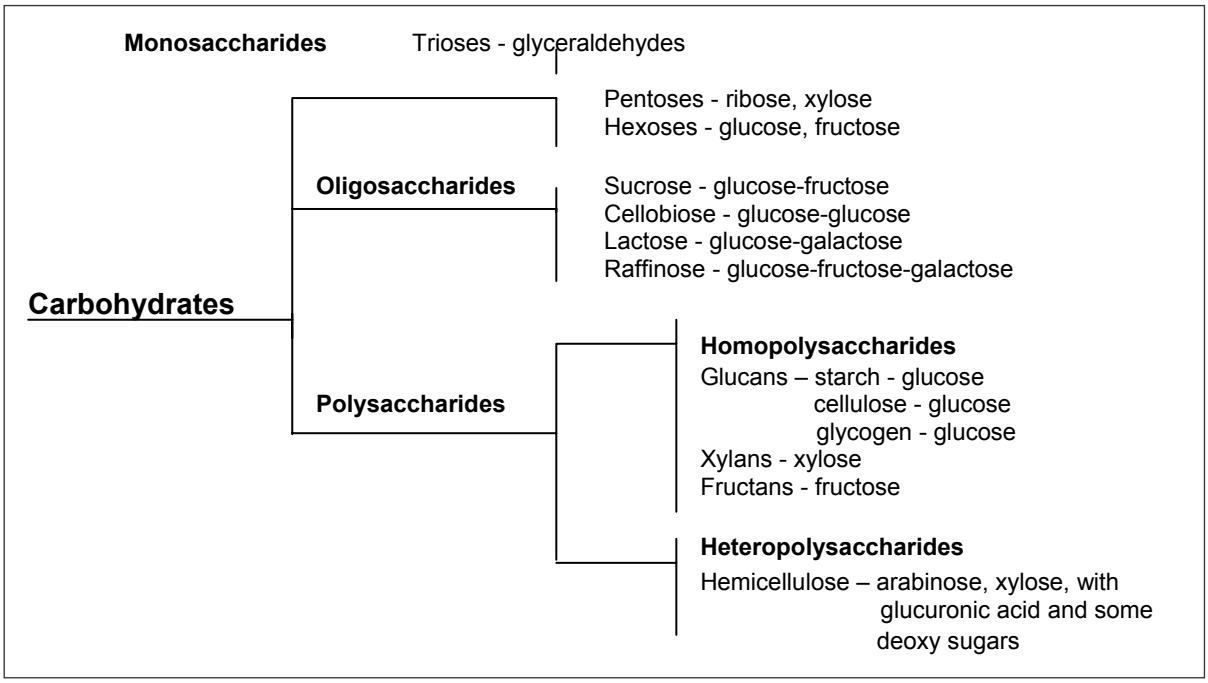

Figure 1.5 Classification scheme of carbohydrates according to the size of the molecule, adapted from (Jobling, 2001)

An alternative classification has been proposed which designates carbohydrates as sugars, starch or fibre, where fibre is composed of a variety of polysaccharides including cellulose, hemicellulose, pectic substances, gums, mucilages, algal polysaccharides and lignin, nonpolysaccharide. Fibre is a complex substance, however, whose single common property is resistance to digestive enzymes. Currently, there is still no precise and universal accepted definition of dietary fibre (Shiau, 1997). In general, aquatic animals utilise carbohydrates less efficiently than do terrestrial animals (Shiau, 1997). The results of investigations into prawn nutrition, however, have shown that they also normally utilise complex carbohydrates, such as starch, more efficiently than simple monomer sugars such as glucose (Shiau, 1998).

1.4.3.3 Homopolysaccharides (amylose, cellulose and xylan)

According to the carbohydrate classification of Jobling, (2001) amylose, cellulose and xylan are classified as homopolysaccharides. Each carbohydrate is composed of repeating units of a single mosaccharide; glucose - amylose, cellulose (Hart *et al*., 1995) and xylan - xylose (McDonald *et al*., 2002).

Amylose

Amylose is a homopolysaccharide (Jobling, 2001) that is a major component of starch (McDonald *et al*., 2002). It is composed mainly as a linear chain of glucose monomers (50 - 300) with α-1,4 linkages (Fig. 1.6) (Hart *et al*., 1995).

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Figure 1.6 Part of amylose molecule (McDonald *et al*., 2002).

Cellulose

Cellulose is the most abundant single polymer in plants and is found in a nearly pure form in cotton (McDonald *et al*., 2002), with significant amounts also found in wood, straw, linen and many other agricultural crops. It is an unbranched polymer of glucose (Fig. 1.7) joined by β-1,4-glycosidic bonds (Hart *et al*., 1995). Cellulose linear molecules contain an average of 5000 glucose units, aggregated to give fibrils which make fibres (Hart *et al*., 1995).

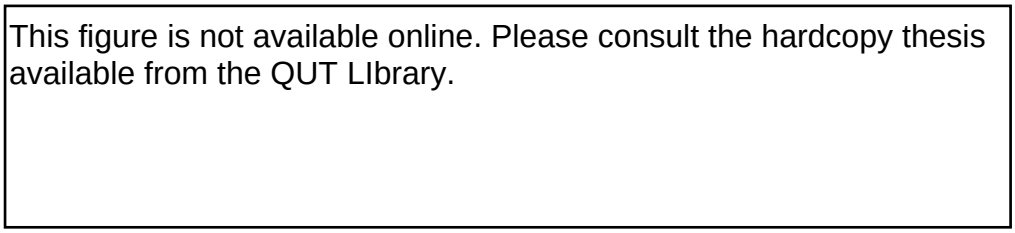

Figure 1.7 Part of cellulose molecule (McDonald *et al*., 2002).

Xylan

Xylan is a polymer of β-1,4 linked α - D - xylose monomers which differs from glucose only by the lack of the C_6H_2OH (Fig. 1.8) (Voet and Voet, 1995) and is found in relative abundance in most plants (Mathews *et al*., 2000).

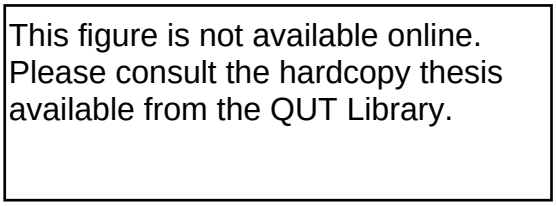

Figure 1.8 α - D - xylose monomers (Voet and Voet, 1995).

1.4.3.4 Lipids

Lipids are a group of heterogeneous organic compounds that are soluble in organic solvents such as chloroform, ether or benzene and which represent a major nutrient source for crustaceans (Jobling, 2001; McDonald *et al*., 2002). As detailed in Fig. 1.9, lipids are divided in two groups, glycerol-based and non-glycerol based. The glycerol-based lipids are subdivided into simple and compound lipids. The compound lipids are again subdivided into glycolipids and phospholipids. The non-glycerol based lipids are divided into steroids, terpenes and waxes. For the crustaceans the simple (fatty acids and triglycerides), compound lipids (glycolipids and phospholipids) and steroids are very important lipids (D'Abramo, 1997; Teshima, 1997).

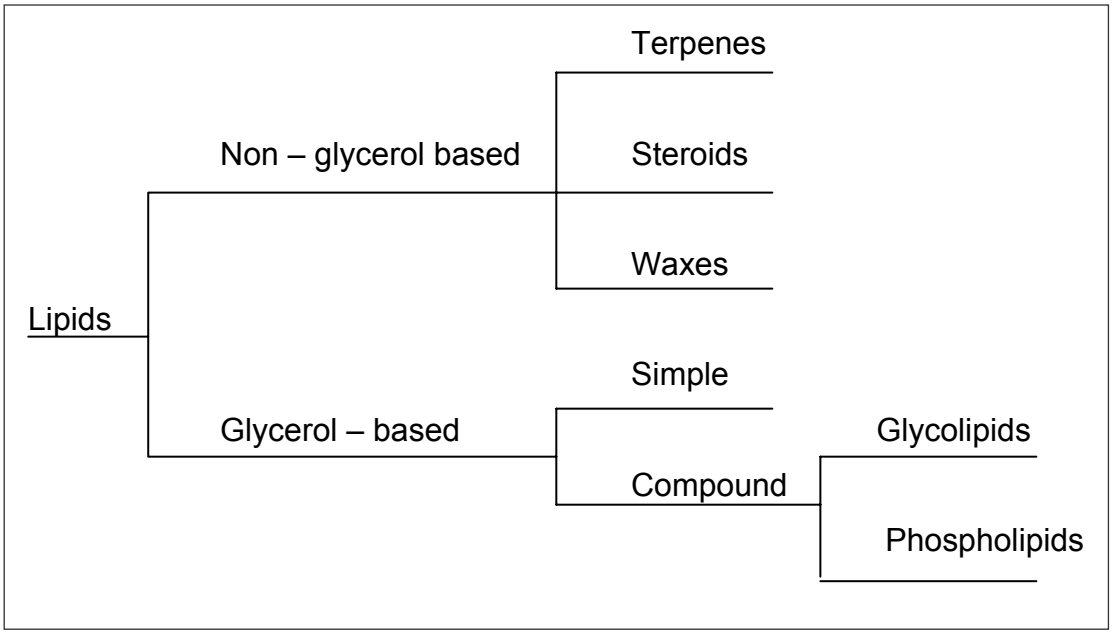

Figure 1.9 Classification scheme of lipids, adapted from (Jobling, 2001).

