OPTIMISING THE NUTRIENT

SPECIFICATIONS OF MANUFACTURED

FEEDS FOR FARMED JUVENILE GREENLIP

ABALONE (*Haliotis laevigata* Donovan).

Submitted by

THOMAS ALBERT COOTE

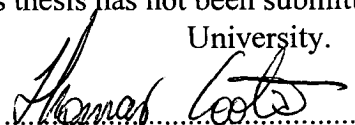B.Sc., B.Med.Sc. (Hons.), Flinders University of South Australia

The experiments reported in this thesis were written as chapters. These chapters are being prepared as a series of papers for journal publication. Accordingly, they are presented in a uniform format based on the "Instructions for Authors" for *the Journal of Aquaculture Nutrition.*

Declaration

All work described here has been performed by the author, except where otherwise acknowledged. This thesis has not been submitted previously for a degree at any University.
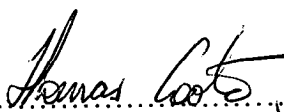
Thomas A. Coote

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ABSTRACT

A series of experiments were conducted to investigate the protein, energy and lysine requirements of greenlip abalone *Haliotis laevigata*. An initial experiment estimated the optimal crude protein requirement. Digestibility coefficients of the protein and energy components of feed ingredients were then estimated and used to formulate diets to investigate the optimal dietary ratio of digestible protein to digestible energy and the effect these parameters have on tissue quality in abalone. Finally, the lysine requirement of greenlip abalone was estimated using two methods of lysine supplementation.

The first experiment involved feeding a series of semi-purified diets in which the essential amino acid profile was similar to that of the soft tissue of abalone. Abalone grew fastest at a crude protein level of 20.1%. Excess dietary protein reduced the growth rate. The optimal digestible protein:digestible energy ratio was estimated at 14.4 gDP.MJ^{-1}DE. Leaching of essential amino acids over 24 hours immersion ranged from 26-54%.

The apparent and true digestibility coefficients of the protein and energy components of five feedstuffs were estimated. Defatted soyflour provided the most digestible protein (96.7% digestible). Pre-gelled starch provided the most digestible energy (80.6%). Cost analysis indicated that defatted soyflour was the most cost-effective source of protein and energy of the feedstuffs studied.

In a factorial experiment, abalone were fed diets at ten levels of protein and two levels of energy. The optimal dietary digestible protein level was estimated at only 13%, and the optimal ratio of digestible protein:digestible energy between 12.3-17.9

g.MJ^{-1}. Abalone grew poorly on diets with fish oil levels of 6.0 - 9.0%, although their growth rate increased when returned to a commercial diet containing less oil. The composition of abalone foot muscle and, to a lesser extent, viscera, was affected by dietary protein level and, in some cases, by dietary energy level. Carcass partitioning revealed that meat yield was also affected by dietary protein level.

The lysine requirement of greenlip abalone was determined using two methods of lysine supplementation; the addition of free L-lysine-hydrochloride to diets and the use of gluten enriched with covalently-bound lysine. Abalone showed a linear response to free-lysine, and a curvilinear response to covalently-bound lysine. The former method was therefore unable to produce an estimate of dietary lysine requirement. Use of covalently-bound lysine predicted that maximal growth occurred when lysine accounted for 3.9% of crude protein.

ACKNOWLEDGEMENTS

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My final thanks must go to my mother for her continual encouragement and support, most especially for when the going was difficult.

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I dedicate this thesis to my mother, Harriet Coote, and my late father, Donald Coote, who gave me the education they never received.

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CHAPTER 1

INTRODUCTION

Australia exported 3,000 tonnes of wild-caught abalone valued at $147.6 million in 1995-6 (ABARE, 1996). Demand is growing, yet, with the exception of the tropical abalone, *Haliotis asinina*, the abalone fisheries of Australian waters are thought to be fully exploited (S. Shepherd, Pers. Comm.). In an attempt to fill increasing demand for this commodity, two species of abalone native to Australian waters, *Haliotis laevigata* and *H. rubra* ("greenlip" and "blacklip", respectively) are now being farmed commercially. Culture of these valuable marine molluscs has the potential to generate significant export income.

Commercial farming of abalone has presented many challenges, particularly in relation to nutrition. Currently, in mainland Australian waters, a moratorium exists on the harvest of wild macroalgae, the natural diet of abalone, for use as abalone feed. Use of macroalgae has the following disadvantages: supply can be variable, may introduce pathogens, predators and competitors to the farmed abalone, requires a high labour input and results in high feed conversion ratios (FCR); in the order of 25:1 (Mozqueira, 1992). These constraints and disadvantages have stimulated research into the development of cost-effective manufactured feeds for farmed abalone. Manufactured feeds may be made all year round, may be produced pathogen-free, result in low FCR (about 1.5:1), constant nutrient values, and automated feeding systems may be designed for their use.

Despite research into the evaluation of ingredients for use in abalone diets (Uki *et al.*, 1985a, Viana *et al.*, 1994) and the testing of manufactured feeds (Uki *et al.*, 1985b, Mozqueira, 1992, Viana *et al.*, 1993, Britz *et al.*, 1994), there has been limited

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definition of the nutritional requirements of the animal. Uki *et al.* (1986a) and Mai *et al.* (1996) both investigated the requirement of abalone for essential fatty acids and Mai *et al.* (1995a) examined the response of abalone to various levels of dietary lipid. Uki *et al.* (1986b), Taylor (1992), Mai *et al.* (1995b) and Britz (1996) evaluated the dietary protein requirement of abalone but all focused on crude protein and only Mai *et al.* (1995b) balanced the amino acid profile of the diet to that of the abalone body. No studies examining the dietary requirements of abalone for essential amino acids or concerning the ability of abalone to digest feed ingredients have been published. Thus, the most basic nutritional information required to optimise the growth of this valuable animal is lacking.

The normal way of assessing the dietary requirement of a nutrient (eg, protein) is by growth experiment, in which the growth response of the animal is measured to increasing levels of the nutrient. This method has limitations, in the case of abalone, as water-soluble nutrients such as free amino acids leach from the feed prior to consumption. Much effort has been directed toward overcoming this problem by methods such as microencapsulation (Villamar and Langdon, 1993, López-Alvarado *et al.*, 1994) but with limited success. The covalent attachment of free amino acids to intact protein sources, such as that used by Fox *et al.* (1995,b) to investigate the requirement of *Penaeus vanamai* for lysine, offers an alternative method to overcome leaching loss.

Ideally, evaluation of the dietary protein requirement of an animal should be carried out on a digestible protein basis at a minimum of two energy levels. This will allow an estimate of the optimal digestible protein:digestible energy ratio. Obviously this cannot be carried out until the ability of abalone to digest feedstuffs is known.

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The objectives of this thesis were to optimise nutrient specifications in manufactured feeds for greenlip abalone. These objectives were to determine: (1) digestibility, (2) optimal dietary crude protein level, (3) optimal dietary protein:energy ratio, (4) optimal dietary lysine level, and (5) effect of different calcium and phosphate levels on growth. This information may then be used to refine and reduce the cost of commercial abalone diets, with the objective of enhancing the viability of the abalone aquaculture industry.

CHAPTER 2

REVIEW OF LITERATURE

2.1 The emergence of abalone aquaculture: the need for a manufactured feed

The decline of wild fisheries and the emergence of spawning technology.

Abalone are marine snails of the genus, *Haliotis*. This genus is characterised by an oval shell and a large, muscular foot. McCormick and Hahn (1983) and Fallu (1991) estimated the number of species at about 100, although Lindberg (1992) suggested only 60-70. All belong to the monogeneric family Haliotidae (Cox, 1960) and are members of the class Prosobranchia. However, Brown and Murray (1992) found large genetic differences among some haliotid species, and suggested a taxonomic review.

Abalone generally inhabit shallow, rocky waters close to land. They occur in the waters off most land masses except South America and Eastern North America (Hahn, 1989). The distribution of species off the coasts of Japan, North America, Australia and Southern Africa was described by Lindberg (1992). Being herbivorous, subsisting by grazing or catching drift algae, abalone are usually restricted to the depth of water that can sustain macroalgal growth. Most temperate abalone occur from the intertidal zone to water of about 20 m depth, but tropical species occur to depths of up to 200 m (Lindberg, 1992). Recently, abalone have been introduced to the coast of Chile (Godoy *et al.*, 1992) and to Iceland (Nielsson, 1994), although in the latter case they are confined to land-based farms.

The meat of the muscular foot of abalone (individuals of *H. rufescens* may attain a weight of one kilogram, yielding over 400 g of meat) has been sought as food by

humans for millennia (Knauer, 1994). In recent times, demand for the meat has escalated, particularly in Japan and China. Increased demand may be due to a combination of human population increase, depletion of wildstocks, a higher profile of the commodity, and an increase in the number of people able to afford it. The meat of abalone is utilised in either canned, dried or frozen forms.

In addition to the meat, the inside of abalone shell has a layer of nacre, with a characteristic iridescent lustre. The shell, (particularly that of the New Zealand "paua"; *H. iris*) is used for the manufacture of jewellery, buttons and for works of art, and is of considerable value (Nie, 1992). Abalone have also been known to produce pearls, and some enterprises now farm various species and are attempting to produce cultured abalone pearls (Fankboner, 1991; 1993).

Commercial demand for abalone meat was originally satisfied by exploiting wild stocks. The most commercially important fisheries are/were located along the coasts of Australia (Prince and Shepherd, 1992), California (Tegner *et al.*, 1992), Canada (Farlinger and Campbell, 1992), China (Nie, 1992), Japan, Mexico (Guzmán del Próo, 1992), New Zealand (Schiel, 1992), and South Africa (Tarr, 1992), with smaller fisheries located in the Channel Islands (Clavier, 1992) and the Middle East (Johnson *et al.*, 1992). In recent times, however, wild stocks in several fisheries have declined (Hooker and Morse, 1985; Genade *et al.*, 1988; Tegner *et al.*, 1992). World production peaked at 27,600 tonnes in 1968, but has since declined to about 12,000 tonnes (FAO, 1973-1988). Australia's production peaked in the early 1970's as virgin stocks were exploited (Shepherd, Pers. Comm.) but the introduction of fishing quotas and restriction on licence numbers has seen production stabilise at about 5,000 tonnes.

Following unsuccessful attempts at the artificial fertilisation of eggs in 1894/5

(Walker, 1990), the first successful attempts to cultivate abalone were made in 1935 (Hahn, 1989) and subsequently by Ino, (1952). As early as the late 1950's hatcheries were operational in Japan, but it was not until the 1970s that intensive research into culture techniques commenced (Grant, 1981). By 1978, Japanese hatcheries were producing 28 million abalone spat (Walker, 1990). By the early 1980's, abalone culture techniques developed by the Japanese were being adopted by other countries. With wild fishery stocks in decline due to over exploitation and pollution, the price of abalone increased to a point where culture of abalone became profitable.

Economics and ecology: why a manufactured feed is sought.

The culture of abalone is usually carried out by one of three methods. The first (stock enhancement), was pioneered by the Japanese and involves raising juvenile abalone until they are about 20-30 mm in length. They are then released into the wild to enhance natural stocks, and are harvested when they attain legal size (Grant, 1981). Survival rates vary widely, with Saito (1984) reporting 20% and McCormick and Hahn (1983) suggesting 3-6% for a 13 mm release size, and 10-30% for 20-30 mm. Tegner and Butler (1985) recorded only 1% survival of seed over one year, (with predator collection to ease pressure) with an estimated mortality of 43%. The remaining animals (56%) were presumed to have left the area. Stock enhancement is still practised in Japan (Hahn 1989) and China (Hooker and Morse 1985). Capital and running costs for this type of culture are low, but losses due to predation, poaching and animal movement are obviously high.

The second method involves holding the abalone in barrels or cages either anchored to the sea floor or suspended in mid-water. This method is particularly popular

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in certain coastal areas of mainland China (Nie, 1992). Advantages of this system are that the animals are usually exposed to good water conditions and there are no pumping costs. Disadvantages are that feed must be supplied to the cages manually, raising labour costs (Nash, 1991) and animals are exposed to potential predators such as starfish, crabs and to infestations, such as mudworm (*Polydora* and *Boccardia* spp.; Lleonart and Handlinger, 1997).

The third method of raising abalone is in tanks or raceways on land, with water pumped from the sea (Hooker and Morse, 1985). Capital costs for this method are usually high (land must be purchased; pumps, tanks and piping installed), but the advantage is that the farmer has more control over such parameters as flow rate, aeration and feeding regimes.

Feed is one of the major operating costs associated with holding abalone in the sea or on land. Prior to attaining a size of about 10 mm, juvenile abalone graze on diatoms (McCormick and Hahn, 1983; Ebert and Houk, 1984; Nash, 1991). However, diatoms quickly become a limited food source due, in part, to the density at which the animals are stocked, and the farmer is required to wean the animals onto either macroalgae or manufactured feed (Knauer *et al.*, 1995). Algae (either drift or harvested) are usually cheap (on a wet-weight basis) and have the advantage of being the natural food of the animal. However, supplying macroalgae to tanks is labour-intensive, their abundance is often seasonal (Mozquiera, 1992), and feeding abalone continuously on one or two species could result in nutritional deficiencies (Britz *et al.*, 1994). The farmer also runs the risk of introducing organisms with the algae that are predators, pathogens or parasites of abalone or competitors for food and oxygen. In addition, in some countries (for example Australia), the harvest of wild macroalgal stocks is limited

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or prohibited altogether. The feed conversion ratio of algae (wet weight of algae consumed to weight gain of whole, wet abalone) is poor, typically 25:1 (Mozqueira, 1992).

In contrast to macroalgae, manufactured feeds can be produced year-round, they can be easily stored and feeding systems can be automated. There is evidence that they result in increased survival rates, better feed conversion and faster growth than natural feed (McCormick and Hahn, 1983; Hahn, 1989; Britz et al., 1994). Manufactured feeds may be adapted to supply the nutritional requirements of abalone of different ages and may also be adapted for use in different culture systems.

Desirable characteristics of manufactured feed for abalone.

Palatability: Any feed manufactured for any cultured species must be palatable but fortunately abalone readily consume a wide range of diets based on ingredients as diverse as semolina, fishmeal and casein without palatability problems (Fleming et al., 1996). However, the candidate did find that greenlip abalone (*H. laevigata*) refuse to eat diets high (20% dry matter) in gelatine. Substances that act as attractants and may increase the palatability of diets have been studied, such as amino acids, phospholipids and dipeptides (Harada et al., 1987; Harada, 1989), as have the attractant properties of feedstuffs themselves, such as fish silage, fresh and dried macroalga, gelatin, and soybean meal (Viana et al., 1994). Currently, no commercial Australian diet includes an ingredient specifically as an attractant or to increase palatability.

Digestibility: An essential characteristic of any feed is that it must supply enough digestible protein, energy and micronutrients to ensure the animal grows at an economic rate. Most farmers report that they require a growth rate of 67-100 $\mu m.d^{-1}$ to ensure their

stock reach a shell length of 70-80 mm in 3 years (Fleming *et al.*, 1996). To accurately meet the dietary requirements of the animal, it is necessary to know the ability of the animal to digest, absorb and metabolise potential feedstuffs. Although some researchers have addressed the (crude) protein requirements of abalone (Uki *et al.*, 1986b; Taylor, 1992; Mai *et al.*, 1995b; Britz, 1996) none have investigated digestible protein requirements. Similarly, some research has been undertaken into the lipid requirements of abalone (Mai *et al.*, 1995a) but no published data on lipid digestibility are available. No data concerning essential amino acid, vitamin or mineral requirements of abalone have been published apart from results arising from this thesis (Coote *et al.*, 1996).

Leaching: Manufactured feeds developed for abalone should be highly stable in water. Abalone are slow feeders that rasp their feed with a radula, unlike finfish, that quickly swallow feed pellets intact. Farmers report a desire to keep labour costs low by feeding as infrequently as possible. Additionally, it is important that excessive leaching of nutrients from the feed be restricted as much as possible to help maintain good water quality and to conserve feed nutrient levels. Highly water-stable feeds would help achieve these goals. Water stability of feeds is related to feedstuffs used, method of manufacture and use of binders. With the exception of Knauer *et al.* (1993), no research results that have focused on abalone diets in any of these areas are published.

Waste: It is essential that manufactured feed produces as little waste as possible. Unutilised digested nutrients exert an energetic demand on the animal to excrete them and, along with food wastes and faeces, provide a food source for other organisms (eg; bacteria and fungi) that may flourish, resulting in sub-optimal conditions for the cultured species. The risk of disease outbreak may also be increased. From an economic point of view, the reduction of waste, in the form of uneaten feed, faeces and metabolic wastes

(eg; ammonia) is highly desirable. Many countries have in place environmental guidelines that limit the amount of waste that can be disposed of in the sea, or impose taxes proportional to the amount discharged (EPA, 1993).

The state of abalone feed development, commercialisation and research

Research into manufactured feeds for abalone has been conducted for over 30 years and has been extensively reviewed by Fleming *et al.* (1996). Practical diets have been produced both in the form of gels (Ogino and Ohta, 1963; Sagara and Sakai, 1974; Koike *et al.*, 1979) and pellets (Gorfine and King, 1991; Morrison and Whittington, 1991; Viana *et al.*, 1993; Britz *et al.*, 1994).

Although considerable effort has been directed toward the development of manufactured feeds for abalone, there are surprisingly few produced commercially. Fleming *et al.* (1996) reported a total of 11 commercial abalone feeds produced. The Chinese and Japanese feeds are for local use, while small amounts of the New Zealand feed have been exported to Australia. In Australia, Deakin University has developed an abalone feed that has been commercially manufactured (Fleming *et al.*, 1996). Since that 1996 review, three companies have commenced the manufacture of abalone feed in South Australia.

Until recently, some Australian abalone farmers were able to supply their needs by making feed on-site. These feeds were usually made on a "trial and error" basis and, consequently, were of sub-optimal formulation. The recent involvement of commercial feed companies has seen the cessation of this practice in Australia.

There are compelling reasons why research should be directed toward the development of a manufactured feed for abalone in Australia. These include:

1/ Diets that have been formulated for exotic abalone may not produce similar results when fed to Australian species.

2/ The importation of large quantities of feed is expensive, and carries with it the risk (however slight) of pathogens.

3/ Australia has a large range of inexpensive feed ingredients used in established feed industries.

4/ There is currently a moratorium on the harvest of wild macroalgal stocks from the sea in the waters off mainland Australia, and only limited amounts may be taken from Tasmanian waters.

5/ There are few published reports on the nutritional requirements of abalone and no workers have established a logical sequence of research to fill the information gaps in this field.

6/ Formulated feeds have a more consistent nutrient content.

7/ Formulated feeds are readily available year-round.

8/ Formulated feeds may have a lower labour cost than harvested macroalgae.

In order to formulate cost-effective, nutritionally complete diets for any cultured species, one requires a pool of potential feedstuffs, knowledge of the nutritional requirements of the species and the apparent digestibility coefficient of the species for the feedstuffs (De Silva and Anderson, 1994). As an impressive range of traditional feedstuffs are available in Australia, the next logical steps are to evaluate the ability of abalone to utilise them and use that information to evaluate their nutritional requirements. This thesis attempts to apply the above strategy by evaluating the dietary protein requirements of abalone, the optimal dietary protein:energy ratio and requirement for at least one of the essential amino acids. The starting point is the

evaluation of feed design and preparation methods in a growth experiment that allowed the candidate to gain experience with husbandry procedures. In relation to protein requirements it was necessary to conduct a "range-finding experiment" on dietary protein and then to assess certain feedstuffs for protein and apparent energy digestibility.

2.2 Digestibility and digestion in abalone.

Determination of digestibility

Feeds for farmed animals can be formulated on a crude basis but this can lead to over-supply of nutrients or nutritional inadequacies when considered on a digestible basis. Clearly, research into digestibility and availability of nutrients has great importance to both the feed manufacturing and farming sectors.

Apparent digestibility may be estimated by the direct method, involving the determination of feed consumed and faeces produced, or by the indirect method, involving either the addition of an indigestible marker to the feed (eg, chromic oxide), or use of an indigestible component of the feed (eg, acid-insoluble ash). Most digestibility work involving abalone has used the indirect method, requiring the collection of only a representative faecal sample (Wee *et al.*, 1992, 1994), although Fleming (1995) used the direct method. Much of this work evaluated the digestibility of macroalgae and hence is of limited use in the formulation of a manufactured feed. Where digestibility data on manufactured feeds were given (Wee, *et al.*, 1992, 1994), the proprietary formulations were not presented. The candidate has generated digestibility data for the protein and energy components of some feedstuffs when fed to abalone and used these data in research to quantify protein and energy requirements for abalone. The focus of this research is directed at the protein and energy requirements of abalone because protein is traditionally the most expensive dietary component due to inclusion level. Energy is considered because adequate supplies are necessary to ensure efficient utilisation of protein (see "Protein deposition and growth"; Section 2.3).

Apparent Digestibility

This is the most crude estimate of digestibility and is defined as the difference between the amount of the component of interest in the diet and that in the faeces, divided by the amount in the diet (Low, 1982). The apparent digestibility of protein does not correct for endogenous losses of protein or amino acids. It is also less accurate at lower dietary protein levels, as endogenous N production accounts for a higher proportion of faecal protein (Eggum, 1973). Thus, Sauer *et al.* (1989) recommended that apparent digestibilities should only be carried out under standard conditions (for example, all test diets should contain similar protein levels). In practice, this is difficult to achieve, when some of the feedstuffs evaluated have a lower protein content, without confounding the experiment by varying the inclusion level of ingredients with higher protein content, in the basal diet.

True Digestibility

True digestibility is determined in a similar way to apparent digestibility, but an attempt is made to account for the endogenous production of certain components such as protein. Endogenous proteins consist of digestive secretions (eg; enzymes) that are usually soluble, cells shed from the digestive tract lining, and bacterial protein contributed by resident microflora (van Barneveld, 1993). Endogenous protein production may be estimated by feeding the animal a protein-free diet, or by regression to zero using a series of test proteins at various levels. However, such methods are

subject to error, as endogenous losses depend, in part, on the composition of the diet and on the intake level (Sauer and Ozimek, 1986; de Lange *et al.*, 1989).