The basic unit of lipids are fatty acids, which are divided into two classes; saturated and unsaturated (Hart *et al*., 1995). Crustaceans can synthesise *de novo* saturated fatty acids and like other animals they possess enzyme systems

(delta - 9 - desaturase), which can convert saturated to mono-unsaturated fatty acids (D'Abramo, 1997). Crustaceans are unable to synthesise polyunsaturated fatty acids however, and therefore they need food that can supply these components (D'Abramo, 1997; Mulford and Villena, 2000).

Steroids are essential components for crustacean nutrition and have a large influence on growth, development, reproduction and survival (Teshima, 1997). Crustaceans cannot synthesise steroids *de novo* so they need exogenous sources. The most important source of steroids for crustaceans is cholesterol (Teshima, 1997). Phospholipids are also essential components in crustacean diets because they are needed for normal growth, development, reproduction and survival, but unlike steroids, crustaceans are able to synthesise phospholipids (Teshima, 1997).

The metabolism of lipids is under the control of adipokinetic and hyperglycaemic hormones (Mulford and Villena, 2000). Lipids taken, as nutrients are degraded by lipase enzymes to diglycerides and monoglycerides for absorption after which they are converted to phospholipids and stored in the hepatopancreas (Mulford and Villena, 2000).

1.4.3.5 Other ingredients (vitamins, minerals and binders)

Other major feed ingredients include vitamins, minerals and binders. While vitamins and minerals are normally required in only very small amounts, they are very important for normal growth and reproduction.

Vitamins

Vitamins are organic compounds, which participate as cofactors in key metabolic reactions and are one of the most important components to be considered in crustacean nutrition. Vitamins are typically required in small amounts for normal health, development, growth and reproduction (DeSilva and Anderson, 1995; Shiau, 1998). They cannot as a rule be synthesised by the organism, and therefore must be supplied as part of the diet (Steffans, 1989). Vitamins can be divided in two groups; water-soluble and fat-soluble vitamins (New, 1987; Halver, 1989).

An adequate supply of fat-soluble vitamins can improve growth, while water-soluble vitamins can also influence growth, health and reproduction of crustaceans (He *et al*., 1992). Inadequate levels or absence of particular vitamins can lead to serious metabolic disorders, which are referred to as avitaminoses. Inadequate supply of vitamins in the diet may result in nutritional disorders, poor growth, stress response, an increased susceptibility to infections or even death (Conklin, 1997)

Minerals

Like most animals, crustaceans require small amounts of minerals, to support a number of key metabolic processes (DeSilva and Anderson, 1995). For example, minerals can act as biocatalysts for enzymes, hormones and proteins or form components for hard-tissue matrices (exoskeleton), soft-tissues, and as cofactors or activators of enzymes. Minerals are also involved in maintenance of osmoregulation, pH balance and membrane potential (Davis and Lawrence, 1997).

Minerals are usually divided in two groups:

-Macro-minerals - Ca, P, Na, K, Cl, Mg

-Micro-minerals - Cu, Fe, I, Mn, Se, Zn

Quantitative mineral requirements for crustaceans have not been formally determined (Davis and Lawrence, 1997). Inadequate levels of minerals, however, can result in a variety of nutritional diseases, poor growth, stress, an increased susceptibility to disease infections or even death (DeSilva and Anderson, 1995).

Binders

Binders are additives used in feed formulation to increase feed durability and water stability. Substances used for pellet water stability can be divided into two groups, water stability for dry pellets and water stability for moist pellets (moist feed). In dry pellet formulation, substances which are used include casein, gelatine, collagen, chitosan, agar, corn starch, potato starch, wheat gluten, carboxymethylcellulose, lignosulphonates, sodium alginate, sodium hexametaphosphate hemicelluloses and bentonites (New, 1987; Lim and Cuzon, 1994).

1.4.4 Feed formulation

Feed formulation is a process in which appropriate feed ingredients are selected for inclusion in a diet to ensure the final diet contains the required quantities of essential nutrients. As described previously, the major nutrients in feeds are proteins, lipids, carbohydrates, minerals and vitamins. Every one of these components has an optimal level for inclusion in feeds to support normal growth and reproduction in targeted species.

One of the earliest methods used for feed formulation is the Pearson square method. The method of calculation involves combining two ingredients with different nutrient concentrations to yield a product with a specified nutrient

concentration (Houser and Akiyama, 1997). In Germany, during the 1870s a chemical method for evaluating feed ingredients was also developed. This method became very common for producing aquaculture feeds. This method includes the analysis of the composition of six feed attributes as detailed in Table 1. 6 (Hardy, 1989).

Table 1.6 Composition of categories in feed (Hardy, 1989).

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An important factor which must be considered in crustacean nutrition is knowledge of nutrient composition and availability in the potential ingredients used in feed formulations (DeSilva and Anderson, 1995). Tables 1.7 and 1.8 provide

examples of compositions of natural and formulated feeds used to feed mud crab broodstock (Millamena and Quinitio, 1999).

Table 1.7 Approximate composition of natural and formulated diet for broodstock of mud crab (Millamena and Quinitio, 1999).

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Table 1.8 Composition of formulated diet for broodstock of mud crab (Millamena and Quinitio, 1999).

This table is not available online. Please consult the hardcopy thesis available from the QUT Library.

1.5 Major digestive enzymes in crustaceans

Enzymes are complex proteins that are able to catalyse specific biochemical reactions with very high efficiency (up to 1 million times more efficient than inorganic catalysts) and are selective. As detailed below, several major classes of enzymes have been identified in crustacean digestive systems which appear to play major roles in liberating essential nutrients from materials ingested during feeding.

1.5.1 Protease

Protease enzymes facilitate the breakdown of proteins and, as detailed below, several major classes of proteases have recently been identified in crustacean digestive systems. Specifically;

- Trypsin is a major proteolytic enzyme which normally exhibits high activity in crustaceans. Interestingly, unlike vertebrate trypsin, crustacean trypsin can hydrolyse native proteins (Dall and Moriarty, 1983).
- Chymotrypsin is a serine protease recently identified in the digestive systems of crustaceans such as prawns (Guillaume, 1997).
- Carboxypeptidases A and B are proteolytic enzymes identified in several crustaceans species, although the levels of activity vary widely among species (Guillaume, 1997).
- Aminopeptidases and dipeptidases are exopepsidases that have been identified in penaeids (Ceccaldi, 1997).
- Astacin is a metallo-protease enzyme with a wide spectrum that has a unique ability to cleave native collagen (Guillaume, 1997).

1.5.2 Amylase

Amylase (α-1,4 glucanase) is a glucosidase, which can hydrolyse the α-1,4 bonds of linear fragments of amylopectin, but cannot hydrolyse α-1,6 bonds from branch points (linear chains are connected at branch points by α-1,6 bonds) (Guillaume and Ceccaldi, 2001). Amylase has been poorly studied in crustaceans (Ceccaldi, 1997).

1.5.3 Cellulase

Efficient digestion of cellulose typically occurs via the synergistic action of several different classes of cellulase enzymes which cleave β-1, 4 linkages between the glucose monomers. Cellulase (endoglucanase) activity has been detected in a number of commercially important crustacean species, including *Penaeus japonicus* (Yokoe and Yasumasu, 1964), *Euphausia superba* (Chen and Chen, 1983), *Homarus gammarus* (Glass and Stark, 1995) and *Cherax quadricarinatus* (Xue *et al*., 1999).

1.5.4 Xylanase

Xylanase (endoxylanase) activity has been reported in *C. quadricarinatus* (Xue *et al*., 1999) and *E. superba* (Turkiewicz *et al*., 2000).

1.6 Aims of project

Currently, trash fish or artificial prawn pelleted feeds are used as the diet for intensively cultured mud crabs. This practice is based primarily on the belief that mud crabs are a carnivorous species which require high levels of animal protein in their diets. If mud crabs are able to readily utilise other dietary

components, such as carbohydrates, then the potential exists to formulate diets which support good growth while minimising the requirement for relatively expensive protein-based feed ingredients.

There are two major aims to the current investigation; firstly to identify and profile overall activity levels and tolerances of selected digestive enzymes (protease, amylase, cellulase and xylanase) from the hepatopancreas (digestive gland) of mud crabs. The second major aim is to determine the effect of altering protein and carbohydrate composition in formulated feeds by assessing the impact on feed digestibility, digestive enzyme activities and growth performance.

2. GENERAL METHODS

2.1 Animals

Experimental animals were supplied by Bribie Island Aquaculture Research Centre Station (BIARC), Bribie Island, QLD Australia. Crabs were initially harvested from the Research Centre's outdoor concrete tanks (9m x 3m x 1.2m). While in the growout tank, crabs were fed EBI Star prawn (*Penaeus japonicus*) feed (50% crude protein, 8% crude fat, 19% crude ash; Higashimaru, Japan) at a rate of approximately 4% body weight per day. Animals selected for the pH and temperature tolerance studies were transported live under moist conditions to the School of Natural Resource Sciences, QUT, and immediately prepared for removal of the hepatopancreas.

Animals selected for the dietary trials were assigned randomly into four groups and housed in individual containment boxes (19.5 x 28 x 22cm) which were then covered by lids (Fig. 2.1). All boxes were supplied with recirculated, aerated seawater that was gravity fed through an electrically heated overhead tank. Water temperature was maintained within a range (27.5 $\pm$ 0.5^{o}C) as recommended for optimal growth of mud crabs (Chen and Jeng, 1980).

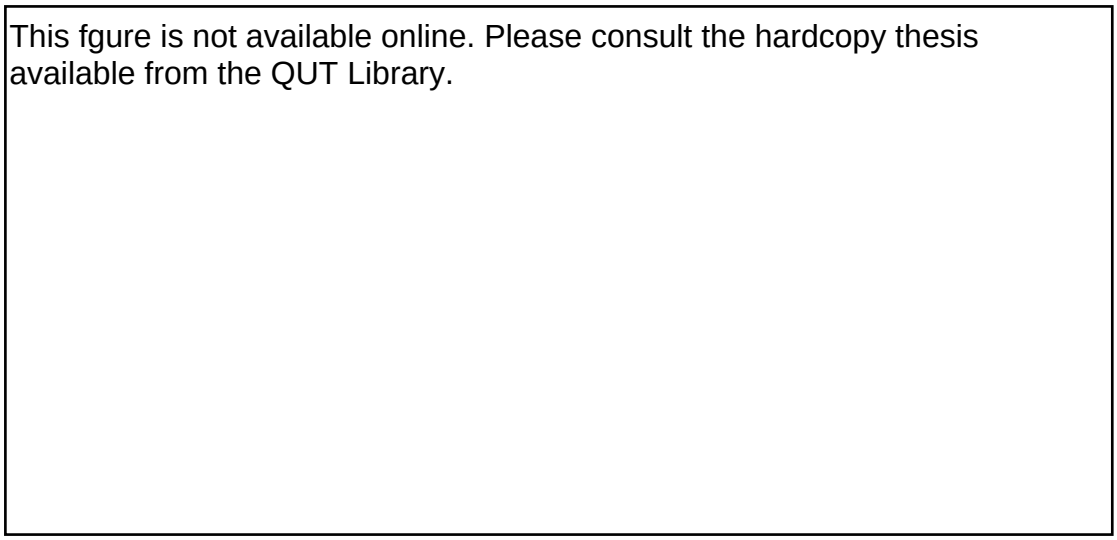

Figure 2.1 Recirculating culture tank system.

2.2 Preparation of hepatopancreas for digestive enzyme assays

After final body measurements were taken, individual crabs were anaesthetised in ice water for approximately five minutes. To minimise the risk of contaminating samples with any microbial enzymes which may have been present in the gut, the hepatopancreas was dissected out, cut into small pieces and washed thoroughly three times in homogenate buffer solution (10Mm sodium citrate / 0.1M NaCl; pH 7.0). After washing, hepatopancreatic tissue was weighed, mixed with equal amounts of homogenate buffer solution then homogenised in an Ultraturrax homogeniser for one minute at full speed. Finally, the homogenate was centrifuged in a microfuge set at 13,000 rpm for 10 minutes at room temperature, after which the supernatant was stored at -20^{o}C until required.

2.3 Digestive enzyme assays

One enzyme unit (U) was defined as the amount of enzyme that catalysed the release of 1μmol of product per minute under the assay conditions. Specific activity was defined as enzyme activity (U) per mg of protein in the homogenate. Protein concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad, USA) using gamma globulin as the protein standard. Briefly, the hepatopancreatic extract was diluted 1:16 in distilled water, then 100μl of the diluted extract was mixed with 5ml of dye reagent (diluted 1:4 in distilled water). After incubation for 15min at room temperature, the absorbance of the solution was measured at 595nm using a Novospec spectrophotometer (LKB).

2.3.1 Protease activity determinations

Total protease determination was performed on hepatopancreatic extracts using an insoluble dye-linked marker, azurine cross-linked casein (AZCL-casein; Megazyme, Ireland) as substrate. Briefly, 250 μl of a 0.75% AZCL-casein solution was adjusted to pH 7.0 using 90 μl of substrate buffer solution (0.1M citric acid / 0.2M Na_2PO_4; adjusted to pH 7.0 with 1M NaOH). A 10 μl volume of hepatopancreatic extract, diluted 1:5 in distilled water, was then added and the mixture incubated at 40^0C for one hour in a water bath. The reaction was stopped by placing the tubes in ice for 10 min. Following this, 700 μl of distilled water was added to the reaction mixture which was then centrifuged at 13,000 rpm for 10 min at room temperature. The absorbance of the supernatant was read at 590 nm. Protease (Subtilisin A; *B. licheniformis*) (Megazyme) was used as the enzyme standard.

2.3.2 Amylase activity determinations

Total α-amylase activity was determined using a Ceralpha (Alpha-Amylase) Assay Kit (Megazyme) as per the manufacturer's description. Briefly, 6.6 µl of a hepatopancreatic extract, diluted 1:20 in distilled water, was added to 600 µl of substrate buffer (0.1M Malic acid / 0.1M NaCl / 2mM $CaCl_2$ $2H_2O$ / 0.01% sodium azide; pH 7.0). The mixture was incubated at 40°C for 20 min after which 133 µl of substrate (BPNPG-7, 54 mg + 10 ml distilled water) was added and the incubation continued for a further 10 min. After incubation, 1.9 ml of stopping reagent (20% (W/V) Trisodium phosphate solution, pH 11) was added to the mixture and the absorbance was measured at 590 nm.

2.3.3 Cellulase activity determinations

Total cellulase determination was performed on hepatopancreatic extracts using an insoluble dye-linked marker, azurine cross-linked hydroxyethylcellulose (AZCL-HE cellulose; Megazyme) as substrate. Briefly, 250 µl of a 0.75% AZCL-HE-cellulose solution was adjusted to pH 5.5 using 70 µl of substrate buffer solution (200mM Sodium citrate – adjusted to pH 5.5 with 1M HCl). A 30 µl volume of hepatopancreatic extract was then added and the mixture incubated at 50°C for two hours in a water bath. The reaction was then stopped by placing the tubes in ice for 10 min. After that, 700 µl of distilled water was added to the reaction mixture which was then centrifuged at 13,000 rpm for 10 min at room temperature. The absorbance of the supernatant was read at 590 nm. Endoglucanase EG11 (Megazyme) was used as the cellulase enzyme standard.

2.3.4 Xylanase activity determinations

Total xylanase determination was performed on hepatopancreatic extracts using an insoluble dye-linked marker, azurine cross-linked xylan (AZCL-xylan; Megazyme) as a substrate. Briefly, 900 μl of a 0.5% AZCL-xylan solution in substrate buffer (50mM sodium citrate; adjusted to pH 5.5 with 1M NaOH) was combined with a 100 μl volume of hepatopancreatic extract (diluted 1:5 in distilled water) and samples incubated at 40^0C for one hour in a water bath. The reaction was stopped by placing the tubes in ice for 10 min. After that, 700 μl of distilled water was added to the reaction mixture which was then centrifuged at 13,000 rpm for 10 min at room temperature. The absorbance of the supernatant was read at 590 nm. Beta-xylanase M6 (Megazyme) was used as the xylanase enzyme standard.

2.4 Glucose assays

The capacity of carbohydrases in extracts from the mud crab hepatopancreas to cleave glucose from polysaccharide substrates (starch or cellulose) was determined using a Glucose Reagent Kit (Sigma, USA) as per the manufacturer's instructions with the following modifications;

2.4.1 Amylase

A 2% solution of starch was prepared in substrate buffer (0.1M Malic acid / 0.1M NaCl / 2mM $CaCl_2$ $2H_2O$ / 0.01% sodium azide; pH 7.0). A 400 μl volume of this substrate solution was then combined with 40 μl of hepatopancreas extract, incubated for 10 min at 40^oC and then placed in ice for a further 10 mins. A 25 μl volume of the starch / hepatopancreatic extract mixture was then added to 5 ml of

Enzyme Colouration Reagent and 475 μl of distilled water. This mixture was incubated for 30 minute at 37°C after which the absorbance was measured at 450 nm. For calibration purposes, negative controls (data not shown) were also made by including all of the above reagents with the exception of the starch substrate solution or the hepatopancreatic extracts.