Real Digestibility

Real digestibility differentiates between endogenous and exogenous amino acids. The ^{15}N isotope dilution technique (de Lange *et al.*, 1990), involves infusion of ^{15}N-labelled amino acids into the body and their subsequent incorporation into endogenous protein. These can then be differentiated in the faeces from dietary amino acids. The chemical transformation method of Hagemeister and Erbersdobler (1985) may also be used. Values for real digestibility tend to be 4-5% higher than those for true digestibility (Low, 1982). However, the high cost associated with the use of ^{15}N isotopes and that of the chemical labelling technique limit the use of these techniques in terrestrial animals. There may be greater potential for their application in the study of animals such as abalone due to the comparatively small quantities of feed they consume.

Brief overview of the digestive tract

The digestive tract of abalone is complex (Fig. 2.1). Four distinct, main regions may be discerned along the tract: the buccal region, the oesophagus, the stomach and the intestine (Crofts, 1929). The radula is rasped against algae, feed pellets or a substrate and edible particles are conveyed into the buccal cavity, probably aided by the backward-pointing teeth (Crofts, 1929). Food particles are mixed with copious amounts of mucus in the buccal cavity and with secretions from the salivary glands (McLean, 1970) as the food passes along the oesophagus. From the oesophagus, the food passes into the large, thin-walled muscular crop, where mixing of food and secretions occurs.
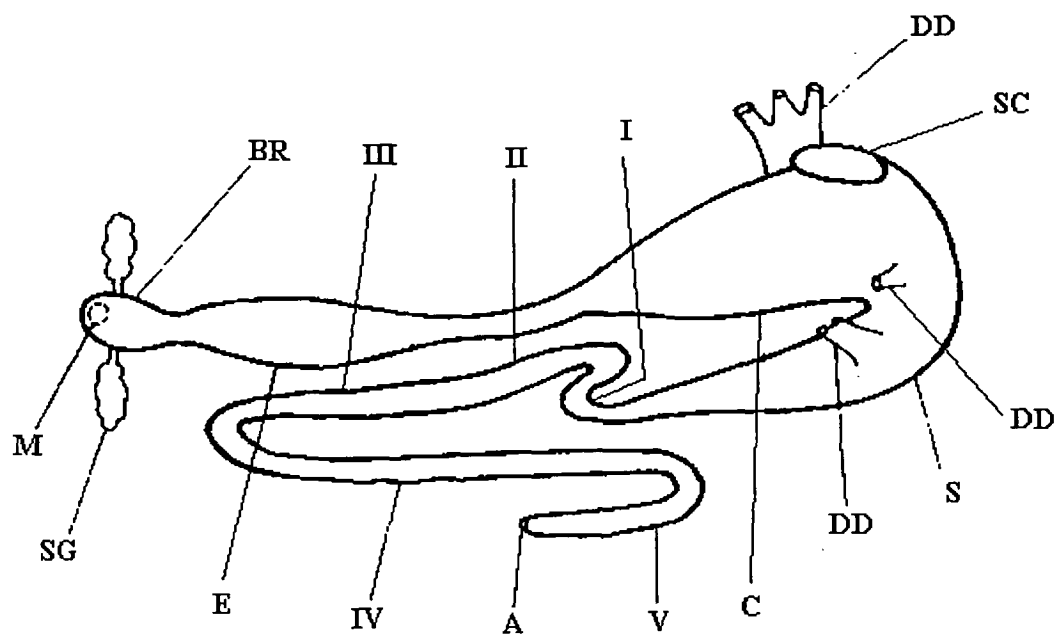
Fig. 2.1: Schematic diagram of abalone digestive tract.
A, anus; BR, buccal region; C, crop; DD, digestive gland duct;
E, oesophagus; M, mouth; S, stomach; SC, spiral caecum
SG, salivary glands; I-V, intestinal regions.

(Adapted from McLean, 1970).

Algae are (at least partially) digested at this point as Foale and Day (1992) reported that some algal types are degraded to an unrecognisable state within six hours of ingestion. Crofts (1929) describes a semi-circular valve guarding the narrow opening leading to the muscular stomach, that has less than half the capacity of the crop. Stomach contents are kept in motion not by peristalsis but by cilia. Digestive glands secrete enzymic secretions into the stomach via ducts guarded by valves (Crofts, 1929). The digestive fluid/food mix is directed by the beating of cilia into "gutters" that lead to the spiral-shaped caecum. Crofts (1929) states that the digestive juice travels up the deep anterior gutter of the caecum and passes back to the stomach via the posterior gutter. A longitudinal fold of the caecum, the typhlosole, keeps the gutters separate. The mixture emerges from the caecum into the first of the five intestinal sections described by Campbell (1965), known as the "sorting area" that is lined with prominent ridges and grooves (Campbell, 1965) and marked by a one-way valve to prevent regurgitation of food (Crofts, 1929). Mucus is secreted into this region, combined with the fine material in the intestine and carried into grooves and formed into fine threads by the action of cilia. Indigestible material is formed into a faecal rod, that is compacted as it passes along the intestine through regions II, III and IV. Material not already in the faecal rod is added to it in regions III and IV. Final compaction and pellet formation takes place in region V (Campbell, 1965). Faecal pellets discharge into the mantle cavity. Fretter and Graham (1976) postulate that compaction of the faecal pellet aids in preventing the disintegration of the pellets in the mantle cavity of the animal.

Protein digestion by abalone

Knowledge of protease activity in the gut of abalone is limited, having been detected in the midgut gland (McLean, 1970), digestive tract (Cho *et al.*, 1983; Yamaguchi *et al.*, 1989) and intestine (Groppe and Morse, 1993) of abalone. Britz, *et al.*, (in press) demonstrated that protease concentration in *H. midae* increased by a factor of approximately 20 to peak at 18 hours after feeding and returned to the pre-feeding level after 24 hours. Similarly, protease activity rose by a factor of approximately seven to peak six hours after feeding, and had returned to pre-feeding levels by 24 hours after initial feeding. Despite a deficiency of information on the ability of abalone to digest protein, manufactured feeds tend to contain higher levels of protein than macroalgae; typically 20-54 percent (Fleming *et al.*, 1996). Much of this protein is of terrestrial origin (eg cereal proteins). The effects of high levels of dietary protein of terrestrial origin on abalone growth and tissue composition are unknown.

Carbohydrate digestion, metabolism and storage in abalone

Abalone have evolved an ability to supply most of their energy requirements from carbohydrates (Britz *et al.*, in press). Knauer (1994) has presented a summary of the mono-, di- and polysaccharide substrates found to be hydrolysed by abalone digestive enzymes. Structural and reserve carbohydrate levels of marine macroalgae can be quite high. The main reserve carbohydrates of macroalgae are laminarin (brown algae) and Floridean starch (red algae). A study by Powell and Meeuse (cited by Percival and McDowell, 1967) revealed that some brown algae of the Pacific coast of North America contain 22-34% laminarin (dw). Structural carbohydrates include cellulose, xylans and mannans, but mucilages such as alginic acid, carrageenan and fucoidin also occur. Carrageenan has been shown to make up to 55% of the dry weight

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of *Eucheuma spinosum*, while cellulose ranges from 0.6% to 10% (dw) for brown algae, with a similar range for red algae (Percival and McDowell, 1967).

It is likely that enzymes acting on reserve carbohydrates (eg; laminarin and Floridean starch) show the highest activity (Hylleberg-Christiansen, 1972; Elyakova *et al.*, 1981), although structural carbohydrates (eg; cellulose) are also hydrolysed to some degree (Bennett and Nakada, 1968; Nakagawa and Nagayama, 1988). Although abalone are undoubtedly capable of digesting marine carbohydrates, the degree to which they are able to digest terrestrial carbohydrates is unknown. Although some commercial abalone feeds contain macroalgae and/or their extracts, others contain high inclusion levels of terrestrial carbohydrates (Fleming *et al.*, 1996). An assessment of the ability of abalone to digest raw and processed terrestrial starches would be a useful addition to the available information on abalone nutrition.

Do bacteria play a role in the digestive processes of abalone ?

It has been suggested that, given the long gut (relative to the size of the abalone) and the fact that it is a herbivore, bacteria may play some part in the digestive processes of abalone, in a way analogous to the symbiotic relationship between gut microflora and ruminants. In brief, ingested microbes may attach to, and proliferate in, favourable regions of the gut, providing benefits to the host. In the case of abalone, benefits may include breakdown of structural carbohydrates of algae via extracellular enzymes, food pre-conditioning (Harris, 1993) and the provision of amino acids (Fong and Mann, 1980) and vitamins (Shiau and Lung, 1993). Some researchers (Garland *et al.*, 1985; Erasmus *et al.*, 1994; Harris, 1994) have found bacteria in the gut of abalone, but their exact contribution (if any) to the efficiency of digestion is yet to be established.

2.3 Dietary protein and amino acid requirements

Protein deposition and growth

Protein deposition in animals is determined by both intrinsic (eg; age, sex, genotype of animal) and extrinsic (eg; protein intake, protein availability and energy intake) factors (Miller and Payne, 1963). However, the relative importance of intrinsic and extrinsic factors is still unclear. Research directed toward the nutrition of terrestrial farm animals resulted in the concept of protein- and energy-dependent phases of protein deposition (Black and Griffiths, 1975). At a constant energy intake, protein deposition (PD) in growing animals increases linearly with protein intake until energy becomes limiting (Fig. 2.2). At this point (the optimal digestible protein: digestible energy ratio (DP:DE)) for that energy level, provision of more protein will result in no further gain in PD. That is, maximum protein deposition (PD_{max}) has been reached. At a higher energy intake, protein deposition continues as dietary protein levels increase until energy once more becomes limiting.

This concept is often described as a linear-plateau relationship, but it has also been reported as linear (Zhang *et al.*, 1984), curvilinear (Fuller and Garthwaite, 1993) and 2-phase linear (Batterham *et al.*, 1990). The slope of the relationship between protein intake and protein deposition represents the efficiency of protein utilisation (Bikker, 1991). This situation is more likely to be seen in young, fast-growing animals, such as the result obtained by Close *et al.* (1983) for pigs of less than 40 kg liveweight.

However, as Whittlemore and Fawcett (1976) predicted, pigs should have a maximum capacity for PD. This PD_{max} is dependent on the protein and energy levels of the feed, the feed intake of the animal and the capacity of the animal for growth.

20
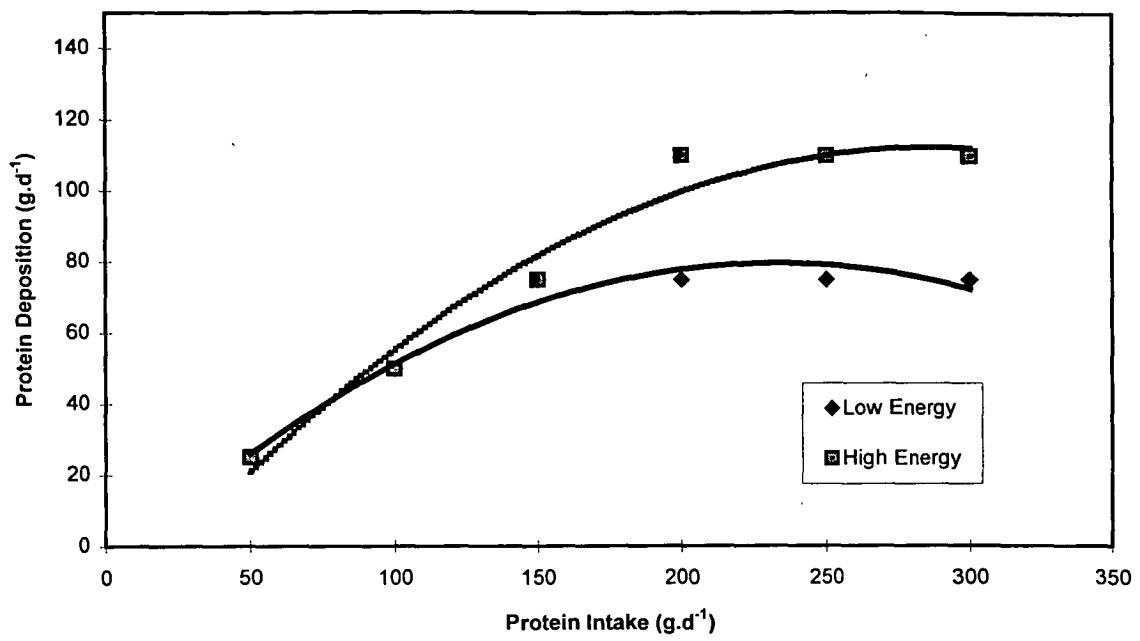
Fig. 2.2: The relationship between protein intake and protein deposition at two levels of energy intake. Adapted from Bikker (1991.)

PD_{max} is more likely to be reached if the pig is older, castrated or has a poor genotype for PD (Bikker, 1991). The linear-plateau responses reported by Campbell *et al.* (1983 and 1985) for pigs indicate that PD_{max} was reached.

Little work of a similar nature has been applied to the study of protein deposition in abalone. This thesis examines the effect of dietary protein and energy levels on protein deposition in abalone.

Dietary protein requirements of abalone

The protein content of macroalgae eaten by abalone is highly variable. Britz *et al.* (1994) gave the protein content of dried *Plocamium corallorhiza* and *Ecklonia maxima* as 20% and 10% respectively, while much lower values (0.1% for *Laurencia papillosa* to 2.8% for *Ulva lactuca*) were reported by Abbas *et al.* (1992) for 15 species of benthic algae from the Arabian Gulf. *Macrocystis pyrifera* was reported by Ma *et al.* (1989) to have a protein content of 20.0%.

Uki *et al.* (1986b) conducted growth experiments on juvenile *H. discus hannai* using test diets containing either casein or white fishmeal as a protein source. Dietary protein levels ranged from 0% to 55%. The highest growth rate was recorded for those animals consuming a diet containing 46.5% crude protein (50% casein). However, when feed conversion efficiency, protein retention and net protein utilisation were taken into consideration, the authors concluded that an optimal protein level for practical diets, using casein, would be 20-30%. Similarly, Taylor (1992), concluded that a diet of at least 30% crude protein (the maximum protein level of the experimental diets was only 30%) was necessary to produce maximal growth in *H. kamtschatkana*. Britz (1996), using fishmeal-based semi-purified diets, found that the specific growth rate of *H. midae*

tended to increase as dietary protein level increased to the maximum dietary protein level (47%) used in this experiment. The author noted, however, the need to acquire data on digestible energy content of feedstuffs in order to define the optimal digestible protein:digestible energy ratio. The main criticism of the above experiments is that the diets relied on a single source of protein. It is unlikely that either casein or fishmeal contains the ideal profile of essential amino acids for optimal growth of abalone. Estimates derived from such studies are likely to be high as the animals fed diets of lower protein content will suffer growth reduction due to a deficiency of essential amino acids.

Mai *et al.* (1995b) improved on the nutritional studies outlined above in that the essential amino acid profile of the experimental diets was adjusted to be similar to that of the soft tissue of the abalone. The dietary protein content for optimal growth was reported to be between 24.0-34.5% and 25.2-36.6% for *H. tuberculata* and *H. discus hannai*, respectively. However, these workers also acknowledged the shortcomings of their results due to the fact that digestibility values for protein were not determined.

The above reports yield a wide range of "optimal dietary protein levels" for abalone. Research at the fundamental level of digestibility of individual feed ingredients is required prior to the elucidation of an optimal (digestible) protein level.

Essential and non-essential amino acids

Of the twenty or so amino acids that are incorporated into protein, ten are unable to be synthesised by most metazoans and are termed "essential", and must be provided in the diet. These are arginine, histidine, lysine, threonine, phenlyalanine, tryptophan, methionine, valine, leucine and isoleucine. The remaining amino acids are termed "non-

essential" and can be synthesised to meet requirements, provided that adequate essential amino acids and energy is available. Using the radiometric technique of injecting ^{14}C-glucose into *H. rufescens*, Allen and Kilgore (1975) concluded that all of these ten amino acids are essential for abalone. No conclusion was drawn concerning the dispensibility of tyrosine, as in many animals it can be synthesised from phenylalanine.

Dietary essential amino acid requirements of abalone

Although the study by Allen and Kilgore (1975) indicated qualitative requirements of abalone for the 10 common essential amino acids (EAA's), no information is available concerning the quantitative requirements of abalone for any of these. Even in the case of finfish, complete requirements for all EAA's have been established for fewer than 10 of the 300 or so species cultured worldwide, although partial requirements are known for several others (Wilson, 1994). These data have been derived from growth experiments on the eels *Anguilla anguilla* and *A. japonicus* (Arai *et al.*, 1972), carp (Nose *et al.*, 1974), channel catfish *Ictalurus punctata* (Wilson *et al.*, 1978 and 1980), Nile tilapia (Santiago and Lovell, 1988) and *Catla catla* (Ravi and Devaraj, 1991). Other methods of quantitative estimation of amino acid requirements, such as serum amino acid levels, or amino acid oxidation, have been found to be of limited use (Wilson, 1994).

Growth experiments typically involve purified diets containing crystalline EAA's to formulate a series of diets containing graded levels of the EAA under scrutiny, but replete in all others. Requirements are estimated based on the conventional growth response curve. An increase in weight gain is normally observed with increasing amino acid intake up to a break-point (corresponding to the requirement of the specific amino

acid), beyond which weight gain levels off. Methods used to estimate the break-point include regression analysis (Akiyama *et al.*, 1985), continuous broken-line models (Wilson *et al.*, 1980) described by Robbins *et al.* (1979), and quadratic analysis (Santiago and Lovell, 1988).

Difficulties encountered with growth experiments to assess amino acid requirements include the leaching of crystalline EAA's into the water from the feed, (this would be especially significant in experiments with abalone, due to their slow feeding behaviour), lack of precision in interpretation of the growth curve (Wilson, 1994) and evidence from prawn nutrition suggesting that free EAA's are incorporated into muscle tissue at very low rates (Deshimaru, 1981). It would be desirable to slow the release of crystalline EAA's into the blood via a method such as encapsulation (Lopez-Alvarado, 1994) or to avoid the problem of leaching altogether by covalent attachment of free EAA's to intact protein known to be limiting in one EAA as developed by Fox *et al.* (1995,a).

Essential amino acid profile of abalone

For some time, the "ideal protein" concept has been advocated as the method to estimate the dietary EAA requirements of animals (ARC, 1981). The "ideal protein" is the amino acid balance of a diet that would most perfectly meet the animal's immediate amino acid needs at all stages of the life cycle. Thus, the "ideal protein" would change with maturity of the animal. One way to approximate the "ideal protein" in a real diet for an animal is to examine the amino acid profile of the whole body, and to formulate a diet that closely resembles the amino acid profile of the body. Although not strictly the "ideal protein", such a diet is likely to be a good starting point for a commercial diet

for abalone as they are grown for a relatively small percentage of their lifespan and are usually sold prior to full maturity. In addition, if one knew the EAA profile of the animal, and the most limiting EAA of the diet (normally lysine for animals fed a cereal-based diet), then one would need only to evaluate the dietary requirement for lysine, and the requirements for the rest could be estimated based on their ratio to lysine (Wilson, 1994).

Cowey and Tacon (1983) applied a similar concept to fish nutrition and found a high correlation between the experimentally determined EAA requirements of carp (Nose, 1979) and the pattern of these amino acids in the body of young, growing carp. Similarly, Wilson and Poe (1985) obtained a regression coefficient of 0.96 when regressing the EAA requirement pattern for channel catfish against the composition of a 30 g catfish.

This approach of tailoring the EAA pattern of the feed to that of the body has been used to develop diets for prawns (Deshimaru and Shigeno, 1972; Colvin, 1976) and finfish (Arai, 1981; Wilson and Poe, 1985). More recently, Knauer et al. (1994) analysed juvenile *H. midae* with a view to using the EAA profile of the body as a base for the development of a weaning diet for that species. Table 2.1 depicts the known amino acid profiles of some species of abalone.

Table 2.1: Amino acid composition of the whole soft body of abalone.

All values are %16N (equivalent to % protein) of soft body (dry wt.)

A A	H. rubra[1]	H. midae[2]	H. rufescens[3]	H.iris[4]	H. japonica[5]	H. laevigata[6]
Cys	1.42±0.12	2.41±0.35	0.69±0.07	1.42	1.18	1.29
Asp	ND	10.54±0.06	11.20±0.72	10.20	10.20	7.96
Thr*	4.13±0.26	4.99±0.05	4.63±0.25	4.30	5.06	3.99
Ser	ND	5.13±0.13	4.29±0.25	5.70	5.30	4.46
Glu	ND	14.82±0.39	13.50±0.52	14.60	15.20	14.04
Pro	ND	5.24±0.03	2.68±0.31	4.30	5.21	4.59
Gly	ND	8.37±0.37	4.96±1.02	8.40	7.81	7.32
Ala	ND	5.82±0.07	5.45±0.14	5.90	6.58	4.72
Val*	4.23±0.21	4.61±0.09	4.72±0.42	4.10	4.36	4.40
Met*	1.96±0.13	2.09±0.06	2.60±0.25	1.53	2.54	1.43
Ile*	3.62±0.21	4.11±0.03	3.78±0.49	3.70	4.18	3.77
Leu*	6.13±0.31	6.93±0.07	7.84±0.53	7.30	8.10	5.55
Tyr[7]	2.88±0.22	ND	3.81±0.65	3.20	3.52	2.60
Phe*	3.12±0.27	3.90±0.14	4.07±0.86	4.20	3.73	3.10
Trp*	0.93±0.06	0.82±0.17	0.38±0.02	1.10	0.88	ND
Lys*	5.30±0.26	6.21±0.03	5.99±0.45	5.90	6.43	5.00
His*	1.78±0.13	1.82±0.10	1.97±0.26	2.10	1.48	1.35
Arg*	9.03±0.47	7.91±0.13	7.26±1.06	10.00	9.48	7.29

* Denotes essential amino acid. (For amino acid names in full, see Appendix 3.) ND = Not determined
1 King et al. (1996)
2 Knauer et al. (1994)
3 Allen and Kilgore, (1975)
4 Promak Technology Ltd. (Fleming et al., 1996)
5 Suyama and Sekine (1965)
6 Coote et al., in prep. NB: These animals were raised on manufactured feed.