2.4.2 Cellulase

A 0.5% solution of Carboxymethyl (CM)-cellulose was prepared in substrate buffer (50mM sodium citrate; pH 5.0). A 400 μl volume of this substrate solution was then mixed with 50 μl of hepatopancreas extract, incubated for 1hour at 55°C and then placed in ice for a further 10 mins. A 25 μl volume of the CM-cellulose / hepatopancreas extract mixture was then added to 5 ml of Enzyme Colouration Reagent and 475 μl of distilled water. This mixture was incubated for 30 mins at 37°C after which the absorbance was measured at 450 nm. For calibration purposes, negative controls (data not shown) were also made by including all of the above reagents with the exception of the cellulose substrate solution or the hepatopancreatic extracts.

2.5 Dry matter digestibility determinations

The indirect method of Furukawa and Tsukahara (1966) was used to calculate the apparent dry matter digestibilities (ADMD) coefficients of all feeds used in the dietary trials. Briefly, 0.5g of feed or faecal material was added to 4.0 ml of concentrated nitric acid (AnalaR grade, 16 M HNO_3) and incubated overnight at room temperature. Samples were then heated to 150°C for an additional hour. After cooling, samples were mixed with 5.0 ml of concentrated perchloric acid

(AnalaR grade, 70% $HClO_4$) then heated to 220^{o}C for 30min and 245^{o}C for a further 30 min. After cooling, the absorbance of each sample was read at 346.5 nm. For calibration purposes, the above protocol was repeated using known quantities of Cr_2O_3. ADMD coefficients were then determined by using the formula described by Jones and De Silva (1998) as follows:

ADMD = 100 – 100 (% marker in feed / % marker in faeces).

2.6 Statistical analysis

Where indicated, for testing the significance of differences, Student's *t* test was applied. For all analyses a significant level was considered for $p < 0.05$.

3. INFLUENCE OF pH AND TEMPERATURE ON DIGESTIVE ENZYME PROFILES IN JUVENILE MUD CRABS

3.1 Introduction

Traditionally, mud crabs have been viewed as carnivores that show a preference for natural diets containing molluscs, crustaceans and dead fish (Hill, 1979). Interestingly, however, preliminary studies on the digestibility of experimental mud crab feeds containing plant materials have shown high digestibilities for all nutrients including fibre and ash (Catacutan *et al*, 2003). It has also been demonstrated that the dietary requirements of this species are not nearly as stringent as those of most Penaeid prawns, with good growth occurring over a wide range of protein and lipid levels (Catacutan, 2002). Findings such as these, raise the possibility that a relatively broad range of plant and animal-based ingredients may be incorporated successfully into mud crab diets.

Current research on the nutritional needs of crustaceans has focused largely on the target species digestive processes and ability to hydrolyse, absorb and assimilate nutrients (cf. Guzman *et al*, 2001). One of the main methods for investigating these processes is to profile a specie's digestive enzyme activities. Crustaceans are known to possess many of the enzymes required to breakdown key nutrients in their diet such as proteins, carbohydrates and lipids. Studies on protein digestion in crustaceans has revealed the presence of trypsin (Zwilling and Neurath, 1981; Lu *et al.*, 1990; Vega-Villasante *et al.*, 1995; Figueiredo *et al.*, 2001) chymotrypsin (Van Wormhodt *et al.*, 1992; Figueiredo *et al.*, 2001), carboxypeptidases and aminopeptidases (Appel, 1974; Vega-Villasante *et al.*, 1995; Ceccaldi, 1997; Figueiredo *et al.*, 2001). Carbohydrase activities in

crustacean digestive tissues have also been documented extensively. For example, relatively high levels of amylase activity have been detected in crabs (Brethes *et al.*, 1994), prawns (Omondi and Stark, 1995) and crayfish (Figueredo *et al.*, 2001).

In recent years, carbohydrases other than amylase have been identified which suggest that non-starch carbohydrate-based substrates, such as those found in fibre, may also have potential as nutrient sources in diets for crustaceans. In particular, cellulase activity has been demonstrated in such species as prawns (Noborikawa, 1978; D'Abramo and Sheen, 1994; Gonzalez-Pena *et al*., 2002), crabs (Brethes *et al*., 1994) and crayfish (Xue *et al.*, 1999; Figueiredo *et al*., 2001). Byrne (1999) reported the isolation of an endo-1,4-β-glucanase encoding cDNA from the red claw crayfish (*Cherax quadricarinatus*) strongly suggesting that, in this species at least, cellulase may be produced endogenously. The identification of carbohydrase activity in a wide range of crustacean species provides a convincing rationale for examining the potential of carbohydrate rich plant-based materials as potential ingredients in formulated mud crab diets. The major aim of this study therefore is to profile digestive enzyme activities in the mud crab hepatopancreas and, in particular, to determine if the necessary enzymes exist to utilise polysaccharides commonly found in plant-based materials.

3.2 Materials and methods

3.2.1 Animals

Experimental animals, raised under culture conditions described previously (2.1), were obtained from BIARC. The crabs selected for these pH and temperature tolerance studies had an average weight of 110.84g ± 9.38g. Crabs

were transported live under moist conditions to the School of Natural Resource Sciences (QUT) after which the hepatopancreas was removed and prepared for enzyme analysis as described previously (2.2).

3.2.2 Effect of pH and temperature on digestive enzyme activities

Extracts of mud crab hepatopancreas were screened for the presence of protease (2.3.1), amylase (2.3.2), cellulase (2.3.3.) and xylanase (2.3.4.) activities as described previously. The tolerance of digestive enzymes to changes in pH was determined by adjusting the pH of substrate buffers used in the following assays:

3.2.2.1 Protease

The substrate buffers used were 0.1M Citric acid / 0.2M Na_2PO_4 for the pH range 3-9 and 0.2 M Glycine (adjusted with 1 M NaOH) for the pH range 10-12.

3.2.2.2. Amylase

The substrate buffer used was 0.1M Malic acid / 0.1M NaCl / 2mM $CaCl_2$ $2H_2O$ / 0.01% sodium azide (adjusted with 4M NaOH) for the pH range 3-12

3.2.2.3. Cellulase

The substrate buffer used was 0.2M Sodium citrate (adjusted with either 1M NaOH or 1M HCl) for the pH range 4-12.

3.2.2.4. Xylanase

The substrate buffer used was 50mM Sodium citrate (adjusted with either 1M NaOH or 1M HCl) for the pH range 4-9.

3.2.2.5. Temperature tolerance

The tolerance of digestive enzymes from the mud crab hepatopancreas to changes in temperature were determined by assaying enzyme activities over the temperature range 4-80^{o}C.

3.2.3 Glucose assays

The capacity of carbohydrases in hepatopancreas extracts was determined as described previously in (2.4).

3.3 Results

3.3.1 Effect of pH on digestive enzyme profiles

The effects of pH on protease, amylase, cellulase and xylanase activities in mud crab hepatopancreatic extracts are shown in (Figs. 3.1a-d). Relatively high protease (Fig. 3.1a) and amylase (Fig. 3.1b) activities were demonstrated across a broad pH range (approximately 5.5-10) with maximal activity for both enzymes observed at pH 7.0. Under these conditions total amylase activity was only about half that observed for protease. Cellulase (Fig. 3.1c) and xylanase (Fig. 3.1d) activity levels increased sharply above pH 4.0 to an optimum at pH 5.5. Thereafter, the activity of both enzymes gradually declined with increasing alkalinity. Overall, activity levels for both cellulase and xylanase were much lower (approximately 100 fold) than those observed for either amylase or protease.

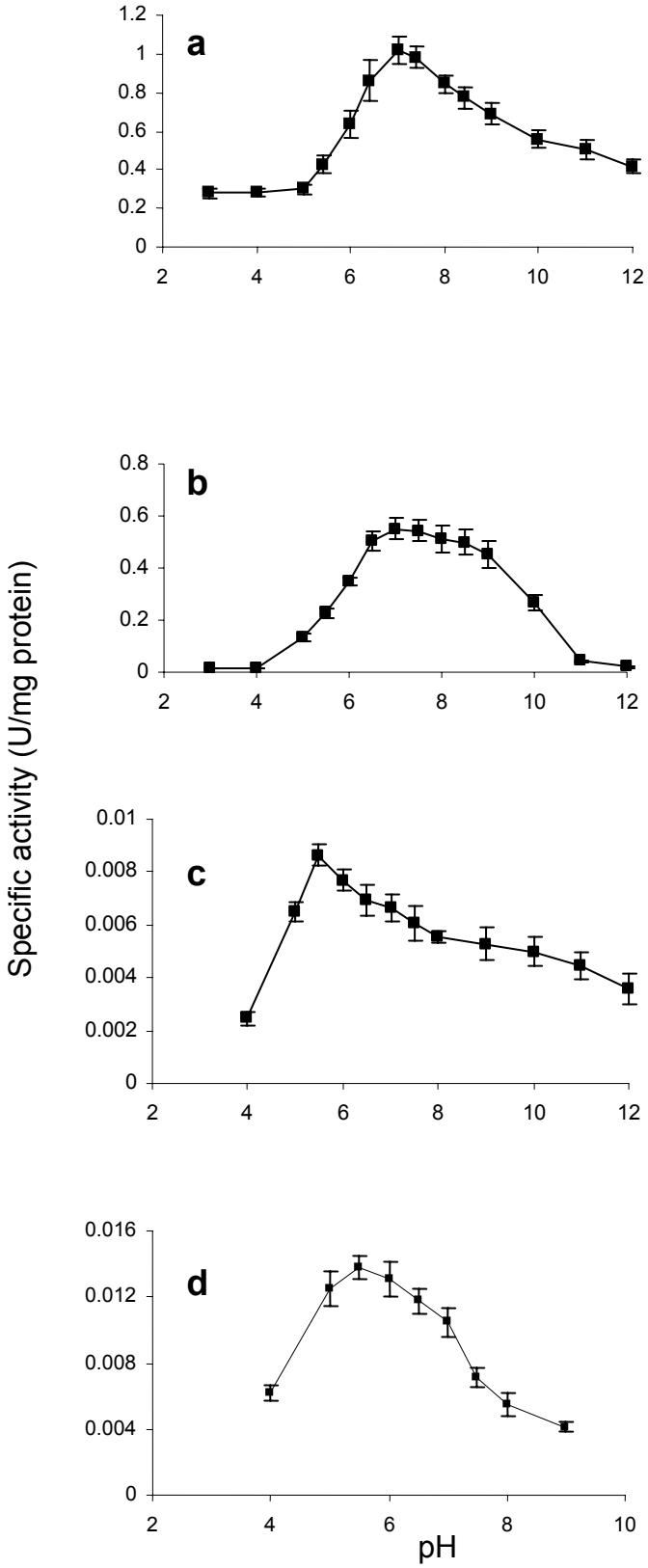

Figure 3.1 Total protease (a), amylase (b), cellulase (c) and xylanase (d) pH tolerances. Data is expressed as units of enzyme activity per mg protein in hepatopancreatic homogenate. Data points represent mean ± SEM; n=8.

3.3.2 Effect of temperature on digestive enzyme profiles

The response of all digestive enzymes tested was basically similar across the sampled temperature range 4-80^{o}C (Figs. 3.2a-d). Specifically, relatively low levels of protease (Fig. 3.2a), amylase (Fig. 3.2b), cellulase (Fig. 3.2c) and xylanase (Fig. 3.2d) activity were observed at 4^{o}C. Thereafter, enzyme activities gradually increased with rising temperature to a peak at 50^{o}C (50-60^{o}C in the case of protease). The activity of all enzymes then rapidly declined as temperature increased. Overall, activity levels for both cellulase and xylanase were much lower than those observed for amylase or protease.

3.3.3 Glucose assays

Evidence of significant glucose production was observed when extracts from the mud crab hepatopancreas were incubated with either starch or CM-cellulose substrate solutions as shown in Table 3. 1.

Table 3.1 Specific activity (U = μM glucose release / min) of hepatopancreatic extracts against starch or CM-cellulose.

SUBSTRATE	**U/mg PROTEIN**
2% Starch	0.029 + 0.0019
0.5% CM-cellulose	0.024 + 0.0026

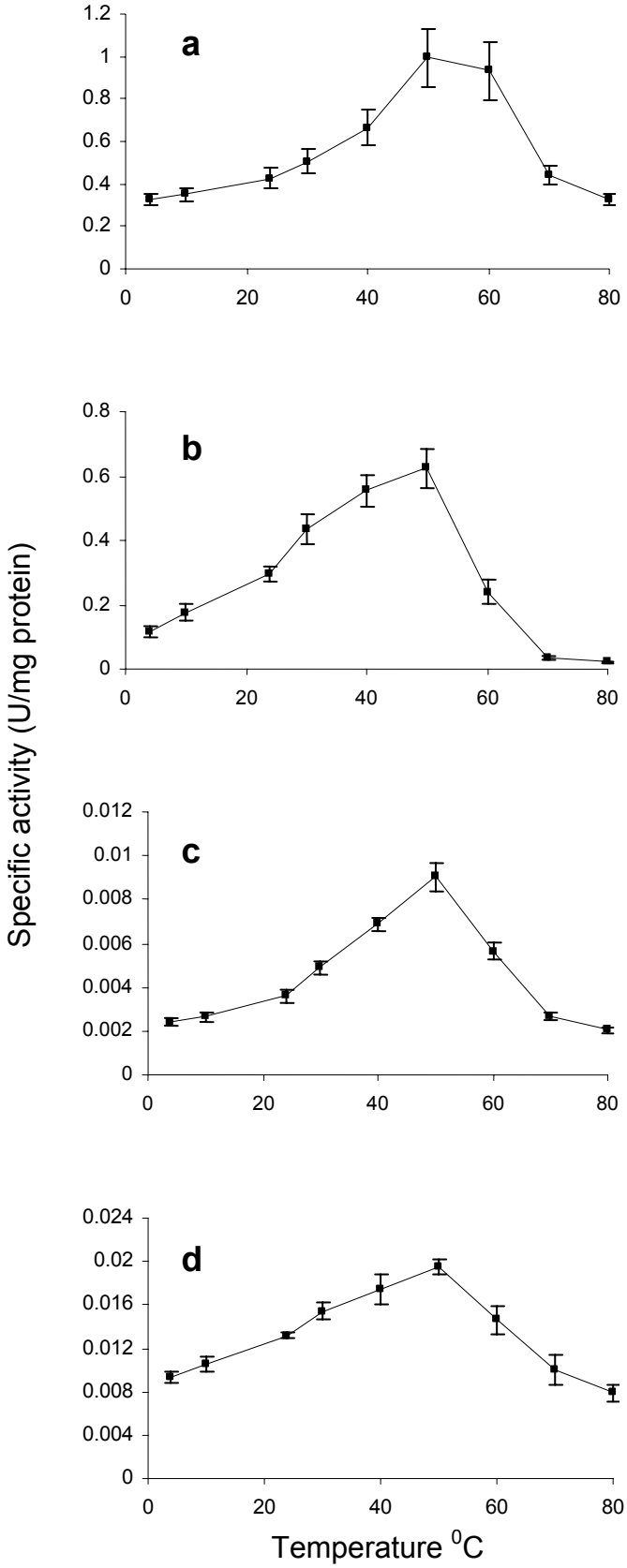

Figure 3.2 Total protease (a), amylase (b), cellulase (c) and xylanase (d) temperature tolerances. Data is expressed as units of enzyme activity per mg protein in hepatopancreatic homogenate. Data points represent mean ± SEM; n=8.

3.4 Discussion

The current study examined the activity profiles of several mud crab digestive enzymes. Overall, enzymes were active over broad pH ranges and all demonstrated significant activity at temperatures well above those experienced under natural conditions. Under optimum conditions, both protease and amylase activities were relatively high although amylase activity was normally only about half that seen for protease. An unexpected finding of this study was that the mud crab hepatopancreas contained enzymes capable of digesting non-starch polysaccharides. Significant cellulase and xylanase activities were detected in hepatopancreatic extracts, although these levels were approximately one hundred times lower than those observed for protease.

The pH and temperature profiles obtained for protease activity in the mud crab hepatopancreas are broadly comparable with those reported for other crustacean species. Specifically, mud crab protease activity was detected over a broad pH range with an optimum between pH 7.0 and 7.4. This finding is consistent with other investigations which have shown that crustacean proteases, such as trypsin and chymotrypsin, are highly active in the range between pH 5.5 and 9.0 (Garcia-Carreno, 1992; Figueiredo *et al.*, 2001). The relatively high level of proteolytic activity detected correlates well with values reported for other carnivorous crustacean species. For example, high levels of proteolytic activity have been detected in the hepatopancreas of several prawn (Le Moullac, *et al*., 1996; Fernandez-Gimenez *et al*, 2001) and lobster species (Johnston and Yellowlees, 1998; Johnston, 2003) which are known to prefer animal-based diets. By contrast, much lower levels of total protease activity have been reported in some crustaceans, such as the red claw crayfish, that prefer diets based on

detritus or plant material (Figueiredo *et al.*, 2001). Based on these findings, we suggest that the high level of protease activity observed in the mud crab hepatopancreas reflects a digestive capacity specialised for hydrolysing animal proteins.