2.4 Energy requirements

Biological partitioning of energy in abalone

The energy requirements of abalone differ to that of mammals and birds in three main respects. Firstly, abalone, being poikilotherms, do not expend energy to maintain a body temperature different to the surrounding water. Secondly, abalone excrete waste nitrogen as ammonia. This requires virtually no energy compared to the production of urea or uric acid, the principal compounds generated in nitrogen excretion by birds and animals (Hainsworth, 1981). Thirdly, abalone spend little time engaged in voluntary movement (eg; foraging) compared to finfish, for example, that spend a considerable part of each day swimming. A flow diagram depicting energy partitioning in abalone can be seen in Fig. 2.3. Note that the energy needs for maintenance and voluntary activity must be met before energy is available for growth.

Energy is lost from the body of an abalone via faeces, ammonia, gill excretions, mucus production and as heat. The energy lost via faeces (FE) and ammonia (UE) has been estimated by Peck *et al.* (1987) as 20.7% of the energy intake (IE) of a 1.0 g (dw) abalone and by Barkai and Griffiths (1988) as 63%. Energy loss due to ammonia excretion is estimated as less than 1% of consumption by Barkai and Griffiths (1988), which correlates well with the estimate of 1.1% for a 1.0 g (dw) abalone by Peck *et al.* (1987). The amount of energy available for metabolic activity (ME) and ammonia excretion is assumed to be approximately equal to the amount of total digestible energy Thus,

$$TDE = ME + UE \dots\dots\dots\dots(1)$$

27

20%

SE

TDE (100%)   RE   50%

HE

30%
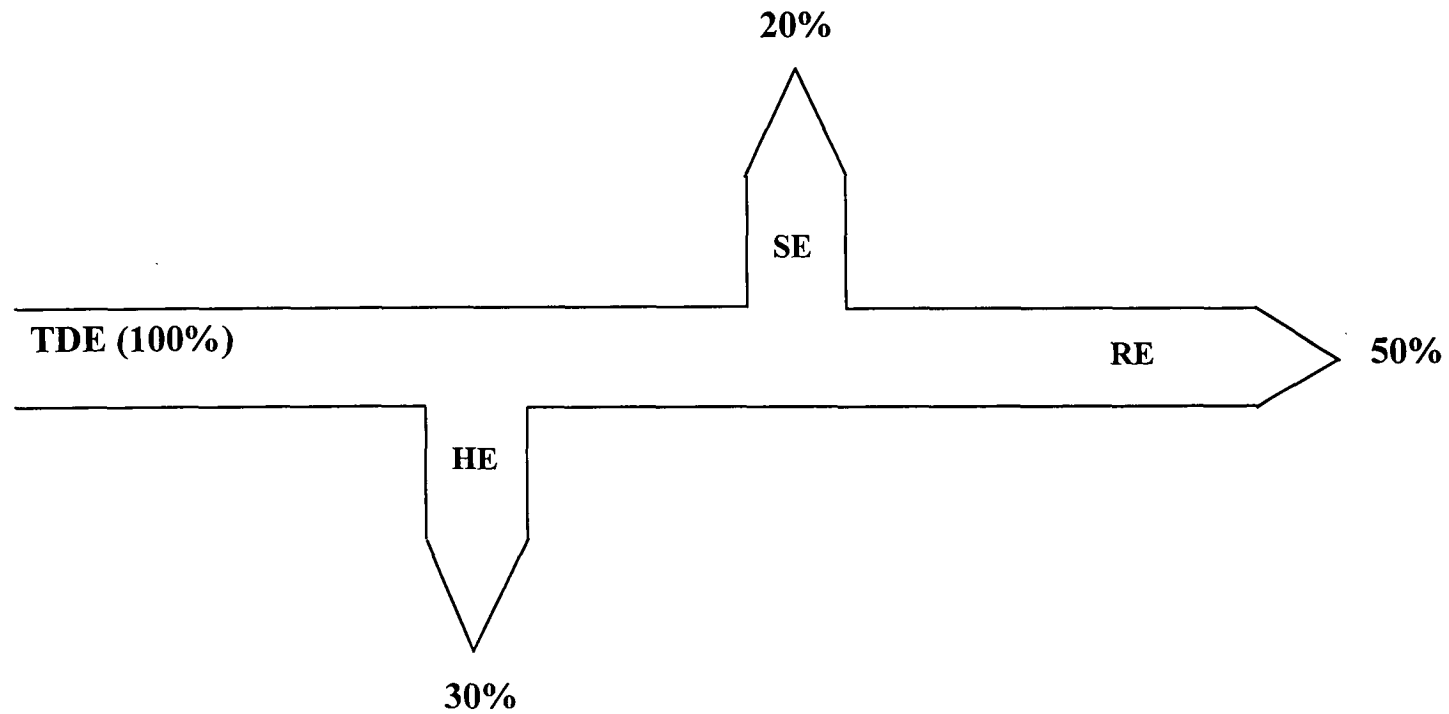
Fig. 2.3: Schematic depiction of energy partitioning in abalone
        (Adapted from Peck et al. 1987)
        TDE: Total Digestible Energy, HE: Heat Energy
        SE: Surface Energy, RE: Recoverable Energy

As UE is negligible (see above),

$$ME \approx TDE\ ....(2)$$

Thus, for abalone, metabolisable energy is approximately equal to dietary digestible energy (DE).

Metabolisable energy can be further subdivided into energy required for growth, reproduction and maintenance, but is ultimately expressed in only two forms, recoverable energy of products formed (RE), for example, meat, eggs or mucus, and heat energy (HE) (Fleming, 1991). Heat generated by abalone by respiration was estimated by Peck et al. (1987) to comprise 21.6%-31.1% of energy intake, depending on animal size. These estimates are in good agreement with the estimate of Barkai and Griffiths (1988), of 32% of the energy budget of H. midae at 19 °C. These authors admit that this value far exceeds laboratory measurements of about 8%, and suggest that these low values probably result from suppression of normal activity within respirometry chambers. So,

$$ME = RE + HE\ ....(3)$$

Mucus, a protein-carbohydrate complex, is secreted from the foot and gills of gastropods and may account for a considerable proportion of the energy budget of these animals (Kideys and Hartnoll, 1991). It is estimated that the whelk, Buccinum undatum expends 27% of its energy intake as mucus (Kideys and Hartnoll, 1991). Mucus production of the abalone H. tuberculata, ranged from 23.3% of the energy intake of a 0.01g (dw) animal to 29.1% of intake in a 50g (dw) animal (Peck et al., 1987). As mucus production in abalone apparently accounts for a large percentage of energy intake, some authors treat it as a separate component, (SE or "surface energy") and add it to equation (3), resulting in:

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$$ME = RE + HE + SE\ldots\ldots\ldots\ldots\ldots(4) \text{ or,}$$

$$DE \approx RE + HE + SE\ldots\ldots\ldots\ldots\ldots(5)$$

As allocations of the energy intake have already been estimated as approximately 30% and 20% for HE and SE respectively (see above), then RE must approximate 50% of DE. As DE can be estimated if intake rates and digestibility of the energy of diets are known, it should be possible to construct a model for growth of abalone. Work undertaken in this thesis may advance knowledge in this area.

Calorific values of food components

Apart from water, most of the components of food can be categorised as protein, lipid, or carbohydrate. Each of these compounds has a distinctly different chemical structure, and consequently, each yields different quantities of energy when oxidised. Mean values generally accepted as reliable for complete combustion of carbohydrate, protein and fat are 17.2 $MJ.kg^{-1}$, 23.6 $MJ.kg^{-1}$ and 39.5 $MJ.kg^{-1}$, respectively. Fish oil, however, being highly unsaturated, may yield a value closer to 36.2 $MJ.kg^{-1}$. The "true" energy yield of protein catabolised *in vivo* is normally considered less (about 19.7 $MJ.kg^{-1}$), when ammonia is produced (Brafield, 1985). Lipids yield more than twice the amount of energy, when oxidised, than either protein or carbohydrate, making them an efficient energy storage mechanism for many animals (eg; migratory birds and hibernating mammals).

Energy storage in abalone

The main storage compounds in molluscs is the carbohydrate glycogen, rather

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than lipids, which, for example, are the main storage compounds in mammals. Storage of energy as carbohydrate or by production of glycogen requires water, and adds more weight to an animal (Hainsworth, 1981). However, these factors, considered handicaps for terrestrial animals, do not pose such problems for marine creatures. Glycogen, a branched polysaccharide, is composed of D-glucose units linked by α-(1$\rightarrow$4) glycosidic bonds, with chains branching off via α-(1$\rightarrow$6) glycosidic linkages. Glycogen is found mainly in the foot of abalone, with concentrations changing according to food supply, time of year, and age and sex of the animal. Levels have been reported to be as high as 41-48% (Knauer et al., 1994). Some workers (Olaechea et al., 1993) have linked tissue glycogen with toughness in abalone meat, although others (Carefoot et al., 1993) found no such correlation. If there is a link between glycogen and toughness it may be of some concern to abalone farmers and processors. The dietary manipulation of tissue glycogen levels has not yet been investigated but this would seem to be an area deserving of some attention.

In contrast to glycogen, lipids constitute only a minor percentage of the foot tissue. Values range from a low of 0.2% (Konosu and Mori, 1959) to 3% (Webber, 1970). However, this study found that visceral crude fat levels ranged from approximately 7.5-10.0% (see Figs. 5.16 a and b). The fact that values change during the growth of the animal is demonstrated by Knauer et al. (1994), who reported values of 2.4% for 10-20 mm shell length specimens of H. midae, but only 0.76% for those of 45-55 mm.

Optimal digestible protein:digestible energy ratio of abalone

As discussed earlier, (Section 2.3) protein deposition in growing animals takes

the form of a two-phase process. As dietary protein levels rise, so too does protein deposition, until limited by the energy content of the diet. When additional energy is provided, growth continues to a new, higher dietary protein level. Irrespective of the energy level of the diet, the point of inflection of the growth function (ie; the point beyond which additional dietary protein results in no additional protein deposition) allows one to calculate the optimal protein:energy ratio of the animal for the conditions of the experiment and the essential amino acid profile. Obviously this value is best expressed as digestible protein:digestible energy. If diets are formulated taking this ratio into account, protein wastage is kept to a minimum, and pollution is reduced. Both factors could translate into significant cost savings for the industry. No information has been published concerning this facet of nutrition for abalone. This thesis aims to evaluate the optimal digestible protein:digestible energy ratio for abalone.

2.5 Conclusions

i) Abalone aquaculture has the potential to generate significant export income. The harvest and feeding of macroalgal wildstocks is not permitted in mainland Australian waters. The development of an economically viable manufactured feed is therefore needed to realise the potential of abalone aquaculture.

ii) Australia has an abundant supply of inexpensive feed ingredients. These ingredients need to be evaluated for their suitability for inclusion in abalone diets.

iii) Very little information is available on the nutritional requirements on abalone. The work done in this field has not always been rigorous and refers to species not found in Australian waters. There is a need for information on species native to

Australia.

iv) All but one of the previous estimates of the dietary protein requirements to sustain optimal growth of abalone were based on experiments in which the animals were fed diets unbalanced in essential amino acids. This may have resulted in elevated estimates of dietary protein requirement. In addition, no studies have been carried out in which the ratio of energy to protein has been taken into account.

v) No published data are available concerning the quantitative dietary requirements of abalone for essential amino acids.

From the above conclusions, the following experimental strategy will provide data that will be of use in the formulation of a commercially viable manufactured feed for abalone.

- The nutritive value of individual feed ingredients should be evaluated to provide a database on which future diets can be formulated.

- The optimal dietary protein inclusion level should be determined at a minimum of two energy levels to provide the optimal ratio of digestible energy to digestible protein.

- An estimate should be made of the dietary requirement of the animal for the most limiting essential amino acid(s). This can be done by formulating a diet limiting only in the amino acid under investigation, and a series of diets with increasing allowances of the limiting amino acid. Abalone are slow feeders, and soluble feed components often leach from the feed before the animal consumes it. Therefore, it will be necessary, in experiments involving species such as free amino acids, to in some way retard or, preferably, eliminate altogether, the effect

of leaching. This may be achieved by encapsulation of the amino acid or, preferably, covalent attachment of the test amino acid to an existing dietary protein.

CHAPTER 3

OPTIMAL PROTEIN LEVEL IN A SEMI-PURIFIED DIET FOR JUVENILE GREENLIP ABALONE (*Haliotis laevigata*)

3.1 Introduction

Utilisation of dietary protein by an organism depends on a number of factors. These may be linked to the diet itself and include the digestibility of the protein, its amino acid profile, the ratio of energy to protein in the diet and the amount of protein supplied. Other factors that affect protein utilisation are animal size, sex, genotype and environmental conditions (ARC, 1981). The provision of dietary protein from a single source is unlikely to provide essential amino acids in the optimal ratio required by the animal for growth. Thus, the protein requirements for abalone, determined by feeding a sole protein source, are likely to be overestimates.

This experiment aimed to determine the optimal protein inclusion level for *H. laevigata*, using a diet with an amino acid profile similar to the soft body profile. Bodyweight (BW) was used as the growth indicator. Digestibility of the protein and energy content of the feeds was also estimated, along with the loss of essential amino acids from the feed due to leaching.

3.2 Materials and Methods

Abalone

Juvenile *H. laevigata* (15-25 mm shell length (SL)) from a commercial abalone farm were used. These had been grown on manufactured feeds for about nine months. Abalone were anaesthetised with 1.0 ml.l^{-1} of a stock solution of benzocaine (Sigma), removed from water and allowed to drain on absorbent paper towel for five minutes to remove excess water. Each animal was weighed to the nearest 10mg before being tagged and assigned to an aquarium. A numbered plastic tag (Hallprint, Adelaide, South Australia) was affixed using a cyanoacrylate glue (Selleys "SupaGlue Gel"). Ten animals were randomly assigned per tank, with duplicate tanks for each treatment.

Aquaria

Rectangular acrylic tanks of 10 L, each containing one U-shaped plastic refuge, were used in a flow-through system. Incoming water was filtered to 30 μm nominal by primary sand filters, then to 10 μm nominal by secondary, composite sand filters. The water flow rate was 0.5 l.min^{-1}.tank^{-1}. Each aquarium was supplied with air via a single airstone at a rate of 0.2 l. min^{-1}.tank^{-1}. The water temperature was adjusted manually to be as close to 20 °C as possible, and was recorded at 0900 on weekdays (range: 14°C - 22°C, mean = 20°C). The tanks were cleaned three times weekly at 1600 hours, after which the abalone were fed at a rate of one gram/aquarium three times weekly. Abalone were allowed to adjust to their diets for 22 days prior to the start of the experiment proper (day 0) that lasted 59 days. Animals were measured on days 0 and 59.

Initially, feeds with nine different protein levels were manufactured. This was achieved by formulating a "basal" diet with a high protein level (Diet 11, Table 3.1). Ingredients were sourced as follows: **semolina**; Allied Milling Co., Adelaide, South Australia, **casein**; New Zealand Milk Products, Rowville, Victoria, **gelatine**; Davis Gelatine, (Australia) Botany, New South Wales, **sodium alginate, calcium sulphate, L-arginine, L-threonine, DL-methionine**; Ace Chemical Co., Camden Park, South Australia, **chromic oxide**; Sigma, **vitamin and mineral premixes and vitamins C and E**; Rhone-Poulenc Animal Nutrition Australia, Carole Park, Queensland. **Wheat starch** (Wade's BO11C, Goodman Fielder Mills Ltd., Lidcombe, New South Wales) was then substituted at increasing levels for protein-contributing ingredients so as to dilute the level of protein for the other diets (Table 3.2) without alteration of the level of oil, vitamins or minerals. The amino acid profile of each feed was identical, and was formulated such that the ratio of each essential amino acid to lysine was at least as great as that of the soft body tissue of *H. laevigata* (Table 2.1). It was necessary to include free essential amino acids to achieve the correct balance of amino acids (Table 3.1). A representative sample of abalone were removed from the cohort of abalone reserved for this experiment. After fasting for two days to allow voiding of gut contents, the abalone were killed by chilling and shucked. The soft tissue was freeze-dried, milled, and a representative sample analysed for amino acid profile using HPLC as per Mason et al., (1978). The feeds were developed using the "Feedmania" program (Version 7.06, Mania Software Pty. Ltd., Armidale, NSW). Dry ingredients (Table 3.1) were weighed on an electronic balance and thoroughly hand mixed. Fish oil (Jack Mackerel oil, Triabunna Fish Oils, Triabunna, Tasmania) was blended into the dry, mixed ingredients.

Sodium alginate and gelatine (binders) were pre-hydrated and dissolved, respectively with hot (95 °C) deionised water using a hand-held electric mixer and added to the dry mix. After preliminary hand mixing, the resultant "dough" was mixed in a spiral-action dough mixer ("Impastrice"; Hill Equipment and Refrigeration, Adelaide, South Australia).

The "dough" was extruded through a commercial pasta-machine ("La Prestigiosa" medium, IPA, Vicenza, Italy). The resultant strips of feed were dried for 12 h in a forced-draught oven at 55°C and stored at −35C until use. Strips of feed were broken into pieces approximately 7mm square prior to feeding. All diets were analysed for crude protein content using methods of the AOAC (1980).

Table 3.1: Ingredient inclusion levels for experimental diets (g.kg^{-1}) on an "as is" basis.

Ingredient	Diet										
	11	10	9	8	7	6	5	4	3	2s	1s
Semolina	495.8	446.2	421.4	396.7	371.9	347.1	322.3	297.5	247.9	198.3	124.0
Casein	290.4	261.4	246.8	232.3	217.8	203.3	188.8	174.2	145.2	116.2	72.6
Gelatine	120.8	108.7	102.7	96.6	90.6	84.6	78.5	72.5	60.4	48.3	30.2
Cr$_2$O$_3$	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Na Alginate	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Mineral PM	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Vitamin PM	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin E	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
CaSO$_4$	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Fish Oil	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
L-Arginine	11.5	11.4	9.8	9.2	8.6	8.1	7.5	6.9	5.8	4.6	2.9
L-Threonine	4.5	4.1	3.8	3.6	3.4	3.2	2.9	2.7	2.2	1.8	1.1
DL-Met	1.4	1.2	1.2	1.1	1.0	0.9	0.9	0.8	0.7	0.6	0.4
Wheat Starch	0.0	92.4	138.7	184.9	231.1	277.2	323.5	369.8	462.2	554.6	693.2
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

"s" denotes supplementary diet. PM = Premix, DL-Met = DL-methionine.

[1] Mineral Premix: (All activities in g.tonne^{-1}) Cobalt 0.025, as CoCO$_3$, Iodine 0.180, as KI, Copper 0.780, as CuSO$_4$, Iron 30.000, as FeSO$_4$, Magnesium 148.000, as MgO, Manganese 4.000, as MnO, Zinc 7.870, as ZnO. All subsequent diets used this premix. Vitamin C provided as "Rovimix Stay-C 35" ® by Roche (L-Ascorbyl 2-Monophosphate). Vitamin E provided as "Rovimix E 50" ® by Roche (500mg synthetic dl α-tocopherol/g).[2] Vitamin Premix: (g.tonne^{-1} except where indicated) Vitamin A 1500 mg retinol equivalents as "Rovimix A-500 Type P" by Roche, Vitamin B1 6.0, Vit. B12 0.009, Vit. B2 5.0, Vit. B6 2.0, Vit. C 200.0, Vit. D3 100 IU, Vit. K3 4.0, Ethoxyquin 40.0, BHT 100, Biotin 0.200, Ca Pantothenate 10.0, Folic Acid 1.5, Inositol 200.0. All subsequent diets used this premix.

Additional treatments

Measurements taken after the acclimation period indicated that the animals were growing better than expected at the lower protein levels. In an effort to obtain data on abalone growth on diets in which protein was limiting, two additional diets (12.2% and 18.8% CP) were formulated (Table 3.1). Duplicate tanks of animals (mean initial SL and BW of 18.55±0.16 mm and 0.78±0.02 g, respectively) from the same cohort were assigned to these diets and to another treatment (diet 3) in common with the experiment already under way. These abalone were smaller than those in the main experiment (Table 3.3). These additional treatments, hereinafter referred to as the supplementary treatments, were run under the same conditions and for the same duration as the original treatments.

Intake

On ten occasions during the experiment, feed from diets 3-11 remaining after three days was removed by siphon onto a filter paper, dried overnight at 55 °C and weighed. Using this data, feed consumption was estimated and used, on an "as is" basis for diets prior to immersion, along with BW increase, to estimate the feed conversion ratio (FCR). The same procedure was carried out on nine occasions for diets 1 and 2.

Statistical analysis

Measurements of BW were used to calculate growth and expressed as specific growth rate (SGR) to minimise the influence of differences in initial size:

$$SGR = 100 * \frac{\ln W(f)/W(i)}{T}$$

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Where W(i) = initial body weight, W(f) = final body weight and T = time in days.

Data were analysed by Systat® (SYSTAT, 1992) using single factor ANOVA on the SGR of bodyweight and expressed as the mean ± standard error. Multiple comparison of means was performed using Tukey's HSD test. Growth rates of abalone fed diet 3 in the supplementary experiment were compared to those fed diet 3 in the main experiment by Student's 2-tailed t-test assuming equal variance. Protein requirements of abalone were estimated from SGR using the second-order polynomial regression model (Lovell, 1989). Linear regressions were also applied to the growth data after the data from the supplementary diets were adjusted downwards by a factor of 0.85 ("Diet 3 correction factor"). This factor reflected the adjustment needed to equate the SGR of supplementary diet 3 with that of diet 3 in the original series (Table 3.2). F-values from ANOVA were used to test the significance of the fit of the regressions.