The potential significance of amylase in the mud crab digestive system is unclear since the natural diet of this species is normally based on animal flesh and therefore probably contains little starch. One possibility is that amylase acts to breakdown any plant-based materials ingested incidentally during feeding. An alternative possibility is that amylase functions to liberate glucose from ingested animal tissues by cleaving α-1,4 linkages in polysaccharide storage molecules. Evidence to support this latter proposal comes from Johnston and Yellowlees (1998) who reported the presence of α-amylases and α-glucosidases in the digestive system of the carnivorous slipper lobster that were capable of hydrolysing glycogen in bivalve muscle tissue.

In the current study, amylase displayed significant activity between pH 5 and 10 with a maximum value at pH of 7. This was unexpected since other investigations into crustacean amylases have shown that optimal activity is typically displayed under mildly acidic conditions. For example, maximal activity in prawns (Moss *et al*., 2001) and crayfish (Figueiredo *et al*., 2001) is normally observed in the pH range between 5 and 6. At present the basis for this difference is unclear. It is important to note, however, that amylase from the mud crab hepatopancreas is highly active around 6.3, the pH value reported for mud crab gastric fluid (Rutledge, 1999). An interpretation of these data are that pH

tolerances for amylase reported in the current study are representative of an enzyme optimised for conditions in the mud crab gastric fluid.

The results of this study clearly demonstrate that mud crabs possess polysaccharide hydrolases capable of degrading components found in dietary fibre. Maximum cellulase and xylanase activities were observed at pH 5.5 which is generally consistent with values reported in other crustacean species (Xue *et al*., 1999; Figueiredo *et al*., 2001; Moss *et al*., 2001). Further analysis of mud crab cellulase activity demonstrated a capacity to liberate glucose from a soluble cellulose substrate suggesting that mud crabs have the potential to utilise cellulose-based substrates as nutrient sources. Overall, levels of cellulase and xylanase activity detected in the mud crab hepatopancreas were much lower than those observed for protease or amylase. This finding is consistent with the results of other studies which have demonstrated that crustaceans which prefer carnivorous diets in the wild, such as the spiny lobster (Johnston, 2003), express less cellulase than do crustaceans which are omnivorous, such as the red claw crayfish (Xue *et al*., 1999; Figueiredo *et al*., 2001).

The detection of any cellulase activity in the mud crab was surprising given the preference of this species for animal-based diets. It is important to note, however, that cellulase activity has been reported in a wide range of aquatic invertebrates whose diets do not normally include substantial amounts of cellulose (Elyakova *et al*., 1981; Johnston, 2003). Dall and Moriarty (1983) have suggested that cellulase activity in some of these species may represent a β-glucosidase with broad specificity that facilitates the digestion of carbohydrates such as laminarin.

4. INFLUENCE OF DIETARY PROFILE ON GROWTH AND DIGESTION IN JUVENILE MUD CRABS.

4.1 Introduction

Formulation of feeds for any animal requires data on the dietary requirements of the candidate species and the nutritive value and digestibility of potential feed ingredients. Digestibility represents a measure for evaluating the nutrient availability for digestion and absorption by an animal (DeSilva and Anderson, 1995). Generally, the digestive tracts in crustaceans appear to possess a relatively well developed system of digestive enzymes which can break down a host of nutrients in the diet, in spite of a lack of pepsin or stomach acid (Dall and Moriarty, 1983; Lee and Lawrence, 1997). In the previous study (3.0) extracts from the mud crab hepatopancreas demonstrated relatively high levels of protease and amylase activity. Significant, albeit low, capacities to digest cellulose and xylan were also demonstrated. Although the full significance of these latter activities remains to be determined, the ability to breakdown soluble cellulose-based substrates to glucose suggests a limited potential to derive metabolic energy from non-starch polysaccharides in plant material.

The identification of a diverse range of enzymes in the mud crab digestive system provides a compelling rationale for considering plant-based ingredients, into diets for this species. Before including any new ingredient in a formulated diet it is important to assess its digestibility to the candidate species, since any ingredient will be of little nutritive value if it is poorly digested or assimilated (Catacutan *et al*., 2003; Guillaume and Choubert, 2001; Lee and Lawrence, 1997; Akiyama *et al*., 1992).

Investigations of crustacean species such as the prawn *Penaeus japonicus* have demonstrated high digestibilities for diets containing fishmeal (80-85% ADMD) but relatively poor digestibilities for diets containing vegetable meal (10-60% ADMD). In a recent study, Catacutan *et al*., (2003) investigated the apparent digestibility of selected feed stuffs (Peruvian fish meal, squid meal, *Acetes* sp., meat and bone meal, copra meal, wheat flour, rice bran, corn meal and defatted soybean meal) by the mud crab, *S. serrata*. High apparent digestibility coefficients were obtained for all diets except for those with meat and bone meal, while protein in all the ingredients tested was highly digestible. Based on these findings, Catacutan *et al.*, (2003) suggests that plant feedstuffs may be utilised as a major source of nutrients in cost effective mud crab diets. Findings such as these raise the possibility that a broad range of both animal and plant based ingredients may now be considered to have potential for use in formulated mud crab diets.

An interesting finding of several studies into crustacean nutrition is that the composition of formulated diets can impact on digestive enzyme production. For example, research on Penaeid prawn species indicates that the activity of proteases and carbohydrases can vary significantly with nutrient levels in the diet (Le Moullac *et al*., 1996; Kumula and Jones, 1995, Le Moullac *et al*., 1994). In a recent study it was also shown that increasing the levels of dietary cellulose in feeds for the prawn *Macrobrachium rosenbergii* caused a substantial elevation in cellulase activity in the gastric fluid (Gonzalez-Pena *et al.*, 2002). Findings such as these raise the possibility that the digestive system of some crustacean species may have the potential to maximise the digestibility of a variety of ingredients by modulating the activities of specific digestive enzymes. The aim of this study was

to determine if changes to the carbohydrate profile in formulated feeds can influence the digestive processes of mud crabs under culture conditions.

4.2 Materials and methods

4.2.1 Animals

Experimental animals, raised under culture conditions described previously (2.1), were obtained from BIARC. While in the growout tank, crabs were fed EBI Star prawn (*Penaeus japonicus*) feed (50% crude protein, 8% crude fat, 19% crude ash; Higashimaru, Japan) at a rate of approximately 4% body weight per day.

4.2.2 Dietary trials

Four dietary treatments (n=9 crabs / treatment) were utilised in this study; three isoenergetic diets were formulated at QUT and prepared to contain 30% or 45% casein with 47% or 29% carbohydrate (starch or CM-cellulose), respectively, to a final energy value of 16.5 MJ/kg (Table 4.1). Dry dietary ingredients were mixed with oil and water added until a crumbly dough consistency was achieved. The dough was then extruded through a 3mm die to obtain pellets 5 to 10mm in length. Pellets were dried at 60^{o}C for 24 hours then stored at 4^{o}C until required. A separate group of crabs was fed the EBI Star *P. japonicus* feed as a reference diet. All diets contained 0.5% chromic oxide (Cr_2O_3) as an inert indicator to allow calculation of apparent dry matter digestibility (ADMD) coefficients. Mud crabs used in this trial had an average initial body weight of 58.6g $\pm$ 3.65g and housed in individual boxes within recirculating system (Fig. 2.1).

Table 4.1 Composition (% dry matter of the diet) of the 3 formulated diets prepared at QUT. Total energy values and protein to energy ratios (P:E) are also indicated.

INGREDIENTS	DIET 1	DIET 2	DIET 3
Casein	45	30	30
Starch	29.4	47	-
CM-cellulose	-	-	47
Gelatine	5	5	5
Cod liver oil	6	6	6
$CaHPO_4$	5	5	5
Vitamin mix	2	2	2
Mineral mix	1.4	1.4	1.4
Fullers Earth	5.7	3.1	3.1
Cr_2O_3	0.5	0.5	0.5
Energy MJ/ kg	16.5	16.5	16.5
P:E (mg/kJ)	30.3	21.2	21.2

Crabs were fed either one of the experimental diets listed in Table 10 or the EBI Star *P. japonicus* diet once daily at a feeding rate of 4% body weight (BW) per day as recommended by Lavina and Buling (1977). Faecal material produced by individual crabs was collected immediately prior to each feeding and stored at -20^{o}C until required for determination of ADMD. The diet trial lasted for 84 days and any crab mortalities across the trial period were recorded and excluded from the results at the point of death. At the end of the trial period, crabs were anaesthetised in ice water for approximately five minutes, then the hepatopancreas was removed and prepared for digestive enzyme analysis as described previously (2.3). From the recorded weight values, the specific growth rates (SGR) of individual crabs were also determined by calculation of the following parameters:

SGR = 100 x (Ln Final weight (g) – Ln Initial weight (g)) / duration of trial (days)

4.2.3 Dry matter digestibility determinations

The indirect method of Furukawa and Tsukahara (1966) was used to calculate the ADMD coefficients of all four feeds used in the dietary trials as described previously (2.5).

4.3 Results

4.3.1 Diet digestibility, survival rates and specific growth rates

Table 4.2 shows the survival rates and specific growth rates for mud crabs exposed to the four dietary treatments. Crabs fed diets containing approximately 50% total crude protein (Diet 1 and the EBI Star *P. japonicus* feed) exhibited the highest specific growth rates. For all dietary treatments the percentage of crabs that survived the trial period was very high, ranging from 89-100%.

Table 4.2 Apparent dry matter digestibility coefficients (ADMD) for the reference and experimental feeds used in the dietary trials. Also shown are the specific growth rates (SGR) and survival rates (SR) of mud crabs fed the different diets. The composition of diets 1 - 3 are detailed in Table 10. Column means $\pm$ SEM with similar superscripts are not significantly different from each other ($p<0.05$).

DIET	ADMD	SGR	SR (%)
1.	$82.58^{ab} \pm 2.68$	$0.77^{ab} \pm 0.09$	89
2.	$90.86^{a} \pm 1.28$	$0.59^{b} \pm 0.08$	100
3.	$78.59^{b} \pm 0.13$	$0.67^{b} \pm 0.06$	89
P. japonicus feed	$88.02^{a} \pm 0.69$	$1.01^{a} \pm 0.11$	100

As shown in Table 4.2, the ADMD of feeds was influenced by the dietary carbohydrate content. The EBI Star *P. japonicus* diet and Diet 2 (30% casein / 47%

starch) were well digested by mud crabs with ADMD values of 88.02% and 90.86%, respectively. The ADMD value for Diet 1 (45% casein / 30% starch), although lower, was not significantly different to those values obtained for the *P. japonicus* diet or Diet 2. The lowest ADMD value (78.59%) was obtained with Diet 3 where CM-cellulose replaced starch as the primary carbohydrate source.

4.3.2 Effect of diet on digestive enzyme profiles

As detailed below, the digestive enzyme profiles obtained from hepatopancreatic extracts were significantly influenced by dietary formulation. Specifically;

4.3.2.1 Protease

In this study, no significant differences were observed when protease activities in hepatopancreatic extracts from the four dietary groups were compared (Fig. 4.1a) with activity values from all treatments falling within the range of 0.82 to 0.86 U/mg protein.

4.3.2.2 Amylase

Amylase activities in hepatopancreatic extracts ranged from 0.35 to 0.69 U/mg protein and were positively correlated with the level of carbohydrate incorporated into the diet. Specifically, amylase levels in extracts from crabs fed 47% starch or CM-cellulose were significantly higher than crabs from other dietary groups (Fig. 4.1b).

4.3.2.3 Cellulase

Cellulase activities in hepatopancreatic extracts ranged from 0.003 to 0.011 U/mg protein and were positively correlated with the level of carbohydrate incorporated into the diet. Specifically, cellulase levels in extracts from crabs fed 47% starch or CM-cellulose were up to four times higher than in crabs from other dietary groups (Fig. 4.1c).

4.3.2.4. Xylanase

Xylanase activities in hepatopancreatic extracts ranged from 0.009 to 0.016 U/mg protein and were positively correlated with the level of dietary cellulose. Specifically, xylanase levels in extracts from crabs fed 47% CM-cellulose were significantly higher than crabs from other dietary groups (Fig. 4.1d).

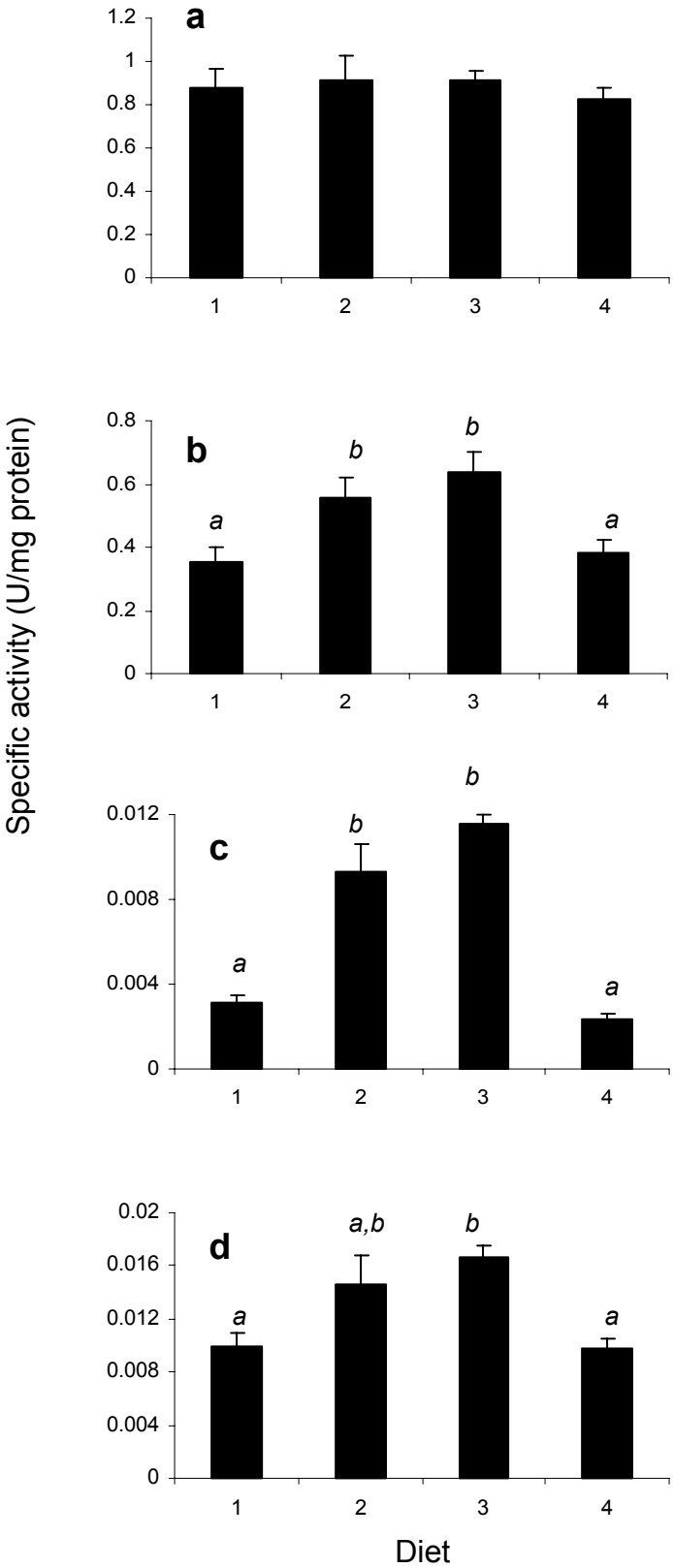

Figure 4.1 Total protease (a), amylase (b), cellulase (c) and xylanase (d) in hepatopancreatic extracts prepared from mud crabs fed experimental diet 1 (1), 2 (2), 3 (3) or the commercial *P. japonicus* diet (4). Data points represent mean ± SEM; n=9. Values noted by different superscript letters are significantly different ($P<0.05$).

4.4 Discussion

This study demonstrated that maximum growth of mud crabs was achieved using the diets containing approximately 50% total crude protein (Diet 1 and the EBI Star *P. japonicus* feed). Interestingly, the protein / energy (P:E) ratio value of Diet 1 is very similar to the optimum value reported in other mud crab growth trials. For example, Catacutan (2002) demonstrated that a dietary treatment of 27.5mg protein / kJ resulted in the highest growth rates in cultured mud crabs. This value is virtually identical to the P:E value of Diet 1 (27.8mg / kJ) which produced one of the highest growth rates in the current study. Another significant finding of this study was that partial replacement of dietary protein with carbohydrate increased carbohydrase activity in the hepatopancreas. In particular, partial replacement of casein with either starch or cellulose stimulated substantial increases in amylase, cellulase and xylanase activities.

The ADMD values reported in the current study indicate that mud crabs can readily digest diets containing up to 47% starch. This finding correlates well with the detection of relatively high levels of amylase activity in the hepatopancreas. Significant amylase activity has also been reported in the digestive systems of prawns (Ribeiro and Jones, 2000; Moss *et al*, 2001), lobsters (Johnston and Yellowlees, 1998; Johnston, 2003) and crayfish (Figueiredo *et al.*, 2001). Dietary trials in the current study indicate that amylase activity was significantly elevated by diets containing high proportions of starch or cellulose. An interpretation of this finding is that the ability of mud crabs to alter amylase activity in the hepatopancreas is not substrate specific but instead represents a more general adaptive response to changes in the level of dietary carbohydrate.

It is tempting to speculate that the presence of non-starch polysaccharide hydrolases in the crustacean digestive system may function to improve the general digestibility of diets. Dietary fibre has the potential to reduce the accessibility of essential nutrients to digestive enzymes in the gut (Potty, 1996). Interestingly, it was demonstrated in the current study that the diet with 47% cellulose was significantly less digestible than the diet containing 47% starch. In a similar study, Gonzalez-Pena *et al.,* (2002) demonstrated that cellulose reduced dry matter and protein digestibility in diets formulated for the prawn *Macrobrachium rosenbergii*. A highly significant finding in both studies was that maximum non-starch polysaccharide hydrolase activities were detected in crustaceans fed diets containing relatively high levels of cellulose. Based on findings such as these, it is tempting to speculate that an ability to modulate non-starch polysaccharide hydrolase activities may represent a general adaptive response in crustaceans which maximises access to essential nutrients when the proportion of dietary fibre changes.