Apparent digestibility

Apparent digestibility of the protein and energy components of some of the diets was estimated after the completion of the growth experiment. Animals were held in the same tanks under the same conditions as the growth experiment. Feeding took place between 1600 and 1800 hours. The next morning tanks were cleaned by siphon at 0900 and subsequently faecal pellets were collected on 200 μm mesh at two hourly intervals. Faeces were dried (55°C overnight) and stored at -20°C until analysed. All feeds contained chromic oxide as an indigestible marker (Table 3.1). Crude protein was estimated using the Macro Kjeldahl method (AOAC, 1981) and GE was determined using a Parr 1261 bomb calorimeter (Parr Instrument Co., Moline, Il.). All CP results are means of duplicate analysis of each replicate sample.

Table 3.2: Crude protein (CP), gross energy (GE), crude protein :gross energy ratio of diets, specific growth rate (SGR) of abalone and experimental diet consumed.

Feed	CP (%)	GE (MJ kg^{-1})	CP:GE (g.MJ^{-1})	SGR (BW)[1]	Consumed[2] (g)
1s	12.2±0.06	17.29	7.08	1.05(0.92)±0.12^{ac}	12.38±2.69
2s	18.8±0.07	16.90	11.14	1.26(1.10)±0.04^{a}	13.74±0.44
3s	23.9±0.04	17.96	13.28	1.13(0.96)±0.04^{ac}	13.03±0.21
3	23.9±0.04	17.96	13.28	0.96±0.07^{ac}	13.23±2.23
4	27.4±0.18	17.99	15.22	1.14±0.17^{ac}	14.67±0.81
5	29.9±0.09	18.32	16.34	0.96±0.00^{ac}	14.76±1.73
6	32.3±0.37	18.08	17.88	0.93±0.09^{ac}	13.35±0.15
7	35.3±0.27	18.73	18.83	1.00±0.04^{ac}	13.71±0.10
8	37.4±0.10	17.35	21.54	0.83±0.07^{bc}	12.97±0.06
9	39.5±0.18	19.02	20.75	0.83±0.18^{bc}	13.63±0.96
10	41.8±0.11	18.70	22.34	0.82±0.02^{bc}	13.82±0.08
11	46.1±0.01	18.92	24.34	0.77±0.15^{bc}	14.08±0.81
F				(4.463)	

[1] Data are means ± sem, n = 2 replicate aquaria. [2] n = 9 for supplementary treatments, n = 10 for all other diets. Values in parentheses are means adjusted by the correction factor. Means with the same superscript are not significantly different (P > 0.05).

s = Supplementary treatment; BW = Bodyweight, F = F statistic for quadratic regression model ($P <$ 0.05).
CP values are mean ± sem, n = 2. GE values are from single analyses.

Dried faecal samples were ground with pestle and mortar. Chromium analysis was performed at the Dept. of Physical Science, University of Tasmania, Launceston, Tasmania using the following method: The sample was dried at 110°C for two hours then 200-500 mg were weighed into a nickel crucible. The crucible was placed in a muffle furnace at 100 °C and the temperature raised to 500°C for one hour to remove organic material. When cooled, the sample was mixed with sodium peroxide and fused over a Bunsen burner. The fusion product was allowed to cool and was then placed in 100 ml of distilled water. To this, 60 ml of hydrochloric acid was added and the reaction allowed to go to completion. This solution was made up to 200 ml with distilled water and chromium levels determined by atomic absorption spectroscopy (Varian, 1989) using an air/acetylene flame.

Apparent digestibility of the protein and energy components of diets was calculated using the following equation (Maynard and Loosli, 1969):

$$ADC = 100 - \left(\frac{N_{(faeces)}}{N_{(feed)}} * \frac{I_{(feed)}}{I_{(faeces)}} \right)$$

Where ADC = Apparent digestibility coefficient

N = Nutrient (protein or energy), I = Indicator (chromic oxide)

Leaching of essential amino acids

Leaching was carried out by placing preweighed feed onto 85 micron mesh screens (Swiss Screens Australia, Brooklyn Park, South Australia) and allowing it to settle to the bottom of aquaria without abalone. Temperature, flow rate and aeration were adjusted to match those of the experiment. At the end of the allotted time, the screens, with leached feed, were removed from the tanks and dried for 12 h at 55°C in a forced-

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draught oven. Representative samples of intact feed and leached, oven dried feed (diet 3) were then analysed for amino acid content.

AMINO ACID ANALYSIS

Samples of feed, feed ingredients, or abalone tissue were analysed for amino acid content using post-column derivatisation HPLC . The process used a Waters machine with 712 WISP injection system, 600E Multi-Solvent delivery system and Millenium Version 2.10 software. Briefly, protein-bound amino acids in the sample are released by hydrolysis with boiling, semi-concentrated hydrochloric acid. The hydrolysate is passed through a cation exchange resin, where chromatographic separation of the amino acids takes place. The individual amino acids are then mixed with ninhydrin, which reacts with them in a heated reaction loop to form a violet dye. This dye is detected at either 570 nm or 440 nm (for proline derivatives). Peak integration software calculates the area under the peak corresponding to each amino acid and compares it to that of the internal standard.

In some cases (eg, where the primary interest was in the lysine content of the sample, such as in Chapter 6), samples were subjected to hydrolysis only, which allowed quantification of lysine, threonine, arginine, isoleucine, leucine, valine, histidine, phenylalanine, tyrosine, glycine, serine, proline, alanine, aspartic acid and glutamic acid. The hydrolysis-only method largely destroys cysteine and, to some extent, methionine. Yields of serine, threonine, isoleucine, valine and tyrosine are often less than 100%. Correction factors (based on extended hydrolysis times for casein) were used to re-calculate values for the latter in this case.

In other cases, samples were subjected to oxidation followed by hydrolysis, in which cases values for cysteine and methionine were available in addition to those above, but tyrosine was not. Neither of the above methods allows tryptophan to be quantified, as it is destroyed by the hydrolysis process.

ACID HYDROLYSIS METHOD

Samples were freeze-dried and ground to less than 1mm particles. Hydrolysis tubes were pyrolysed for 2 hours at 500°C, then allowed to cool prior to sample weighing. Samples of between 0.5 and 4mg of protein were added to duplicate tubes. Duplicate samples of lysozyme were used as controls. To each tube was added 1 ml of redistilled 6M HCl containing 0.1% phenol. The tubes were degassed and the evacuated tubes sealed and placed in an oven at 110°C for 24 hours. After 24 hours, the cooled tubes were centifuged at 3,000 rpm for 3 minutes.

The tubes were then cracked open and norleucine was added (50μl of 4mM norleucine to tubes containing less than 2 mg of protein and 50 μl of 40mM norleucine to tubes containing more than 2 mg of protein. Samples were dried down using a Savant Speedvac and 2ml of loading buffer was added. The amino acids were dissolved by sonication and vortexing and the samples filtered through a 0.45 μm Millipore filter into a Waters 4 ml vial using a syringe. Vials were capped with teflon liners and loaded onto the HPLC.

OXIDATION STEP

Duplicate samples were weighed into tubes and cooled in an ice-bath to ice temperature. To each tube was added 1 ml of freshly-made, ice-cold performic acid. Tubes were allowed to incubate in the ice-bath for 16 hours. Ice-cold HBr (150 µl) was added to each tube and the samples were allowed to sit on ice for 10-30 minutes or until bubbling had subsided. Samples were then dried in the Savant Speedvac and hydrolysed by the addition of redistilled 6M HCl as above and processed as described above.

3.3 Results

Survival and growth

Survival rates were 100% for all treatments. Of the original nine diets, the diet containing 27.4% CP yielded the highest SGR (1.14±0.17) and best FCR (1.22:1±0.02). Neither SGR(BW), FCR, nor diet consumed differed significantly (P > 0.05) between any of the treatments (Table 3.2). When data from the supplementary treatments were included in the analysis, significant differences (P < 0.05) in SGR(BW) were noted (Table 3.2). The mean SGR of abalone fed diet 3 was 15% higher (n = 20) in the supplementary trial than in the main trial. This difference was significant (P < 0.05). Quadratic regression of unadjusted data (Fig. 3.1), for both trials combined, predicted maximal growth (Y_{max} = 1.103) occurs at 17.0% CP (X_{max}). Quadratic regression of the data, adjusted by the "diet 3 correction factor" (0.85) predicted maximal growth (Y_{max} = 1.033) at 27.0% CP (Fig. 3.2). The F-value (4.463) from the ANOVA for adjusted growth data used in Fig. 2 indicated that the R^2 value (0.4318) for the regression was significant at the 0.05 level. When data from the supplementary treatments is included,
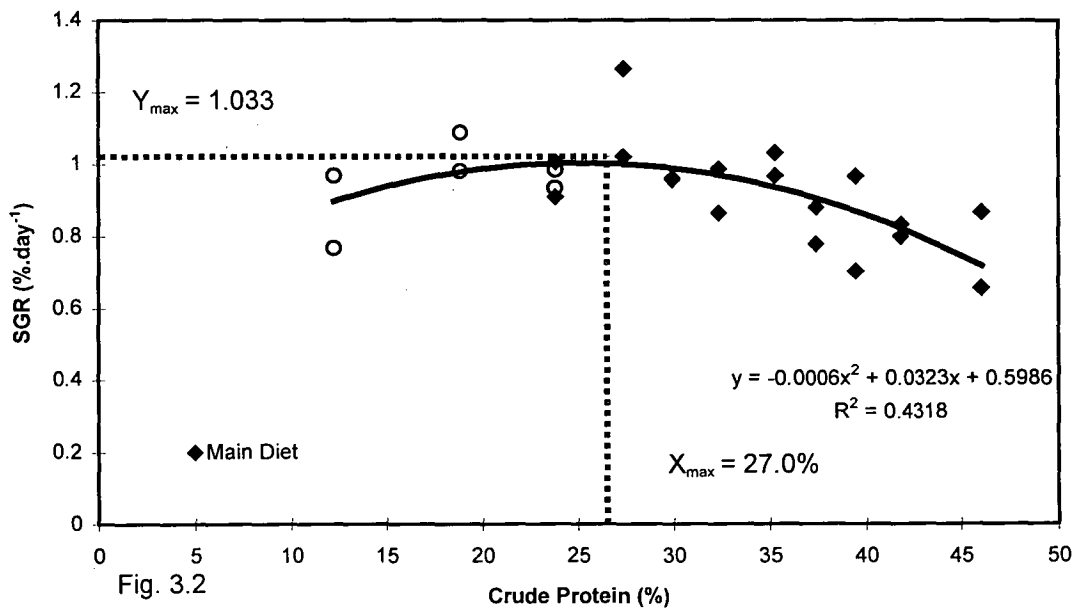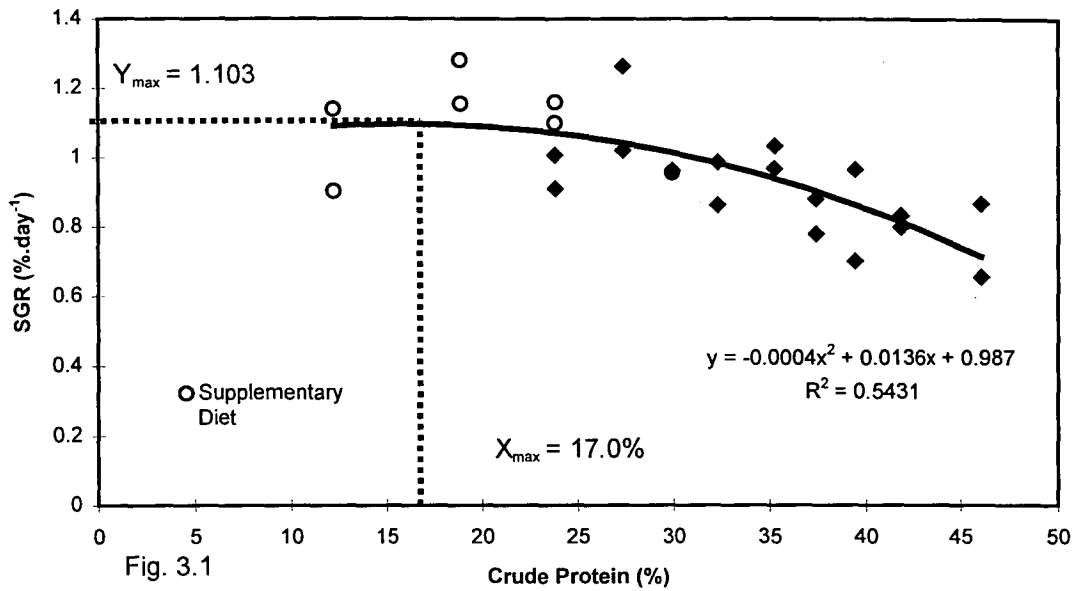
Fig. 3.1: Quadratic regression of specific growth rate (SGR) of abalone.
Fig. 3.2: Quadratic regression of SGR with data from supplementary treatments
adjusted downward by a factor of 0.85, the "diet 3 correction factor".

abalone fed diet 2 (18.83% CP) experienced the highest SGR (1.26±0.04). Feed consumption ranged from an estimated 12.38±2.69 g for diet 1 to 14.76±1.73 g for diet 5 (Table 3.2), but there was no significant difference between treatments. FCR values were highest for diets with both lowest and highest protein levels (Fig. 3.3), although FCR values did not differ significantly (P > 0.05) across treatments.

There was no significant difference (P > 0.05) in BW or SL among the abalone allocated to the original nine diets on days 0, 26, or 85 (Table 3.3). The abalone of the supplementary treatments also showed no significant difference (P > 0.05) in BW or SL on day 0, but by day 26, significant differences (P < 0.05) had appeared. These differences were still present on day 85 (Table 3.3). Significant differences (P < 0.05) in body weight gain (BWG) over the period from days 26-85 were seen for data from supplementary and main experiments (Table 3.3).

The figure shows a scatter plot with FCR on the y-axis (0.5 to 2.5) and Crude Protein (%) on the x-axis (0 to 50), with a fitted curve and the equation:

$$y = 0.0024x^2 - 0.136x + 3.3071$$
$$R^2 = 0.7944$$
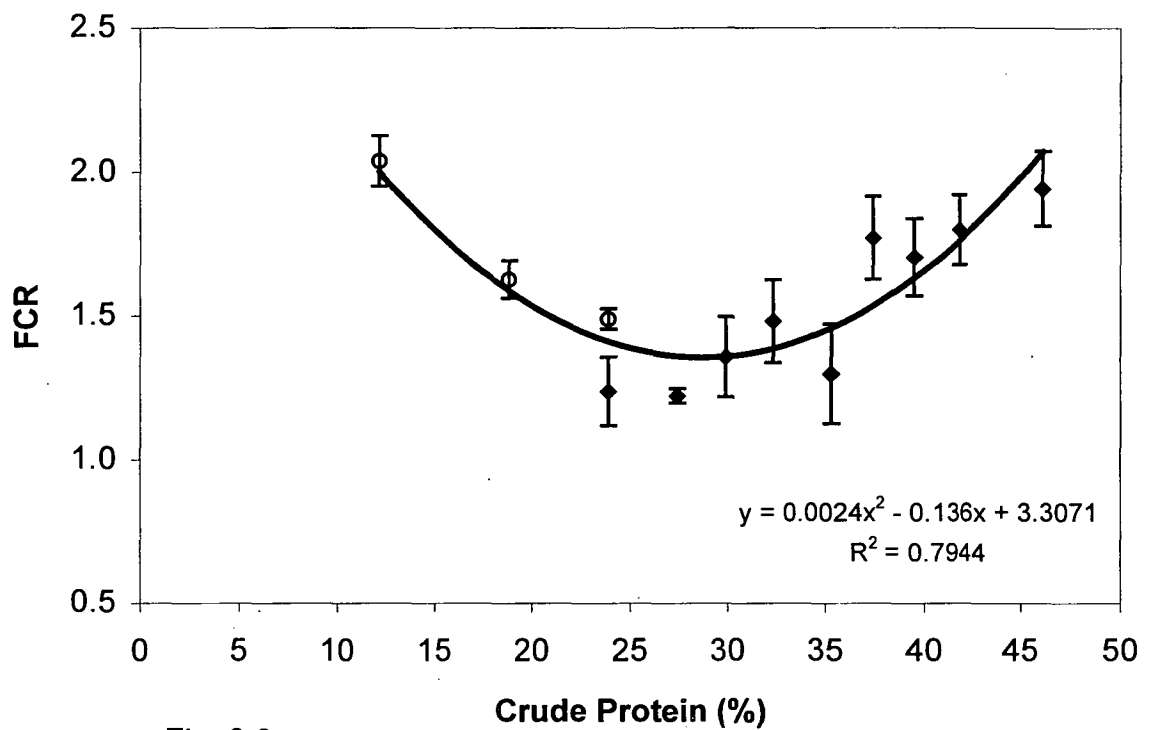
Fig. 3.3

Fig. 3.3: Feed conversion ratio (FCR) of experimental diets.
FCR = Feed consumed /wet weight gain
Open symbols indicate supplementary diets, closed symbols indicate original diets.

Table 3.3: Abalone body weight (g.abalone^{-1}) and shell length (mm) at each measurement point of the experiment, and body weight gain for the post-acclimation period (days 26-85). (Mean ± sem, n = 2 replicate aquaria).

Diet	BW (day 0)	BW(day 26)	BW(day 85)	BWG(days 26-85)	SL(day 0)	SL(day 26)	SL(day 85)
1s	$0.55^a \pm 0.01$	$0.69^a \pm 0.01$	$1.30^a \pm 0.12$	$0.61^{ce} \pm 0.11$	$15.76^a \pm 0.01$	$17.73^a \pm 0.05$	$22.85^a \pm 0.66$
2s	$0.56^a \pm 0.03$	$0.78^b \pm 0.02$	$1.62^b \pm 0.01$	$0.85^{ae} \pm 0.01$	$16.04^a \pm 0.24$	$18.50^b \pm 0.24$	$24.81^b \pm 0.23$
3s	$0.63^a \pm 0.04$	$0.88^c \pm 0.01$	$1.75^b \pm 0.01$	$0.88^{ae} \pm 0.01$	$16.63^a \pm 0.29$	$19.47^c \pm 0.05$	$25.58^b \pm 0.13$
3	0.96 ± 0.04	1.43 ± 0.02	2.49 ± 0.10	$1.07^{ad} \pm 0.08$	19.80 ± 0.21	22.69 ± 0.08	28.02 ± 1.03
4	0.88 ± 0.11	1.29 ± 0.13	2.49 ± 0.09	$1.20^a \pm 0.04$	19.22 ± 1.27	22.27 ± 1.24	28.22 ± 0.08
5	0.94 ± 0.16	1.44 ± 0.28	2.54 ± 0.52	$1.10^{ab} \pm 0.24$	19.70 ± 1.20	22.93 ± 1.05	28.54 ± 1.89
6	0.86 ± 0.24	1.29 ± 0.29	2.19 ± 0.37	$0.91^{ae} \pm 0.08$	19.05 ± 1.99	22.09 ± 1.99	27.09 ± 1.33
7	0.91 ± 0.06	1.39 ± 0.15	2.45 ± 0.28	$1.07^{ad} \pm 0.13$	19.50 ± 0.47	22.62 ± 0.62	28.43 ± 0.78
8	0.87 ± 0.01	1.21 ± 0.02	1.94 ± 0.04	$0.74^{bde} \pm 0.06$	19.14 ± 0.34	21.67 ± 0.48	26.09 ± 0.14
9	0.91 ± 0.05	1.30 ± 0.17	2.11 ± 0.05	$0.81^{ae} \pm 0.12$	19.57 ± 0.47	22.41 ± 0.78	27.00 ± 0.24
10	0.90 ± 0.04	1.29 ± 0.03	2.06 ± 0.08	$0.77^{bde} \pm 0.06$	19.36 ± 0.06	22.29 ± 0.04	26.57 ± 0.47
11	0.93 ± 0.15	1.32 ± 0.22	2.04 ± 0.23	$0.73^{bde} \pm 0.01$	19.69 ± 1.05	22.40 ± 1.01	26.71 ± 0.66

BW = Body weight, SL = Shell length, "s" denotes supplementary diet. Means with no superscript or the same superscript are not significantly different (P > 0.05). Note that means for supplementary diets have been analysed by single factor ANOVA and Tukey HSD test separately to data from the main experiment, with the exception of the data for BWG(days 26-85).

Protein and energy digestibility

Three diets (diets 5, 6 and 7) were selected for apparent digestibility evaluation and yielded similar results. The mean protein digestibility was 71.7± 1.9% and the mean energy digestibility was 55.6%± 1.7%. Thus, a diet containing 25% CP and 18 MJ kg^{-1} GE would contain approximately 18% and 10MJ kg^{-1} digestible protein and energy, respectively.

Amino acid loss due to leaching

Loss of essential amino acids (EAA's) supplemented in crystalline form exceeded 44% over 24 hours leaching, while loss of unsupplemented EAA's ranged from almost 26% (leucine) to 40% (isoleucine) (Table 3.4).

Table 3.4: Essential amino acids lost from diet 3 after 24 hours immersion in

seawater

EAA	Intact Feed (g kg^{-1})	Leached Feed (g kg^{-1})	Supplemented (g kg^{-1})	% Loss
Met	5.21	2.74	0.68 (13.1)	47.4
thr	9.85	5.49	2.27 (23.0)	44.3
arg	14.87	6.92	5.75 (38.7)	53.5
val	11.95	7.41	0.00	38.0
ile	9.70	5.82	0.00	40.0
leu	15.67	11.62	0.00	25.9
lys	12.23	8.87	0.00	27.5

EAA = Essential amino acid

NB: Histidine, phenlyalanine and tryptophan not analysed.

Values in parentheses are supplementation as a percentage of the total for that amino acid.

3.4 Discussion

Many previous studies have chosen growth, usually as an increase in length or body weight, to quantify the dietary protein requirements of aquatic species (Lovell, 1989). Sometimes, measurements such as protein efficiency ratio, net protein utilisation and feed conversion efficiency are also employed (Uki *et al.*, 1986b). In this work, growth is reported principally in terms of weight gain (as SGR) but the FCR and BWG of each diet are also reported. SGR was chosen as it generally reflects protein utilisation. In fact, Mai *et al.* (1995b) reported that protein gain was highly correlated with weight gain (r = 0.99) for two species of abalone.

Quadratic regression models fitted to the data predicted that diets containing 17-27% CP would elicit maximal growth (Figs. 3.1 and 3.2). This experiment indicated that young *H. laevigata* grew best when fed a semi-purified diet containing 27.4% CP, 18.0 MJ kg^{-1}GE and a CP:GE ratio of 15.2 g.MJ^{-1}. The higher optimal protein levels recommended by Uki *et al.* (1986b, 38% for *H. discus hannai* using casein as a protein source) and Taylor (1992) (at least 30% for *H. kamtschatkana* also using casein) and the much higher value (47% for *H. midae*) reported by Britz (1996) may have been due to the use of experimental diets with a sub-optimal EAA profile. If this was the case, optimal growth would tend to occur at higher levels of dietary protein than if the diet contained a balanced EAA profile, assuming the animals on lower CP levels were unable to compensate by higher intake rates. There was no evidence in the present trial to suggest that animals on the feeds with lower protein levels consumed more feed to compensate (Table 3.2).. The optimal dietary protein level (20.1%) estimated by quadratic regression (without the diet 3 correction factor) is lower than any previously

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published for abalone.

The results of this experiment were complicated by the addition of the supplementary treatments. The abalone fed diet 3 in the supplementary experiment grew significantly (P < 0.05) better than those fed diet 3 in the original experiment (Table 3.2). This may be due to the smaller initial size of the abalone used in the supplementary treatments, as SGR (weight) tends to be biased in favour of smaller animals, when comparing growth rates of animals of unequal sizes. Consequently, it was necessary to re-examine the data by adjusting downward the growth rate of animals on supplementary diet 3 to equal that of the animals on the original diet 3. An equal downward adjustment of the SGR of those fed diets 1 and 2 was also made prior to regression analysis (Fig. 3.2). This adjustment may compensate for the difference in initial size of the abalone, and resulted in a shift of X_{max} from 17.0% (Fig. 3.1) to 27.0% (Fig. 3.2). The estimate given by analysis of the adjusted data lies within the optimal ranges of 24-34.5% and 25.2-36.6% given for *H. tuberculata* and *H. discus hannai* respectively by Mai *et al.* (1995b).

Accretion of protein by an organism is a two-phase process, limited firstly by protein availability. If protein is not limiting, energy becomes the limiting factor. The CP:GE ratios for the two diets that yielded the best growth in this experiment were 11.1 g MJ^{-1} for diet 2 and 15.2 g MJ^{-1} for diet 4 (Table 3.2). These are slightly lower than the estimates provided by Mai *et al.* (1995b) of about 50-60 kJ g^{-1} in casein/gelatin based diets (equal to a CP:GE ratio of 16.7-20 g MJ^{-1}). A more accurate estimate of the levels of dietary nutrients that are required for growth and their ratios would have been achieved using diets formulated on a digestible basis. Although digestibility data for abalone were not available for the feed ingredients used, whole-diet protein and energy

digestibilities can still provide valuable estimates.

While the digestibility of the diet that produced the best growth overall (diet 2) was not evaluated, the digestible protein and energy content can be estimated using the data from the diets that were evaluated (diets 5-7). Thus, diet 2 contained approximately 13.5% digestible protein (18.8 x 0.717) and 9.4 MJ.kg^{-1} digestible energy (16.9 x 0.556). Thus, an estimate of the optimal dietary DP:DE ratio is approximately 14.4g MJ^{-1}. For diet 4 the ratio is even lower at 10.9g MJ^{-1}.

A notable result from this experiment is the marked decrease in SGR as the dietary protein level increased. At least two explanations exist for this observation, which was also made by Mai *et al.* (1995b). Firstly, as the dietary CP level increased, the GE of the diets did not increase proportionately (Table 3.2). Consequently, protein may have been catabolised to provide energy to digest and then excrete excess protein. Secondly, Mai *et al.* (1995b) suggested that the utilisation of gelatin by some aquatic species is poor. In the diet series used here, the inclusion rate of gelatin increased with CP level. We have observed that greenlip abalone at this facility reject feed containing high (approximately 20% w/w) gelatin levels. The possible negative effects of gelatin in abalone diets warrants further investigation.

Despite the use of both alginate and gelatine binders and the binding effect afforded by the high inclusion rate of semolina, a large proportion of EAA's were leached from the feed in 24 hours (Table 3.4). Essential amino acids supplemented in crystalline form were, not surprisingly, especially prone to loss. This loss should have been proportionately equal across treatments and should not, therefore, have affected growth more in one treatment than another. However, it raises some concerns about the economic benefit of addition of free amino acids to commercial abalone diets. Video

evidence of abalone feeding at this facility reveals that abalone mostly complete feeding activity six hours after the addition of feed (S. Madigan, Pers. Comm.). During this time, nutrient loss is assumed to be small, but, if feed is given, for example, only every third day as in many abalone farms, the nutritional profile of the feed is likely to have undergone significant change. This is of concern not only to growers and feedmakers, but also to researchers. It is important to investigate the process of nutrient loss further and, if necessary, to evaluate strategies aimed at reducing it.

In the wild, abalone are herbivores, and have adapted to live and grow on a diet relatively low in protein. For example, Mercer *et al.* (1993) evaluated the nutritional value of eight algal diets for abalone and reported that the single species yielding best growth for *H. tuberculata* and *H. discus hannai* were *Palmaria palmata* and *Alaria esculenta,* respectively, with respective protein levels of 15.93-18.38% and 11.06-15.73% (on a dry matter basis).

The results presented here suggest that high dietary protein levels (in excess of 30%) in manufactured feeds (Fleming *et al.*, 1996) may be unnecessary for abalone fed diets with appropriate amino acid balance. These findings should lead to cheaper feeds for the abalone aquaculture industry in Australia. Future work in this area could build on and extend these findings by establishing a database of the digestible protein and energy content of feedstuffs and by investigating different dietary ratios of protein to energy. Formulation of feeds on a digestible basis may help reduce farm effluent, increase feed conversion efficiency and lower production costs.

CHAPTER 4

ESTIMATED TRUE DIGESTIBILITY
OF THE PROTEIN AND ENERGY OF POTENTIAL INGREDIENTS FOR
USE IN MANUFACTURED ABALONE FEED

4.1 Introduction

A diverse range of feed ingredients are available in Australia for use in stockfeeds. However, there is no information on the nutritional value of these feed ingredients for abalone, and the utilisation of corresponding data from terrestrial animals or even finfish in the formulation of diets for abalone would be unwise. In the case of abalone, much of the published data (Fleming, 1995) relates to macroalgae and is of little assistance in the formulation of a diet based on terrestrial materials. Other research (Wee *et al.*, 1992, 1994) has investigated the digestibility of protein, energy and lipid in whole manufactured feeds, to compare the efficacy of inert markers, and, again, is of limited use for feed formulation. Therefore, the compilation of a digestibility database for feed ingredients for use in abalone diets is of paramount importance. Highly digestible diets will also reduce the faecal load from farms, thereby diminishing their environmental impact. In addition, information on digestibility is itself a tool to aid in the determination of more detailed nutritional information. This research is the first step in the compilation of digestibility data for abalone with the aim of producing better commercial and research diets.

4.2 Materials And Methods

Feed Ingredients

Semolina, Peruvian fishmeal, casein, soyflour and pre-gelled starch (PGS) were chosen for evaluation as all except PGS are currently used in commercial abalone feed. Semolina and PGS were obtained from Allied Milling Co., Mile End, South Australia. Casein was from Alaco Food Ingredients, Rowville, Victoria. Solvent extracted soyflour was purchased from Ace Chemical Co., Camden Park, South Australia and the fishmeal, of Peruvian origin, from Agribusiness, Macclesfield, South Australia.

A diet consisting largely of PGS and therefore containing negligible protein served two purposes. Using this diet, both the digestible energy of PGS and the endogenous protein content of abalone faeces could be estimated.

Diets

Five diets were used in this experiment (Table 4.1). All ingredients were finely ground prior to purchase, with the exception of fishmeal, which was re-ground and sieved through 1 mm mesh on arrival. All feedstuffs were analysed for CP and GE prior to formulation (Table 4.2). All diets were produced in the form of flat, well bound "chips" as described in Chapter 3. A diet consisting largely of semolina was used as a reference diet. Other ingredients were substituted into the reference diet to make the test diets. Each diet contained chromic oxide (0.50%), or, in the case of the PGS diet, n-hexatriacontane (3.2 g kg^{-1}) as an indigestible marker.

Table 4.1: Composition (g kg^{-1}) and analyses of experimental diets

Diet:	Semolina	Casein	Fishmeal	Soyflour	PGS
Semolina	952.5	732.5	-	503.5	-
Casein	-	220.0	-	-	-
Fishmeal	-	-	550.0	-	-
Soyflour	-	-	-	454.0	-
PGS	-	-	415.0	-	970.0
Mineral Mix	2.0	2.0	2.0	2.0	2.0
Vitamin Mix	3.0	3.0	3.0	3.0	3.0
CaSO$_4$	2.5	2.5	-	2.5	-
Na-Alginate	10.0	10.0	-	10.0	-
Fish Oil	25.0	25.0	25.0	25.0	25.0*
Cr$_2$O$_3$	5.0	5.0	5.0	5.0	-
Total (%):	1000	1000	1000	1000	1000
CP(%)	9.86	28.17	33.94	27.76	0.3
GE (MJ kg^{-1})	17.19	18.45	18.47	17.88	16.42

PGS = Pre-gelled starch

CP = Crude protein; GE = Gross energy

*Contained 3.2 g.kg^{-1} n-hexatriacontane by analysis.

Digestibility of nutrients was calculated by difference, using equation (4.1)

(R. van Barneveld, Pers. Comm.)

$$DI(\%) = 100 * \left[\left(NUT._{diet} * AD_{diet} \right) - \sum \left(NUT._{other} * IL_{other} * AD_{other} \right) \right] * \frac{1}{ILI} * \frac{1}{NUT.I}$$

Where

DI(%) = Digestibility of ingredient

$NUT._{diet}$ = Nutrient level of diet expressed as 18 MJ.kg^{-1} or % CP

$AD._{diet}$ = Apparent digestibility expressed as a decimal. (eg, 0.67)

$NUT._{other}$ = Nutrient level of ingredient other than test ingredient

$IL._{other}$ = Inclusion level of ingredient other than test ingredient

$AD._{other}$ = Apparent digestibility of non-test ingredient

ILI = Inclusion level of ingredient expressed as a decimal (eg, 2.5% = 0.025)

Nutrient I = Nutrient level of ingredient expressed as 18 MJ.kg^{-1} or % CP

When estimating the protein digestibility of test ingredients, ingredients other than the test ingredient were assumed to contribute negligible protein. When estimating the energy digestibility of test ingredients, fish oil was assumed to have a digestibility coefficient of 70%. This value was kindly supplied by Mr. Graeme Dunstan of CSIRO Marine Laboratories, Hobart, Tasmania. Alginate was also assigned an estimated digestibility coefficient of 70%. All other ingredients were assumed to contribute negligible energy to the diet.

Abalone

Juvenile *H. laevigata* (shell length 30-40 mm) were raised on diatoms from

settlement to about 10 mm shell length, then grown on a manufactured abalone feed containing 26% crude protein (CP) and 18 MJ kg^{-1} gross energy (GE).

Tanks and collection system

Conical tanks (n = 4) were fitted with faecal collection and drainage plumbing (Fig. 4.1). A bucket (20 L capacity) with a mesh bottom (4 mm mesh) was employed to hold the abalone within the tank whilst permitting faeces to drop into the collection tube, that was packed in ice during collection. This system allowed easy cleaning of the tanks with minimal disturbance to the animals. Seawater, filtered to 30 μm by primary sand filters, then to 10 μm by secondary composite sand filters, was supplied to each tank at approximately 2 L min^{-1}. Water temperature was held at 20 °C (±0.2 °C) by a computer-controlled mixing system and aeration (0.5 L min^{-1}) was provided by one air stone per tank. Each tank contained a piece of plastic sheeting to increase the available surface area.

Feeding

Abalone (approximately 100 per tank) were pre-conditioned on each diet by feeding daily for one week before faecal collection. Animals were fed to slight excess at 1700 h each evening and lights were switched off at 1800 h. Following collection of an adequate sample (5-7 days), abalone were returned to their commercial diet for one week, prior to acclimation on the next test diet.

Water Inflow

Air Line

Bucket Housing Abalone

Water Outflow

Support Fins

Benchtop

Drain Valve

Collection Valve

Insulated Ice Box
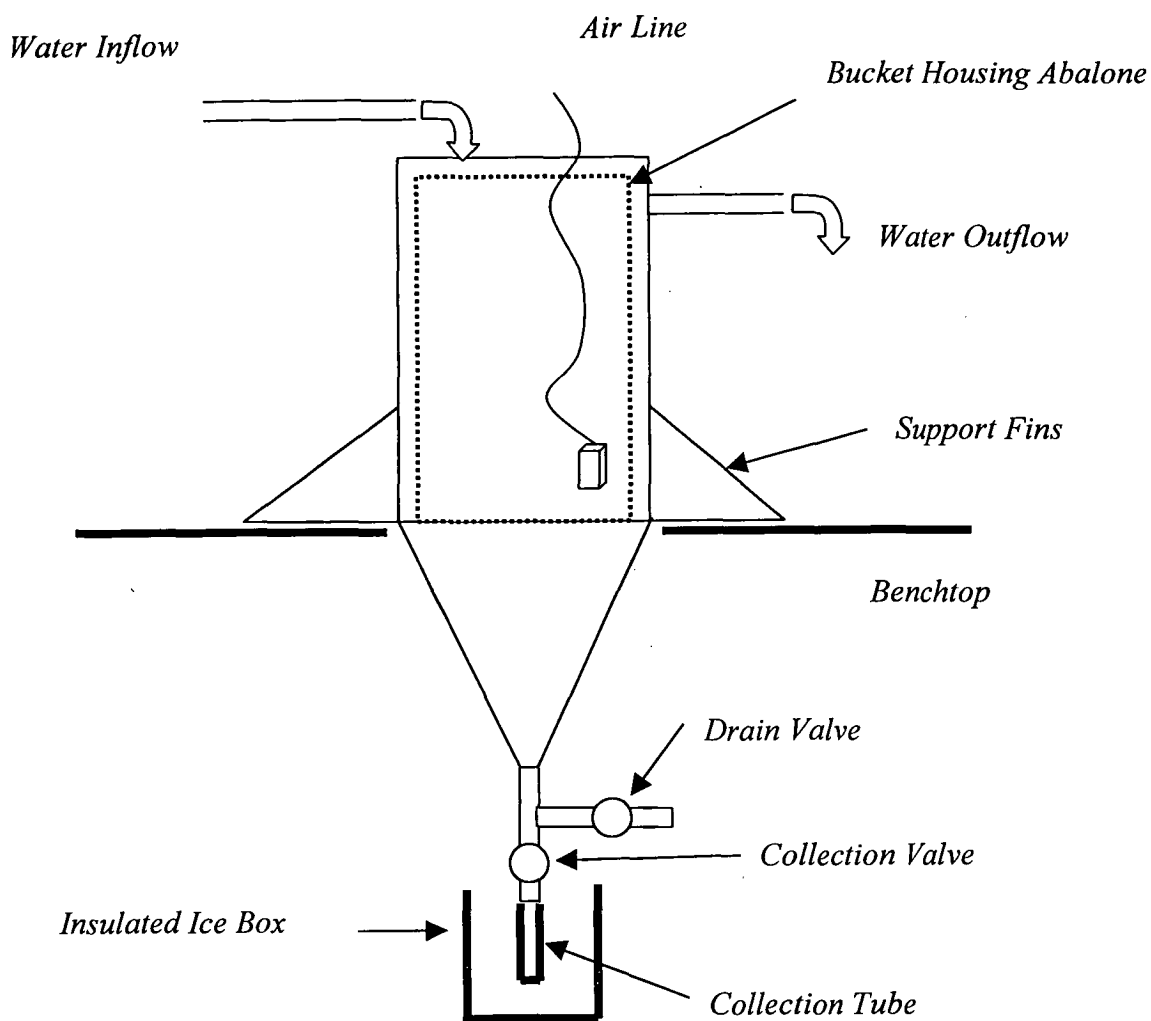
Collection Tube

Fig. 4.1
Diagram of faecal collection tanks used for
abalone digestibility work.

Faecal Collection

Following the acclimation period, faeces were collected daily in the following manner: the buckets were removed and the tanks and collection pipes cleaned thoroughly using ethanol, a bottlebrush and paper towel. Buckets were cleaned of faecal matter and uneaten feed by hosing with seawater. Abalone were out of water for approximately 8 min. each day. The tanks were rinsed and refilled, and the buckets replaced. Collection tubes were packed in ice by 0930 h, when the collection began and continued until 1600 h, when the tubes were removed and the contents poured over 1 mm mesh. Fines were discarded due to concerns that they would have leached excessively. Intact pellets trapped on the mesh were dipped in freshwater to remove salt, and air dried overnight at 22°C. The pellets were then placed in a vacuum desiccator at 15" of mercury for 24 h at room temperature to ensure that faeces were dried without heat damage.

Each diet was fed to all four tanks simultaneously. Each tank contributed one subsample per day, and a further subsample consisting of equal contributions from all four tanks was collected. A sample providing enough material for both crude protein (CP) and gross energy (GE) analyses required a total of 5-7 subsamples. Only three replicates of PGS faeces were available for CP analysis. These samples were not large enough to permit GE determination as well. A larger, pooled sample was collected for GE analysis, all of which was used for this purpose, thus, a CP value was unavailable for this sample. Due to unexpected results (not shown) obtained from the first fishmeal diet, a second diet, based on the PGS reference diet, was tested. This resulted in time constraints, so four replicate samples were analysed for CP only and one pooled sample was collected for GE evaluation only.

Analysis

Dried faecal samples were ground with pestle and mortar. Crude protein and GE were determined using methods of the AOAC (1980). All CP results are means of duplicate analyses of each replicate sample, except where indicated. Chromium analysis was carried out using the method detailed in Chapter 3.

Analysis of n-hexatriacontane was determined with the following method: The dried sample was weighed into a tube to which was added internal standard (1 mg.ml^{-1} $C_{24}H_{70}$ in dodecane), 94% ethanol and 7.5M KOH. The tubes were stirred at 80°C for one hour and then cooled. N-heptane and water was added and the samples were extracted with shaking for 10 min. The tubes were centrifuged and the heptane layer was collected while the samples were warmed in a water bath at 40°C. The extraction was repeated. The tube with the collected heptane was evaporated to dryness, made up to 1 ml with heptane and eluted through a Florisil column into another tube. This tube was evaporated to dryness and made up with enough n-hexane to inject into the HP-GLC. GLC parameters were: SGE BP1, 6m x 0.33mm ID, 0.25 micron film thickness, FID, 300°C isothermal, Helium carrier, split-splitless injector.

4.3 Results

Three samples of faeces from abalone fed the protein-free diet based on PGS contained a mean 4.31(±0.57)% CP. This provides an estimate of the endogenous protein content of abalone faeces, and was used to convert apparent protein digestibilities to estimated true digestibilities. Soyflour was the most digestible protein source (96.7%) followed by semolina (89.2%), casein (77.9%) and fishmeal (58.8%)

(Table 4.2). Pre-gelled starch was the most digestible source of energy (80.6%) followed by fishmeal (79.4%), soyflour (78.6%), semolina (55.5%) and casein (51.9%).

Using the lowest prices available at the time for the feedstuffs, cost per kilogram of DP and per megaJoule of DE were estimated (Table 4.2). Semolina was by far the most expensive protein source ($73.67 kgDP^{-1}), followed by casein ($13.21 kgDP^{-1}), with fishmeal ($3.00 kgDP^{-1}) and soyflour ($1.76 kgDP^{-1}) more economical. Casein was the most expensive source of digestible energy ($0.800 MJDE^{-1}), followed by PGS ($0.156 MJDE^{-1}), with little difference between semolina ($0.077 MJDE^{-1}) and fishmeal ($0.073 MJDE^{-1}). Soyflour was the cheapest source of DE ($0.055 MJDE^{-1}).

Table 4.2: Crude protein, mean (± sem) apparent digestibility of protein, true protein digestibility, digestible protein content, gross energy, apparent digestibility of energy, digestible energy content and dry matter digestibility of feedstuffs with feedstuff cost and cost per kilo of digestible protein and per megajoule of digestible energy. (n = 5 except where indicated).

Feedstuff	CP Content (g.kg^{-1})	APD Coefficient (%)	TPD Coefficient (%)	DP Content (g.kg^{-1})	GE Content (MJ.kg^{-1})	DE Coefficient (%)	DE Content (MJ.kg^{-1})	DMD Coefficient (%)	Cost $.tonne^{-1}	Cost $.kgDP^{-1}	Cost $.MJDE^{-1}
Semolina	104	64.96 ± 1.91	89.2 ± 1.57	92	16.00	55.5 ± 2.70	8.88	46.8 ± 1.98	680	73.67	0.077
Casein	860	67.61 ± 2.00	77.9 ± 1.33	670	21.30	51.9 ± 4.78	11.05	44.6 ± 1.47	8850	13.21	0.800
Fishmeal	630	54.65 ± 1.39[a]	58.8 ± 1.13[a]	370	19.25	79.4[b]	15.28	30.2 ± 4.35	1110	3.00	0.073
Soyflour	470	83.68 ± 0.33[a]	96.7 ± 0.49[a]	454	18.50	78.6 ± 1.01	14.54	54.0 ± 0.55	800	1.76	0.055
PGS	-		-	-	16.00	80.6[b]	12.80	71.0[b]	2000	NA	0.156

a: n = 4; b: n = 1
CP = Crude Protein; APD = Apparent Protein Digestibility; TPD = True Protein Digestibility; DP = Digestible Protein; GE = Gross Energy; DE = Digestible Energy;
DMD = Dry Matter Digestibility

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4.4 Discussion

Methodological Issues

One interesting finding of this work has been the estimate, using a diet based on PGS, of the amount of protein in the faeces of abalone fed a protein-free diet (Section 4.3). Naturally, concerns exist about the feeding of nutritionally deficient diets such as this one for any length of time. However, this was only done in this case to obtain an estimate (albeit crude) of endogenous protein loss.