5. GENERAL DISCUSSION

The traditional producers of mud crabs in South-East Asia continue to use trash fish diets in production systems combined with very low stocking densities. Prospects for increased trash fish availability or affordability in South East Asia, are not promising (ACIAR, 2004a). As a consequence, the development of a cost effective, nutritionally adequate formulated feed has been identified as an urgent priority to support further sustainable development of the mud crab aquaculture industry (ACIAR, 2004b).

The formulation of diets that support intensive production of any aquatic species requires detailed information about the animal's nutritional requirements. An extensive body of knowledge has been developed that documents the nutritional requirements of penaeid prawns resulting in the development of highly intensive production systems. Interestingly, within this group some species have been reported to require high levels of protein in their diets (eg. *P. japonicus* diet contains 50% of protein) while other species require significantly lower levels (eg. *P. monodon* diet contains 40% protein) (Cuzon and Guillaume, 2001). Requirements for carbohydrates are also reported to vary significantly among different species of prawns. For example, the recommended levels of starch vary from 20 – 45% in diets for *P. monodon*, while the recommended level for *P. japonicus* diets is only 10% (Shiau, 1998). While lipids are a major nutrient, no major differences in requirements have been reported among species of marine prawns and the recommended levels range from 4 to 10 % (Cuzon and Guillaume, 2001).

For any potential ingredient to be incorporated into a formulated diet it is first

important to demonstrate that the ingredient can be readily digested by the candidate species. This study investigated the potential for endogenous enzymes from the mud crab digestive system to degrade a range of protein and carbohydrate-based ingredients. Specifically, *in vitro* assays were used to determine protease and carbohydrase activities in mud crab hepatopancreatic extracts. The effect of altering the type and amount of dietary carbohydrate was also evaluated for growth, diet digestibility and digestive enzyme activity in intensive culture feeding trials.

Overall, mud crabs showed significant activity for all digestive enzymes assayed. The highest activity levels were observed for protease which is broadly consistent with the results obtained for other Decapod crustaceans that exhibit a primarily carnivorous feeding habit (Le Moullac, *et al*., 1996; Fernandez-Gimenez *et al*., 2001; Johnston and Yelowlees, 1998; Johnston, 2003). Enzymes from the hepatopancreas also possessed a substantial capacity to digest carbohydrates with α-1,4 glycosidic linkages and to also yield glucose. This finding is similar to that observed for other typically carnivorous marine decapods (Elyakova *et al*., 1981; Johnston, 2003).

As yet it is unclear, however, if the primary role of amylase activity in the mud crab is to cleave α-1,4 linkages in plant-derived starch molecules or to access animal-derived glycogen molecules. Interestingly, the study of Catacutan (2002) showed an apparent minimum requirement of mud crabs for dietary carbohydrates based on α-1,4 glycosidic linkages. Specifically, when lipid levels were fixed at 6%, a diet containing only 10% bread flour produced a lower body weight gain than did diets containing higher levels of this ingredient. This in spite of the fact that the

crude protein content in the diet containing only 10% bread flour was substantially higher than the other diets tested. An interpretation of findings such as these is that the provision of α-1,4 linked carbohydrates may be a limiting factor in mud crab feed formulation.

A novel finding of the present study was the detection of significant, albeit low, enzyme activity in the mud crab hepatopancreas with the capacity to degrade non-starch polysaccharides. Temperature and pH profiles for cellulase and xylanase were obtained and were found to be broadly similar to those obtained for other crustacean species. The relatively low levels of cellulase and xylanase in the mud crab is consistent with the findings of other investigations that have shown that the activities of these enzymes appear to be correlated with dietary preference and are typically low in carnivorous crustaceans.

The detection of cellulase and xylanase activities in extracts from the mud crab hepatopancreas has significant implications for future attempts to formulate diets for this species. Exogenous dietary supplements are often added to animal diets to reduce diet viscosity and other negative impacts of non-starch polysaccharides in formulated feeds (e.g. Batterham, 1992; Farrel, 1992). The detection of both cellulase and xylanase enzymes in the mud crab digestive system suggest that this species may already be adapted to tolerate the inclusion of non-starch polysaccharides in the diet and as a consequence may not necessarily gain significant benefit from the inclusion of exogenous enzyme supplements in artificial diets. This assumption is supported by the relatively high ADMD value (78%) obtained for mud crabs fed a diet containing 47% CM-cellulose.

The mud crab feeding trials demonstrated an interaction between the activity of the three carbohydrase enzymes assayed and the proportion of carbohydrate in the diet. By contrast, secretion of proteolytic enzymes from the hepatopancreas of mud crabs used in the growth trial did not appear to be influenced by variable levels of dietary protein (30-50%) as total protease activity remained high across all treatments groups. This result is in apparent disagreement with the results of other investigations conducted on several species of penaeid prawns which have provided evidence of adaptive enzyme production when protein levels in the diet are altered (Kumulu and Jones, 1995; Guzman *et al*., 2001). It is important to note, however, that these other studies focused on the relationship between dietary protein and proteolytic activity during larval or post larval stages, making comparisons with the current study, where sub-adults were used, difficult.

A major finding of the current study was that carbohydrase activities differed significantly across dietary treatments. Specifically, the partial replacement of casein by either starch or cellulose paralleled increased amylase, cellulase and xylanase activities. An assumption drawn from the positive correlation between dietary carbohydrate levels and carbohydrase activities is that the mud crab is capable of adaptive enzyme production in response to changes in dietary carbohydrate content. Similar relationships have been observed in other decapod crustaceans such as the giant freshwater prawn, *Macrobrachium rosenbergi* (Gonzalez-Pena *et al*., 2002).

In an attempt to assess if a relationship exists between digestive enzyme levels in the hepatopancreas and the digestive potential of mud crabs, ADMD

values were determined and correlated with dietary treatments. ADMD was used in this study since it was a convenient method for determining nutrient availability (Shnider and Flatt, 1975). Overall, ADMD values were generally high, the most digestible diet was the one containing 47% corn starch while the least digestible diet contained 47% CM-cellulose. This finding was not unexpected, since much higher levels of amylase activity were detected in mud crab hepatopancreas extracts than were observed for cellulase. Interestingly, the SGR obtained for mud crabs fed the diet demonstrating the highest ADMD value (47% corn starch) was significantly less than the SGR obtained using the commercial *P. japonicus* diet (Table 11). A possible explanation for this result is that while the experimental diet containing high levels of corn starch was highly digestible, it lacked other important dietary ingredients such as attractants which may have encouraged a higher intake of the feed provided.

It is significant to note, that while an ADMD value of 78% was obtained for the diet containing 47% CM-cellulose and 30% casein, this value was not significantly lower than that obtained for the experimental diet containing much higher levels of protein (45% casein, Table 11). This result is in apparent disagreement with the findings of other nutrition studies where it has been shown that increasing the content of non-starch polysaccharides in the diet can significantly reduce diet digestibility. For example, Gonzalez-Pena *et al.,* (2002) reported that the ADMD obtained for prawn diets was reduced by 30% following the incorporation of only 10% α-cellulose. Likewise, Tuan *et al.,* (2004) reported a 25% reduction in ADMD following the partial replacement of a high protein reference diet with α-cellulose. It is important to note that in the current study a

soluble derivate of cellulose, CM-cellulose, was used while the investigations of Tuan *et al.,* (2004) and Gonzalez-Pena *et al.,* (2002) utilised an insoluble cellulose derivative. Elsewhere it has been shown that while crustacean cellulases have a high capacity to digest soluble cellulose derivatives they are relatively inactive against insoluble forms (Xue *et al*., 1999). Clearly, if attempts are to be made to incorporate cellulose into crustacean diets consideration must be given to the relative solubility of the cellulose derivative and the potential impact it may have on diet digestibility and nutrient availability.

5.1 Conclusions

The major conclusions and findings of this study were;

1. High levels of protease and amylase activity detected in the mud crab hepatopancreas suggest that this species is well adapted to efficiently digest protein and carbohydrates based on α̃-1,4 glycosidic linkages.

2. There was significant evidence of cellulase and xylanase secretion by the hepatopancreas of the mud crab digestive system.

3. There was a positive correlation between carbohydrase activity in the hepatopancreas and the carbohydrate content of diets.

4. Whilst a capacity to digest non-starch polysaccharides may exist in mud crabs, this may be limited as indicated by the relatively low levels of cellulase and xylanase detected and the reduced apparent dry mater digestibility values obtained in the mud crabs fed the high cellulose diet.

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