Bush (1988) suggested that the proteinaceous sheath secreted by the limpet *Patella vulgata* around faecal pellets prevented their disintegration while still within the animal's mantle cavity. The coating observed to cover abalone faecal pellets may serve the same function. The estimate of endogenous protein loss for abalone given here does not include soluble proteins such as enzymes, that are undoubtedly lost from the faeces prior to collection and hence, must be an under-estimate. Nor did this study take into account the fact that endogenous protein excretion is almost certainly influenced by feed intake. Studies with pigs (de Lange *et al.*, 1990) and rats (Moughan and Rutherfurd, 1990) have demonstrated that endogenous amino acid secretion from the small intestine is greater in animals fed a diet containing protein than from those fed a protein-free diet. It has been proposed that this is a result of the stimulatory action of dietary peptides on the gut wall. Nevertheless, inspection of Table 4.2 shows that the endogenous protein content of the faeces can clearly have a profound effect when converting apparent protein digestibility coefficients to true digestibility coefficients, particularly those of feedstuffs with a relatively low protein content, such as semolina.

Initial data gathered from this work were substituted into the equation proposed by

Cho and Slinger (1979) to evaluate digestibilities of test ingredients substituted into a reference diet. The answers provided were often in excess of 100%. This phenomenon, in which the digestibility of a mixture of ingredients is greater or lesser than the mean digestibility of the individual ingredients has been encountered before in digestibility studies of red swamp crayfish (*Procambarus clarkii*; Brown *et al.*, 1989; Reigh *et al.*, 1990). The equation initially used (Cho & Slinger, 1979) does not account for possible associative effects between dietary ingredients. This equation was subsequently replaced by equation (4.1), that has been used for some time in swine nutrition. Digestibility results obtained using this equation were always less than 100%.

Initially, diets were made containing n-hexatriacontane as a digestibility marker. However, the high analysis cost of this marker compared to that for chromic oxide forced a change to the latter marker. Consequently, only the PGS data reported here were derived from the use of n-hexatriacontane.

Two factors that could affect the end result of digestibility estimates are leaching of nutrients from the feed prior to consumption and leaching of nutrients from the faeces prior to collection and analysis. These two processes will tend to have opposite and, therefore, compensating effects. No attempt was made to quantify these effects in this work.

Overall, the standard errors of measurement (s.e.m.) were small, the exceptions being for the DE of semolina and casein and the DMD values (Table 4.2). In a study of the experimental parameters that affect digestibility data in prawn diets, Ellis (1995) concluded that the number of samples collected and the number of days of collection were of approximately equal importance, and that the number of tanks was less so. Ellis (1995) showed that the s.e.m. decreased greatly when the number of days of collection increased

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from two to six, but further increase to 10 days resulted in little extra reduction. Double grinding and extrusion of feed also resulted in a substantial reduction in the s.e.m. of feed samples. When evaluated by the recommendations of Ellis (1995), the faecal collection time and number of replicates of this study appear satisfactory, but the s.e.m. might have been further reduced by more exhaustive analyses of feed samples.

Thomas (1995), drawing on the work by Ellis (1995), recommends that faeces should be collected for at least six days and preferably for ten. However, Thomas (1995) also demonstrates the effect of inclusion level of the test ingredient into the reference diet on the variance of the digestibility. Thomas (1995) argues that the estimation of ingredient digestibilities is not feasible at inclusion levels of 0.2 (ie. 20% of the reference diet replaced by test ingredient) and should be based on inclusion levels of 0.5. In this study semolina and PGS were evaluated at inclusion levels in excess of 0.95, fishmeal at 0.55, and soyflour at 0.45. Only the digestibility of casein, with an inclusion level of 0.22 appears to have been evaluated in less-than-ideal manner. This was done in order to avoid an excessively high dietary protein level.

Digestibility of ingredients

Evaluation of the digestibility of PGS proved difficult and time consuming, as the faecal matter produced by animals on this diet resembled the stringy "type C" faeces described by Wee *et al.* (1992) and were almost neutrally buoyant. Thus, they were difficult to collect in the apparatus. Abalone apparently digested the energy component of PGS with an efficiency of over 80%. Although not as high as the values for carp for hydrated corn (90.3%±2.3) and wheat starch (90.9%±3.4) respectively (Schwarz & Kirchgessner, 1991), this figure may have been influenced by the high level of starch in the

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diet (97%). Starch digestibility has been shown to be negatively correlated with the incorporation level of starch in fish (Inaba, 1963; Chiou and Ogino, 1975; Shimeno *et al.*, 1977). Bergot (1991) reported that rainbow trout (*Oncorhynchus mykiss*) were able to digest extruded or gelatinised starch with an efficiency of nearly 96%, but their ability to digest raw starches varied from less than 5% for potato starch to 52-58% for waxy maize and wheat starches.

Greenlip abalone apparently digest the protein component of semolina with an efficiency (corrected for endogenous protein) of 89%. Failure to account for endogenous protein reduces the apparent digestibility to approximately 61%. The corrected value compares favourably with those reported for rainbow trout of 88-91% (Bergot, 1991), and suggests that digestion of the protein component of feedstuffs such as semolina may well be independent of the digestion of the carbohydrate component. Abalone were unable to digest the carbohydrate component of semolina very efficiently, with an apparent digestibility of only 55.5% (±2.70). This is similar to the ability of rainbow trout (*O. mykiss*) to digest raw starch from wheat (Bergot, 1991). The fact that abalone digested PGS with an efficiency of more than 80% suggests that the physical structure of the starch grains reduces the ability of the enzymes to act on the starch molecules.

Processed soybean meals have been suggested as possible partial replacements for fishmeal in finfish diets (Oliva-Teles *et al.*, 1994) and this study strongly suggests that is also possible in abalone feeds, with abalone apparently digesting almost 97% of the protein and 79% of the energy in solvent extracted soyflour. These values, combined with widespread availability and low cost make soyflour extremely useful for commercial abalone feeds. Table 4.2 shows that defatted soyflour is by far the most economical source of digestible protein for an abalone diet, among the few ingredients evaluated here. It

should be noted, however, that soluble proteins leaching from the faeces prior to collection may have contributed to the high protein digestibility value obtained here. No effort was made to quantify the amount of protein lost from the faeces by leaching.

Casein, like soyflour, has also been included in many experimental (Uki *et al.*, 1985a, b; Viana *et al.*, 1993; Britz *et al.*, 1994) and some commercial abalone diets (Fleming *et al.*, 1996). However, despite the fact that it is very high in protein (around 86%) and that the protein is highly digestible (79%), its high cost and restricted availability must limit its inclusion rate in a commercially viable feed (Table 4.2). In addition, casein returned a very low value for energy digestibility of 51.9%.

Fishmeal, like PGS, proved difficult to evaluate, but for different reasons. The first diet (not shown) was similar to those for casein and soyflour, yet yielded digestibility coefficients that were well in excess of 100% (not shown). This may have resulted from associative effects among feed ingredients. A second diet was formulated (Table 4.1) using PGS in place of semolina in an attempt to reduce associative effects. The results indicated that this was achieved and that abalone do not digest the protein present in this particular fishmeal with high efficiency. It is possible that overheating during processing might have negatively affected protein digestibility of this feedstuff.

Economic Evaluations

The ingredients investigated in this study were compared to each other by costing their digestible protein and digestible energy content (Table 4.2). Feedstuff values were applicable, in Adelaide, Australia, at the time of the experiment, and will, no doubt, change from time to time, depending on availability, for example. Defatted soyflour supplied digestible protein and digestible energy more cheaply than the other feedstuffs. Fishmeal

was the second-cheapest source of digestible protein, followed by casein and semolina. However, semolina is primarily included to supply energy and function as a binder. Fishmeal and semolina supplied digestible energy at almost equal cost, while casein was almost ten times as expensive per megajoule of digestible energy. PGS was almost twice as expensive as semolina when compared by digestible energy cost.

4.5 Conclusions

Defatted soyflour is strongly recommended as an ingredient in manufactured feeds for abalone, not only on a digestibility basis, but also on economic grounds. Peruvian fishmeal, as a crude protein source, although not expensive, is less economical than soyflour due to its lower digestibility. Casein, although rich in highly digestible protein, is largely uneconomical due to its high cost and limited availability. Semolina, although costly per kg of digestible protein, is still likely to be utilised as an ingredient, as it is readily available, functions as a binder and supplies non-protein energy. Processing by extrusion or enzymic treatment may increase the digestibility of the non-protein energy component of this feedstuff for abalone. PGS is a source of readily digestible carbohydrate and helps bind pellets, but may be too expensive to be included as a bulk ingredient.

This work has been a small step in the compilation of a digestibility data base to aid in the formulation of cost-effective feeds for abalone. Data obtained from this work will be used to formulate diets on the basis of DP and DE for an experiment investigating the independent effects of energy and protein intake on protein deposition in growing abalone.

CHAPTER 5
THE EFFECT OF DIFFERENT DIETARY DIGESTIBLE PROTEIN:DIGESTIBLE ENERGY RATIOS ON THE GROWTH AND BODY COMPOSITION OF JUVENILE GREENLIP ABALONE (*Haliotis laevigata*)

5.1 Introduction

Growth in any animal is highly dependent on protein deposition rate which is, in turn, controlled firstly by the intake of dietary digestible protein and secondly by the intake of digestible energy (Pond *et al.*, 1995). This results, typically, in a two-phase growth response as depicted in Fig. 2.2. In the first phase, digestible protein is the limiting factor and, once the intake of digestible protein that supports maximal growth has been attained, digestible energy becomes limiting and no further increase in growth rate is possible at that energy level. In fact, growth rate may decline if excess protein is ingested (Sugahara *et al.*, 1969). If an additional quantity of protein-free energy is made available, a further increase in growth may be possible beyond the level of protein that previously elicited maximal growth.

Regression analysis of experimental data by either the broken line method described by Robbins *et al.* (1979) or the quadratic method described by Lovell (1989) can be used to ascertain the nutrient level required to elicit maximal growth. The quadratic method can also be used to estimate the lowest dietary nutrient level that elicits a growth response within the 95% confidence limits of the maximum. This can be termed the optimal level.

A growth experiment at numerous levels of DP and two levels of DE may be used to estimate the optimal DP:DE ratio, assuming the dietary energy level and essential amino acid levels are high enough to promote maximum potential growth. Feeds formulated to

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this ratio should result in more efficient growth, less effluent and more cost-effective production. The aim of this experiment was to estimate the optimal dietary digestible protein:digestible energy ratio for an abalone diet and to note the changes (if any) to the meat caused by deviations from the optimum ratio.

5.2 Materials and Methods

Experiment I: Growth

Abalone

Young, growing juvenile *H. laevigata* were purchased from South Australian Abalone Developments Pty. Ltd. for use in this work. These animals had been raised on diatoms to about 10 mm shell length (SL), then grown on a commercially manufactured feed. The animals were held until needed in a flow-through holding tank and were fed *ad-libitum* their usual commercial diet. Abalone were size-graded prior to selection for the experiment to reduce the variance as much as possible. At the commencement of the experiment, the mean shell length was 29.66 ± 0.06mm, and bodyweight was 3.52 ± 0.03g (n = 800). Details of the control animals that were sampled from the initial cohort are provided in Table 5.1.

Table 5.1: Analyses of the foot and viscera of control abalone.

	Foot	Viscera
Crude Protein (% dw)	67.05(±0.24)	52.47(±0.91)
Gross Energy (MJ.kg^{-1})	17.81	18.34
Moisture (% ww)	69.7	76.0
Ash (% dw)	9.21	12.16
Crude Fat (% dw)	2.81	6.80
Glycogen (% dw)	7.41	1.24

(n = 1 for all analyses except crude protein, where n = 2).

Values in parentheses are standard errors.

Dw = dry weight

Each sample was based on a pool of 20 abalone.

Feed formulation and manufacture

A range of feedstuffs were assessed previously to determine digestible protein (DP) and digestible energy (DE) (Chapter 4) for use in experimental diets. Two pairs of diets were formulated on the basis of DE. Each pair consisted of one diet high in DP and one diet with negligible protein (Table 5.2). Blending differing amounts of the isocaloric pairs produced a range of 10 DP levels at two discreet levels of DE, referred to as high energy (HE) and low energy (LE). The DP:DE ratio of individual diets can be seen in Table 5.3. The essential amino acid (EAA) profile was identical for all diets at both energy levels, and was based on the EAA profile of the soft body tissue of greenlip abalone obtained by analyses (Table 2.1). Diets contained chromic oxide at 0.5% as an indigestible marker, and were produced in the form of flat "chips" as described in Chapter 3.

Treatments

The 20 different diets (10 protein levels each at two energy levels) were each fed to four replicate tanks of 10 abalone. Abalone were anaesthetised, tagged, weighed and measured as previously described in Chapter 3, and randomly assigned to treatments. There was no significant size difference between replicates at the beginning of the experiment, that lasted 61 days

Aquarium system

For a description of the aquarium system see Chapter 3. In this experiment the flow rate was 0.2 L.min^{-1} and air was supplied via a single airstone at 0.5 L.min^{-1}. Water temperature was recorded on 45 of the 61 days at 0900 (range; 15.5-21.0°C, mean = 17.8 ± 0.2°C). The water supply for the experiment was taken into the facility via a pipeline 1.5km offshore.

Salinity (typically 36 ppt), pH (8.1), ammonia (<1 mg.l^{-1}) and oxygen (8.2mg.l^{-1}) were

monitored regularly and changed little during the experiment.

Table 5.2: Ingredient composition, DP (g.kg^{-1}) and DE (MJ.kg^{-1}), IL (g.kg^{-1}) and total dietary DP (g.kg^{-1}) and DE (MJ.kg^{-1}) of the four base diets

Ingredient	DP	DE	IL(1)	IL(2)	IL(3)	IL(4)
Na Alginate	0.0	9.5	5.0	5.0	5.0	5.0
Semolina	91.0	8.9	0.0	320.0	0.0	320.0
PGS	0.0	13.2	840.0	105.0	785.0	50.0
Fish Oil	0.0	26.5	55.0	90.0	6.0	40.0
Vitamin Mix	0.0	15.0	3.0	3.0	3.0	3.0
Mineral Mix	0.0	0.0	2.0	2.0	2.0	2.0
Kaolin	0.0	0.0	90.0	0.0	194.0	105.0
Casein	678.0	11.1	0.0	268.0	0.0	268.0
Fishmeal	370.0	15.3	0.0	60.0	0.0	60.0
Soyflour	454.0	14.5	0.0	120.0	0.0	120.0
Chromic Oxide	0.0	0.0	5.0	5.0	5.0	5.0
Arginine*	1000	15.0	0.0	17.0	0.0	17.0
Threonine*	1000	15.0	0.0	5.0	0.0	5.0
DE Total			12.65	12.66	10.63	10.61
DP Total			0.0	31.0	0.0	31.0

DP = Digestible protein (Refer to Table 4.2)
DE = Digestible energy (Refer to Table 4.2)
IL (1) = Inclusion level in diet 1 etc.
PGS = Pre-gelled starch
* Free amino acids are assumed to be 100% digestible.
Vitamin and mineral mixes were as described in Table 3.1
Vitamin mix was assumed to contain 15.0 MJ.kg^{-1} DE
Alginate was assumed to contain 9.5 MJ.kg^{-1} DE (see section 4.2)

Table 5.3: Digestible protein and energy levels of the experimental diets and their DP:DE ratios.

DP (g.kg⁻¹)	DP:DE Ratio LE (g.MJ⁻¹)	DP:DE Ratio HE (g.MJ⁻¹)
40	3.8	3.2
70	6.6	5.6
100	9.4	7.9
130	12.3	10.3
160	15.1	12.7
190	17.9	15.1
220	20.8	17.5
250	23.6	19.8
280	26.4	22.2
310	29.2	24.6

DP = Digestible protein
DP:DE = Digestible protein to digestible energy ratio
LE = Low energy
HE = High energy

Abalone were fed their allocated diet three times weekly to satiation level with slight excess.

Carcass analysis

After the final measurement, 20 abalone from each replicate were selected randomly, drained of excess water, placed in labelled bags and killed by freezing while still anaesthetised. Control animals selected randomly at the start of the experiment were also kept for carcass analysis. Frozen animals were allowed to thaw slightly before shell and soft-tissue were separated. The soft-tissue was weighed and further separated into meat and visceral components. Meat, shell and viscera to whole body ratios (w/w) were calculated to indicate the nutritional status of the animals. Meat and viscera samples were then freeze-dried prior to analyses for moisture, ash, crude protein (CP), gross energy (GE), crude fat and glycogen content.

Crude protein, moisture, ash and crude fat were determined using the methods of the AOAC (1980) and GE was determined using a Parr 1261 bomb calorimeter (Parr Instrument Co., Moline, Illinois). Glycogen content was estimated using the method of Murat and Serfaty (1974).

Feed Consumption

On three occasions during the growth experiment, uneaten feed was removed from the aquaria, dried, and weighed to estimate feed consumption. Accurate data was obtained for diets containing 40 - 160 g.kg⁻¹ DP at both energy levels. At higher levels of DP, the feed tended to create excessive fines that were difficult to separate from faeces. An estimated consumption rate was used to calculate protein retention where accurate feed

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recovery was not possible. The consumption rate was assumed to be equal to the mean value for those diets for which consumption was accurately determined. Protein retention was calculated using equation 5.1.

$$PR = 100 * \left(\frac{CPv * W_v(\Delta BW * \%V) + CP_m * W_m(\Delta BW \%M)}{\%FC * \left(\sum FEED * DP\right)} \right) \dots\dots\dots\dots\text{Equation 5.1}$$

Where PR = Protein Retention, CP_v = Crude Protein of viscera, W_v = Dry Matter content of viscera, ΔBW = Change in body weight of replicate, $\%V$ = Viscera as percentage of whole abalone for that treatment, CP_m = Crude protein of meat, W_m = Dry matter content of meat, $\%M$ = Meat as percentage of whole abalone for that treatment, $\%FC$ = Percentage of feed consumed, $\sum FEED$ = Total feed given, DP = Digestible protein content of that feed.

Experiment II: digestibility

Diets containing 40, 190 and 280 g.kg^{-1} DP from both energy levels were assessed for protein digestibility using abalone of a similar size to those in the growth experiment. These three diets were chosen to because they almost span the range of dietary protein content used in the experiment. The methodology and chemical analysis were as described previously (Sections 3.2 and 4.2).

Experiment III: diet change

In an effort to determine whether abalone grown on low protein diets and/or high energy diets suffered permanent impairment, those that were not sacrificed for carcass analysis from diets 40, 70 and 130 g.kg^{-1} DP (HE and LE) were returned to aquaria and fed a commercial growout diet for a period of 43 days, after which they were again measured. Stocking density was maintained as per the growth experiment by halving the number of

replicate tanks.

Statistical analysis

Gain in body weight was expressed as specific growth rate (SGRBW) calculated using the formula from Chapter 3. Protein requirements of the abalone were estimated from weight gain using the second-order polynomial regression analysis model (Lovell, 1989). All percentage data were arcsine transformed prior to analysis. All statistics were calculated using Systat (1992). Tukey's HSD test was used in pairwise mean comparisons. A two-tailed t-test was used to test for significant differences in growth rate due to diet change in Experiment II.

5.3 Results

Survival and growth rates

Due to a system failure in one tank (out of 80), all 10 animals of that replicate died. Apart from this mishap, only one other animal died during the experiment. The data for SGR based on BW of the abalone fed the LE and HE diets are shown in Figs. 5.1(a) and (b), respectively. The SGRBW of abalone fed LE diets was significantly affected by DP:DE ratio ($P < 0.001$), while that of abalone fed HE diets was not ($P > 0.05$). The SGRBW of abalone fed the LE diets increased significantly ($P < 0.05$) from 3.8 g DP.MJDE^{-1} to a maximum at 17.9 g DP.MJDE^{-1} and then decreased significantly ($P < 0.05$) as DP:DE ratio increased to 29.2 g DP.MJDE^{-1}. Tukey's HSD test revealed that the SGRBW of abalone was not significantly different from 12.3 - 17.9 g DP.MJDE^{-1} inclusive ($P > 0.05$). Second-order polynomial regression of the data (Fig. 5.1a) gave an estimate of

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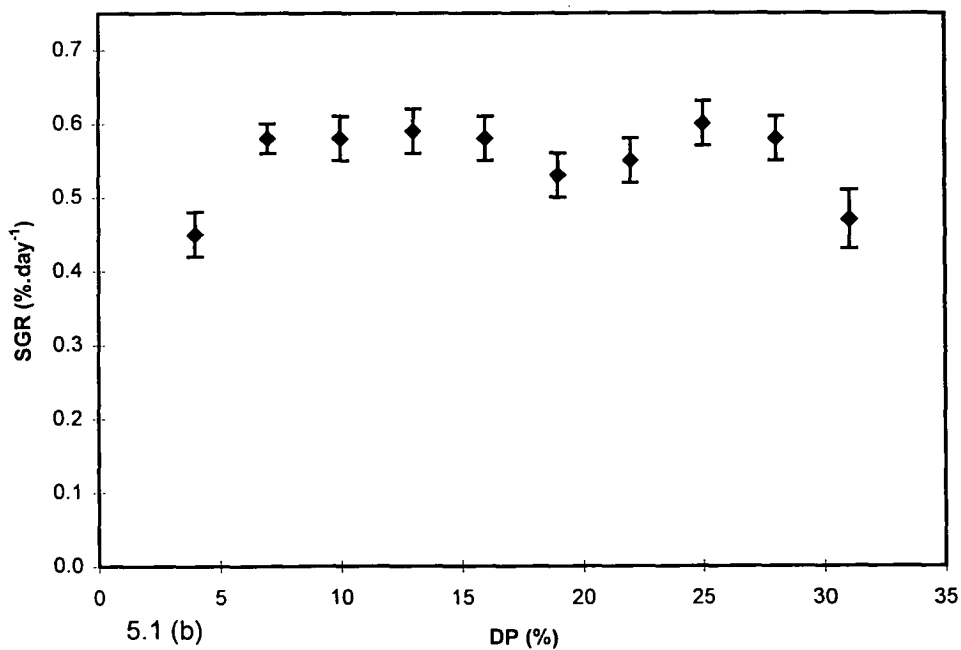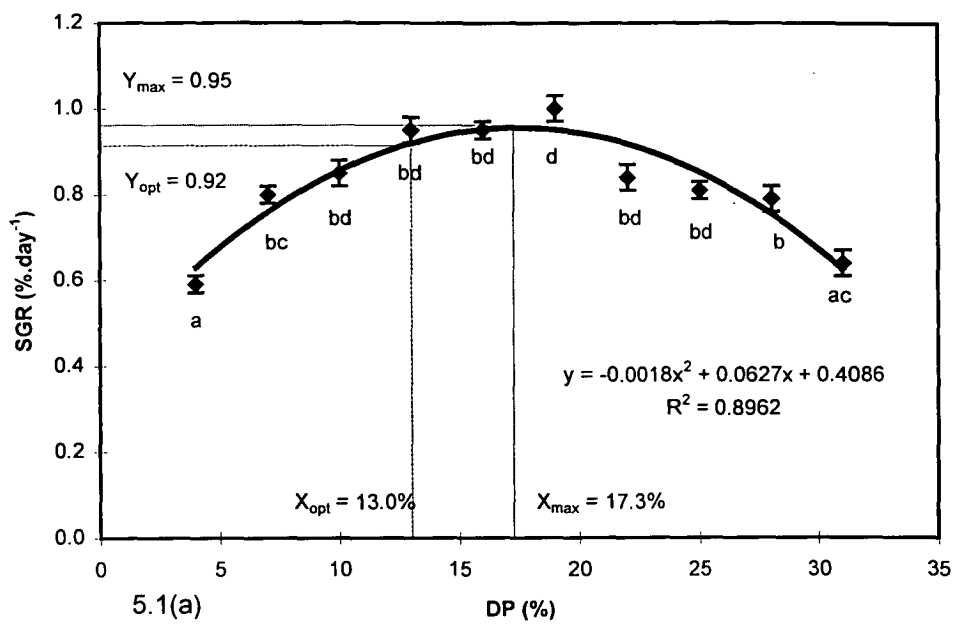
Fig. 5.1: Specific growth rate (SGR) of abalone. (a): Fed LE diets. (b): Fed HE diets. (Mean ± s.e., n = 4) Means with the same subscript are not significantly different (P > 0.05).

X_{max} of 16.3 g DP.MJDE^{-1}, with Y_{max} = 0.95. The lower 95% confidence limit of Y_{max} was bounded by Y_1 = 0.92, with the corresponding DP:DE ratio of 12.3 g DP.MJD E^{-1}.

Abalone fed HE diets exhibited significantly lower growth rates than those fed LE diets (Fig. 5.1b). SGRBW of these animals reached a maximum at a DP:DE ratio of 19.8 g DP.MJD E^{-1}. Due to the growth restriction imposed by the energy level of this diet, these data were not analysed further by broken-line or quadratic regression.

Relationship of muscle, viscera and shell to total weight

Foot muscle, expressed as a percentage of the body weight of animals (ww) fed LE diets, increased from 34.0% for those fed the 3.8 g DP.MJDE^{-1} diet to plateau at about 36.5% for diets with 6.6 - 15.1 g DP.MJDE^{-1} and declined at higher DP levels (Fig. 5.2a). Opposite trends were evident in shell weight and visceral weight (Figs. 5.3a and 5.4a) (as a percentage of body weight). Trends for the three body component indices were similar for HE diets although foot muscle, shell and viscera indices were lower, higher and lower, respectively (Figs. 5.2b, 5.3b, 5.4b).

Carcass analyses results

Results of the CP, GE, ash, moisture, glycogen and crude fat content of the meat and viscera of abalone can be seen in Figs. 5.5-5.16. The crude protein content of the foot muscle of abalone fed LE diets increased linearly from a low of 44.2% for the diet containing 3.8 g DP.MJDE^{-1} to a high of 70.6% for the diet containing 29.2 g DP.MJDE^{-1} (Fig. 5.5a). A similar trend was noted for the abalone fed the HE diets (Fig. 5.5b), with protein increasing from a minimum of 45.2% (3.2 g DP.MJDE^{-1}) to the maximum of 72.3% (24.6 g DP.MJDE^{-1}). In all cases except one (250 g.kg^{-1}DP) the protein content of the foot
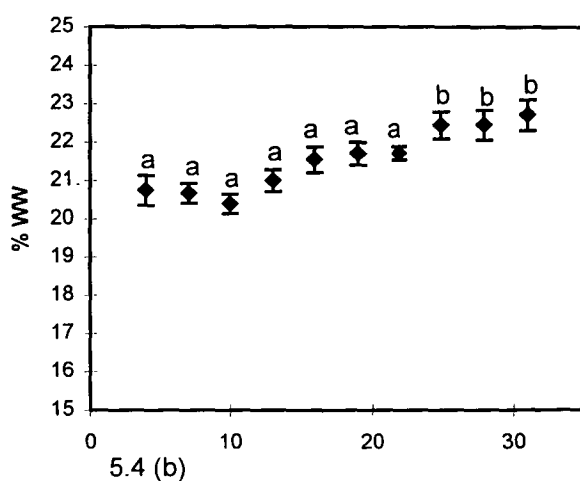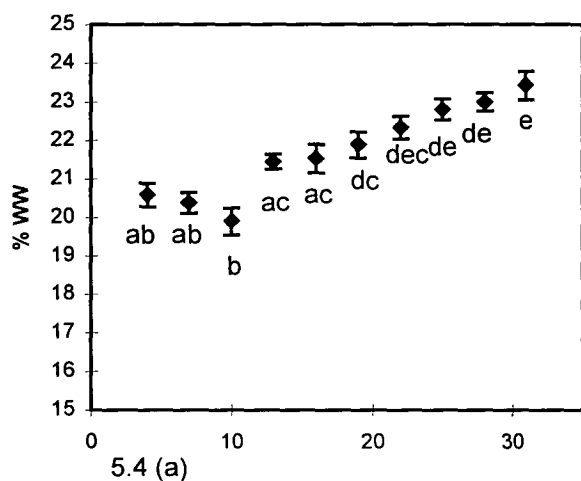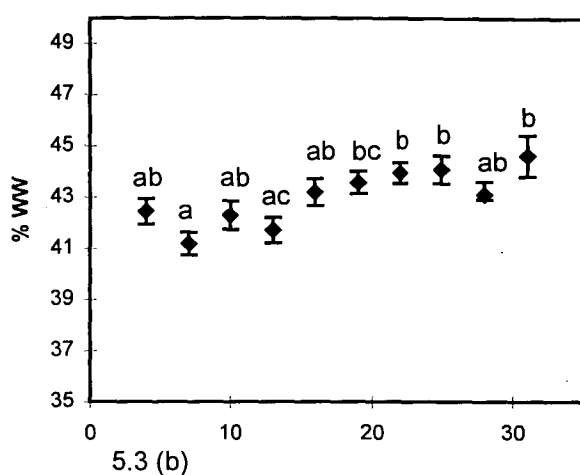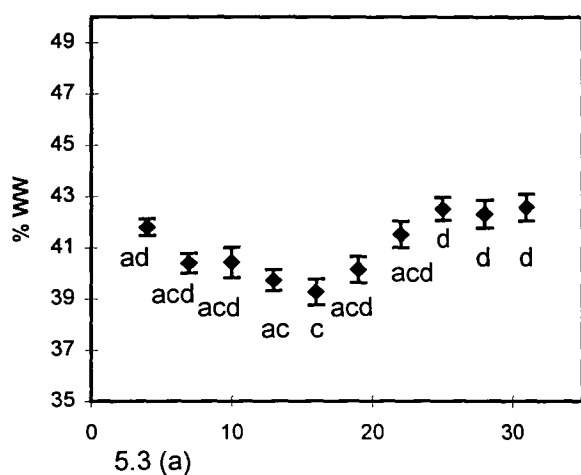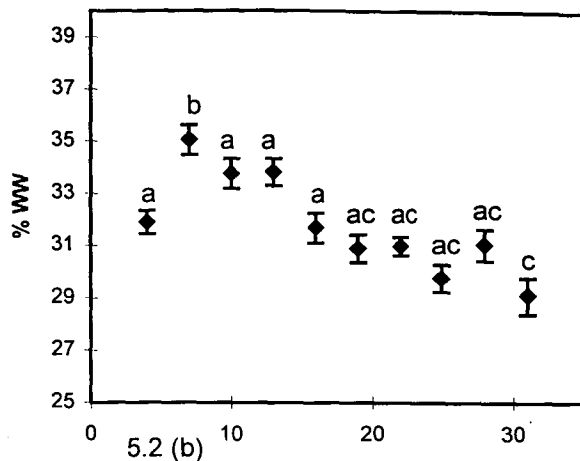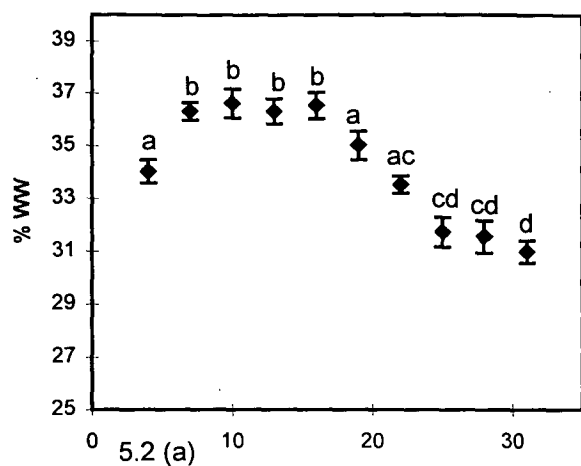
Fig. 5.2: Foot muscle as percentage of whole abalone. (a) Fed LE diets. (b) Fed HE diets.

Fig. 5.3: Shell as percentage of whole abalone. (a) Fed LE diets. (b) Fed HE diets.

Fig. 5.4: Viscera as percentage of whole abalone. (a) Fed LE diets. (b) Fed HE diets.

(Means ± s.e., n = 20) Means with the same sub- or superscript are not significantly different (P > 0.05). Units of X-axes are "percent dietary digestible protein".
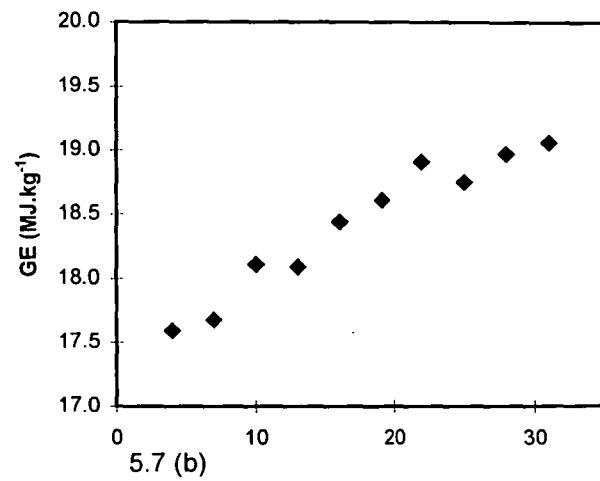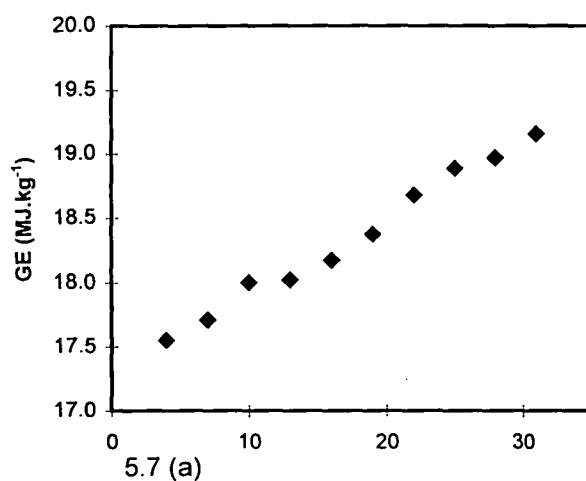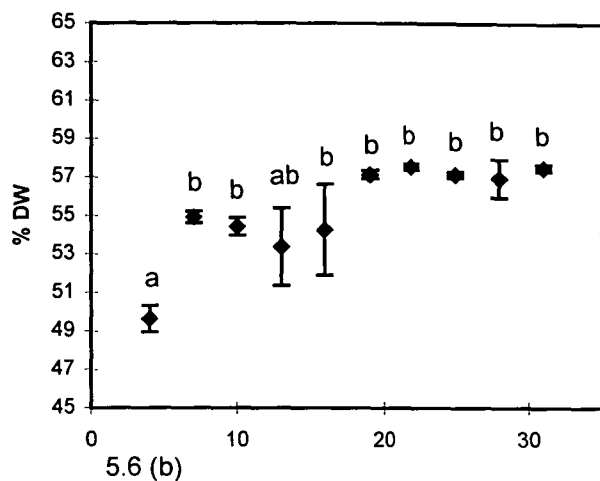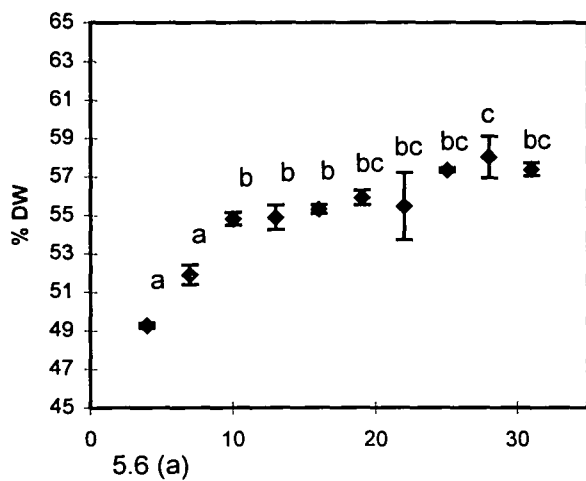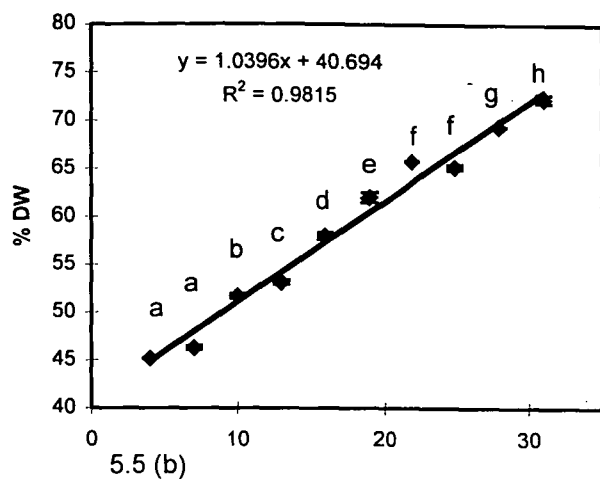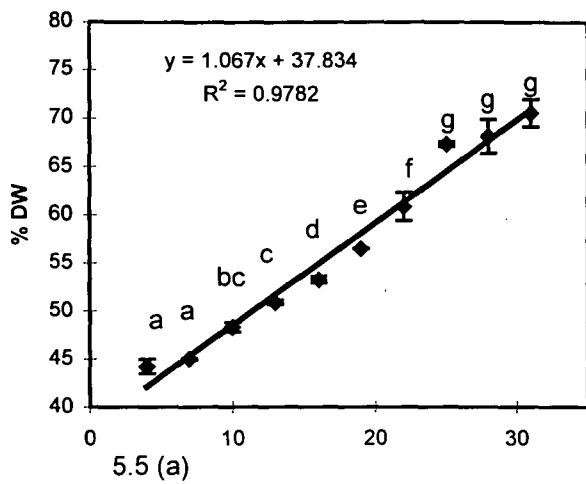
Fig. 5.5: Crude protein of abalone foot muscle. (a) Fed LE diets. (b) Fed HE diets. (n = 2)

Fig. 5.6: Crude protein of abalone viscera. (a) Fed LE diets. (b) Fed HE diets. (n = 2)

Fig. 5.7: Gross energy of abalone foot muscle. (a) Fed LE diets. (b) Fed HE diets. (n = 1)

Units of all X-axes are "percent dietary digestible protein".

Means with the same superscript are not significantly different (P > 0.05).

muscle of animals fed HE diets exceeded that of those fed the LE diets. Trends for visceral protein content were less clear, (Figs. 5.6a and 5.6b) with protein content increasing significantly ($P < 0.05$) with dietary DP:DE ratio from 3.8 - 9.4 g DP.MJDE^{-1} for LE diets and from 3.2 - 5.6 g DP.MJDE^{-1} for HE diets.

The GE content of foot muscle was similar for both HE and LE diets (Figs. 5.7a and b), increasing from approximately 17.6 MJ.kg^{-1} (40 g.kg^{-1}DP) to approximately 19.1 MJ.kg^{-1} (310 g.kg^{-1}DP). The GE content of viscera tended to be greater for abalone fed LE diets. The GE of viscera from animals fed LE diets increased steadily from 3.8 - 17.9 g.kg^{-1}DP, then declined slightly (Fig. 5.8a), while the GE of viscera from abalone fed HE diets increased initially, then tended to plateau beyond 7.9 g.kg^{-1}DP (Fig. 5.8b).

Diets with higher DP:DE ratios (20.8 - 29.2 and 17.5 - 24.6 g.kg^{-1}DP of LE and HE diets, respectively) produced higher foot muscle moisture content (Figs. 5.9a,b) but there were no clear trends in the visceral moisture content data (Figs. 5.10a,b) other than the viscera of abalone fed HE diets contained more water than those fed LE diets.

Trends for ash content (Figs. 5.11 and 5.12) were similar to those for moisture. Trends for glycogen (Figs. 5.13 and 5.14) were the inverse of those for protein. Foot muscle contained little fat, although it was elevated at the highest levels of DP and was usually lower for the HE diets (Fig. 5.15a,b). Viscera fat content was much higher (typically 8-10%) and largely unaffected by DP or DE (Figs. 5.16a,b).

Results of the analyses of the control animals can be seen in Table 5.1. Partitioning of the carcass of control animals showed that they yielded 30.72% (+0.41, -0.39) foot muscle, 40.90% (+0.48, -0.47) shell and 19.96% (+0.61, -0.51) viscera (mean +s.e., -s.e.)

Protein retention (%) (net protein gain/digestible protein consumed) was calculated for each treatment. Protein retention for animals fed LE diets ranged from almost 90% for

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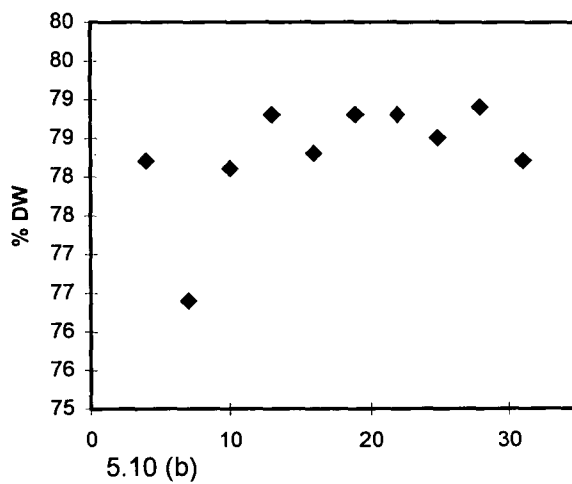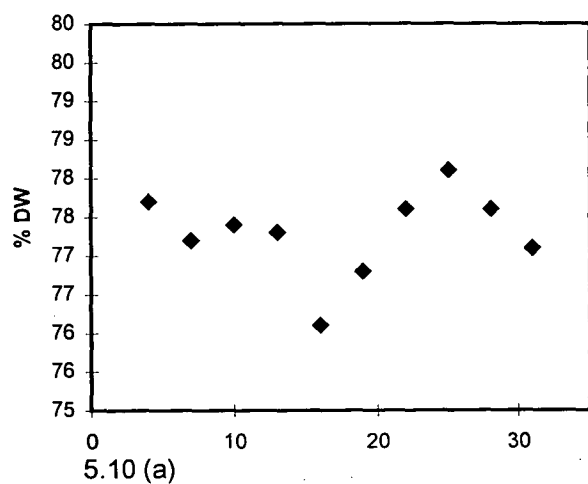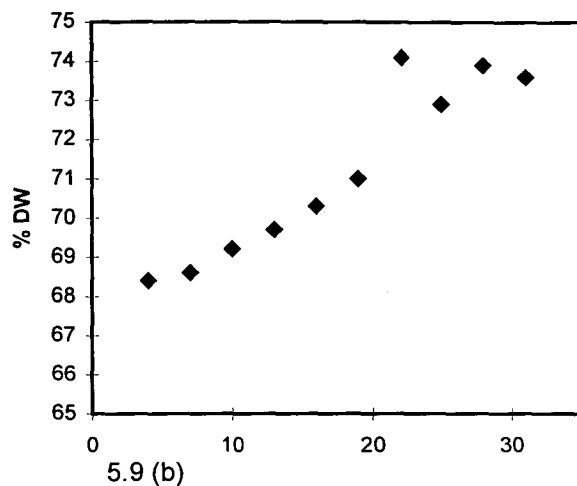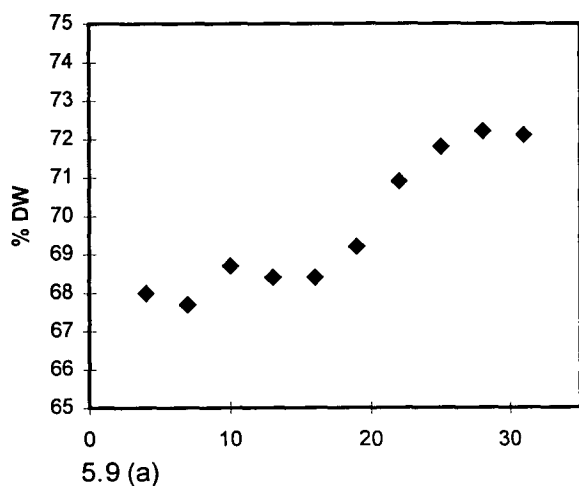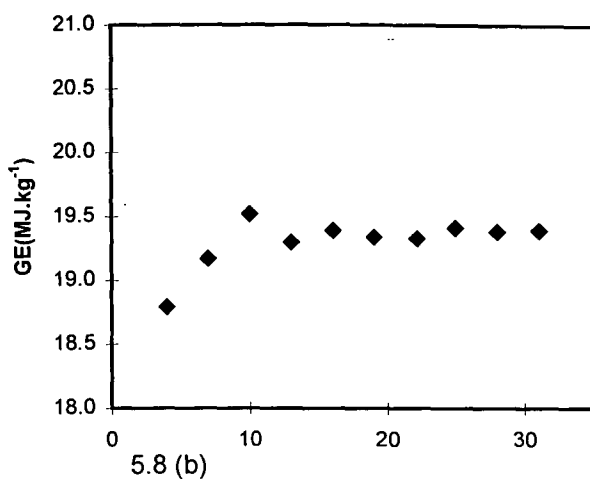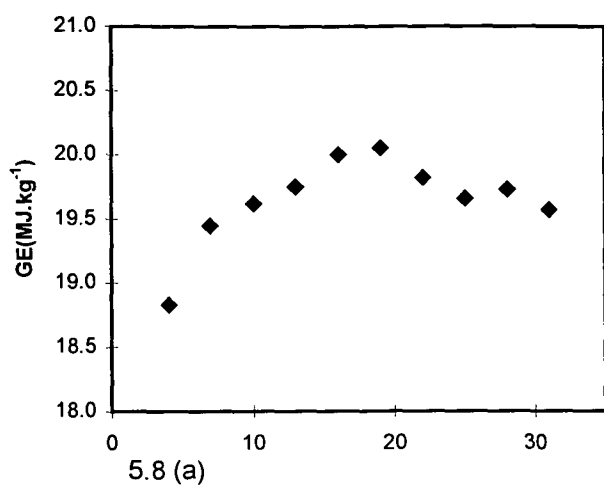
Fig. 5.8: Gross energy of abalone viscera. (a) Fed LE diets. (b) Fed HE diets.

Fig. 5.9: Moisture content of abalone foot muscle. (a) Fed LE diets. (b) Fed HE diets.

Fig. 5.10: Moisture content of abalone viscera. (a) Fed LE diets. (b) Fed HE diets.

Units of all X-axes are "percent dietary digestible protein".
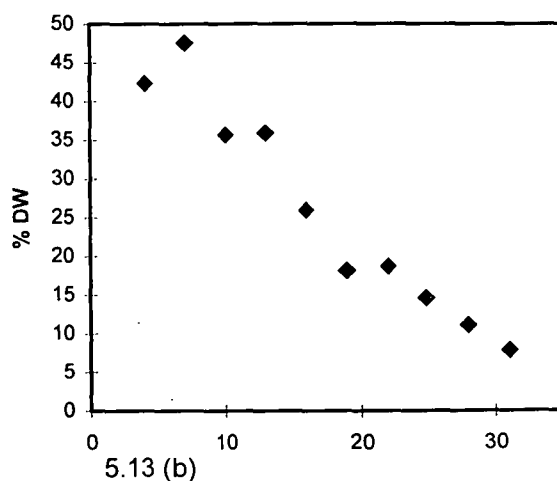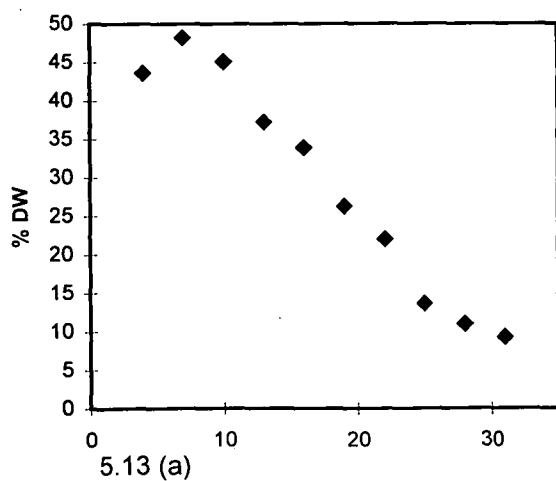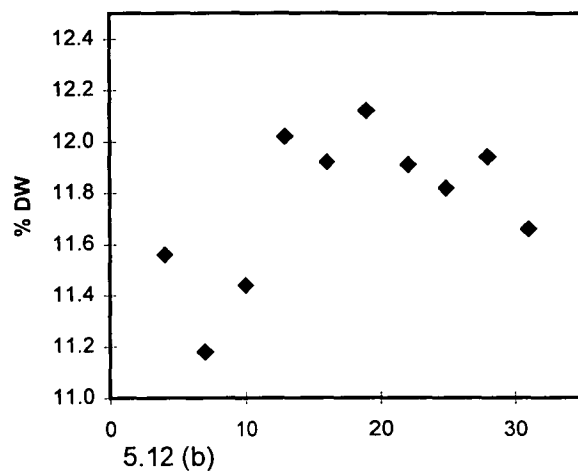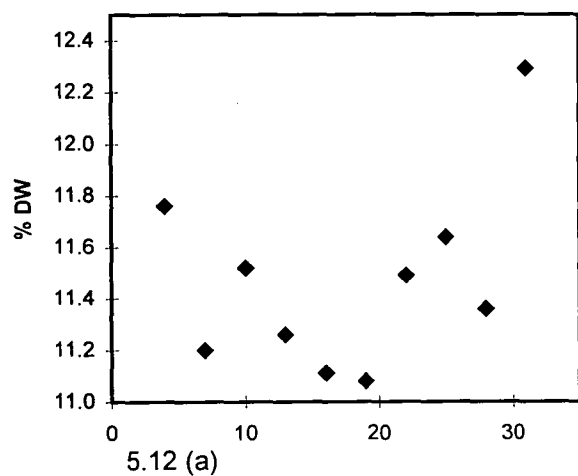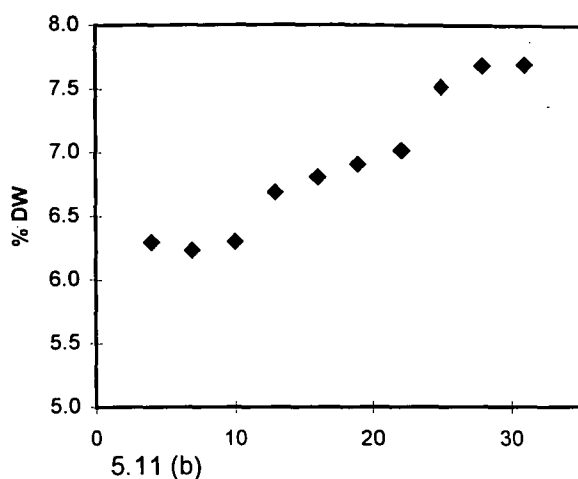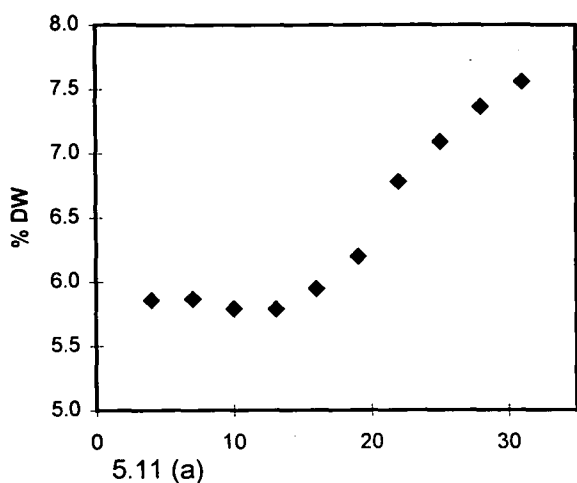
Fig. 5.11: Ash content of abalone foot muscle. (a) Fed LE diets. (b) Fed HE diets.

Fig. 5.12: Ash content of abalone viscera. (a) Fed LE diets. (b) Fed HE diets.

Fig. 5.13: Glycogen content of abalone foot muscle. (a) Fed LE diets. (b) Fed HE diets.

Units of all X-axes are "percent dietary digestible protein".
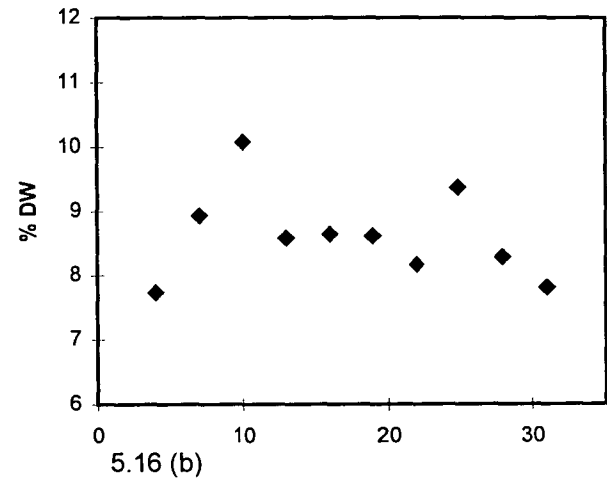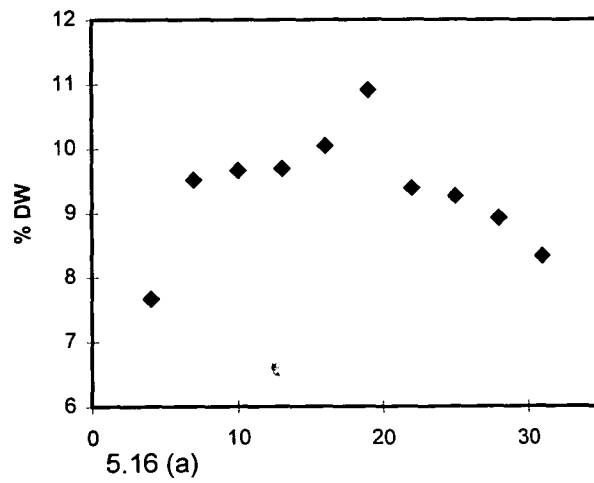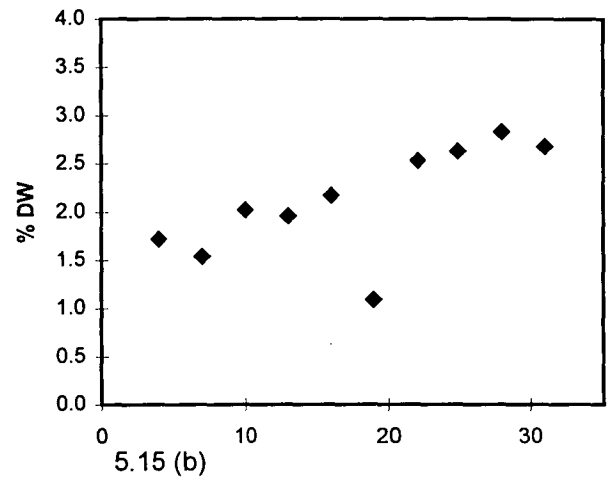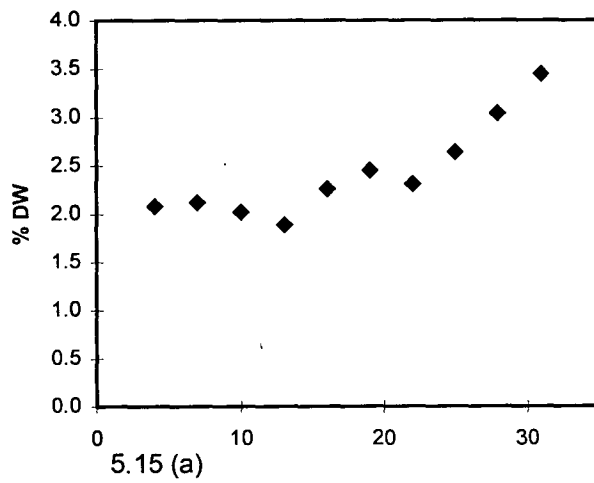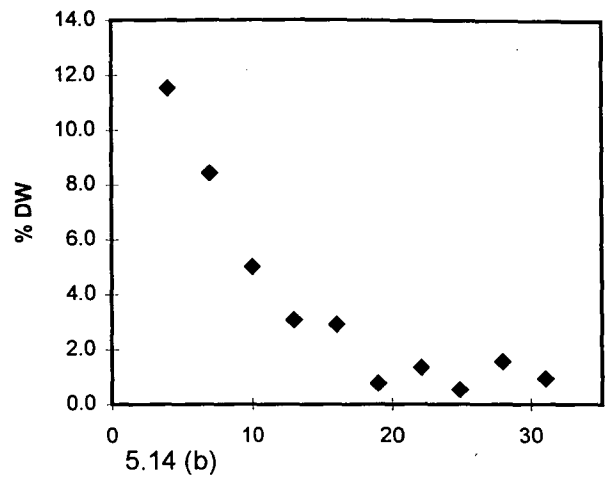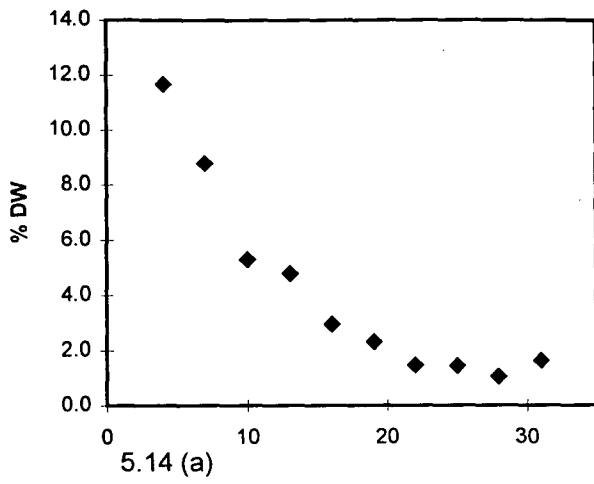
Fig. 5.14: Glycogen content of abalone viscera. (a) Fed LE diets. (b) Fed HE diets.
Fig. 5.15: Crude fat content of abalone foot muscle. (a) Fed LE diets. (b) Fed HE diets.
Fig. 5.16: Crude fat content of abalone viscera. (a) Fed LE diets. (b) Fed HE diets.
Units of all X-axes are "percent dietary digestible protein".

the 3.8 g.kg⁻¹DP diet to 13% for the 29.2 g.kg⁻¹DP diet (Fig. 5.17a). A polynomial function accurately described the relationship between retention and DP. Similarly, retention for abalone fed HE diets ranged from almost 80% (3.2 g.kg⁻¹DP) to 10% (24.6 g.kg⁻¹DP) (Fig. 5.17b).

Experiment II: digestibility

Mean protein digestibilities ($\pm$ se) of the 70, 190 and 280 g.kg⁻¹ DPLE diets were 84.7% ($\pm$0.72), 90.7% ($\pm$0.26) and 77.3% ($\pm$1.97), respectively. Mean protein digestibilities for the corresponding HE diets were 88.4% ($\pm$0.43), 81.4% ($\pm$0.79) and 77.5% ($\pm$1.88). (n = 6 for all treatments except 280 g.kg⁻¹ LE, where n = 5). There was no significant difference in mean protein digestibility between the HE and LE diets at the 70 and 280 g.kg⁻¹DP level ($P > 0.05$). However, protein digestibility at the 190 g.kg⁻¹DP level was significantly lower for the HE diet (81.4 $\pm$ 0.8%) than for the LE diet (90.7 $\pm$ 0.3%) ($P < 0.05$). The digestibility of the dietary protein (irrespective of energy or protein levels) was calculated using digestibility estimates and inclusion levels of individual ingredients (Chapter 4) and was found to be 80%.

Experiment III: diet change

When changed from experimental diet to commercial feed, abalone previously fed the 40 g.kg⁻¹, LE diet showed a significant increase in growth rate ($P < 0.001$). Abalone switched from the 70 g.kg⁻¹ LE diet to the commercial feed experienced a slight, non-significant increase in growth rate ($P > 0.05$). In contrast, abalone fed the 130 g.kg⁻¹ LE diet experienced a slight, non-significant reduction in growth rate ($P > 0.05$) when returned to the commercial diet (Fig. 5.18a). Abalone fed the 40, 70 and 130 g.kg⁻¹ HE diets all
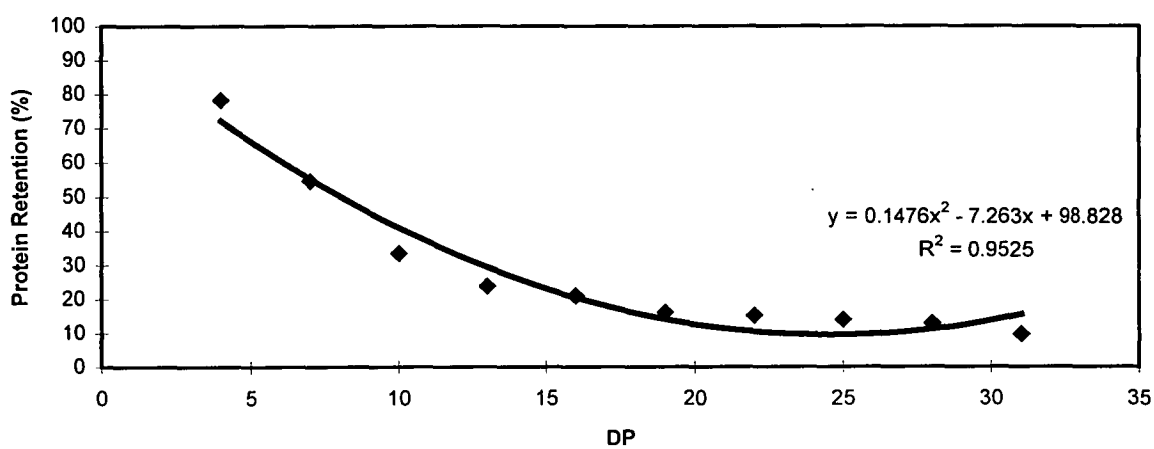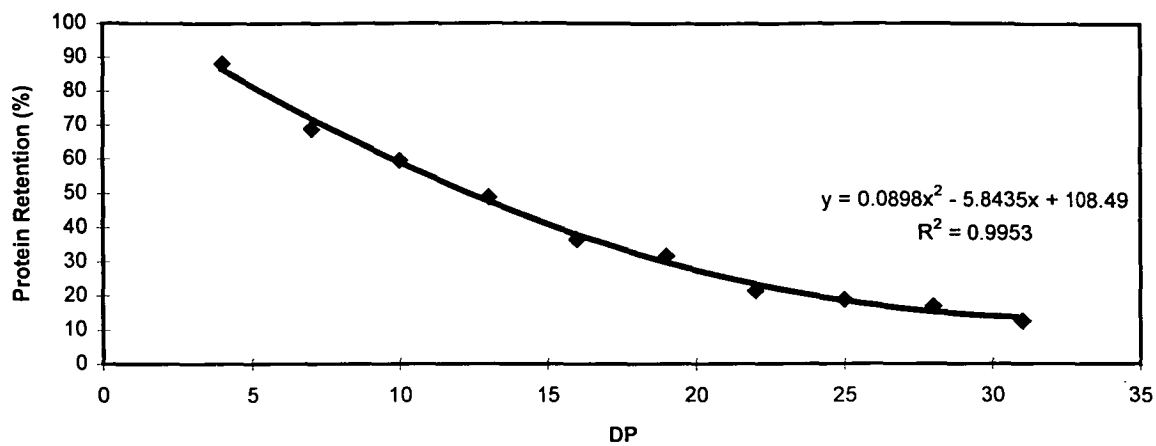
Fig. 5.17: Protein retention (%) calculated for abalone. (a) Fed LE diets. (b) Fed HE diets.

experienced a significant increase in growth rate when changed to the commercial diet (P < 0.001, P < 0.05, P < 0.001, respectively) (Fig. 5.18b).

5.4 Discussion

Experiment I: Growth Experiment

The optimal dietary DP:DE ratio of juvenile *H. laevigata* was evaluated by growth data in this experiment, where growth was expressed as SGR of whole body weight and was found to be 12.3 g DP.MJDE^{-1}. Unlike the use of criteria such as net protein utilisation and feed conversion efficiency, growth response avoids the necessity of having to determine the feed consumption, a difficult task in species such as abalone, as noted by previous workers in this area (Mai *et al.*, 1995b). Although protein gain may yield a more reliable estimate of protein requirement than growth, recent work has shown that protein gain is highly correlated with weight gain and yielded a very similar estimate (Mai *et al.*, 1995b). The dietary protein requirements of abalone have previously been estimated by both broken-line and quadratic regression models. Mai *et al.* (1995b) found the broken-line model gave a closer fit to the data from *H. discus hannai*, whereas the quadratic model was more suited to the data of *H. tuberculata*. In this study only the quadratic regression model was used. It predicted that maximal growth of *H. laevigata* occurs at a DP:DE ratio of 16.3 g DP.MJDE^{-1}, corresponding to a digestible protein level of 17.3% DP, when the dietary digestible energy content is 10.6 MJ.kg^{-1}, while reduction of the DP:DE ratio to 12.3 g DP.MJDE^{-1} will result in a 5% likelihood of growth rate reduction. As there are no other published data on the DP:DE ratio or DP requirements of abalone, these results can only be compared indirectly with other reports of crude protein requirements. If an average
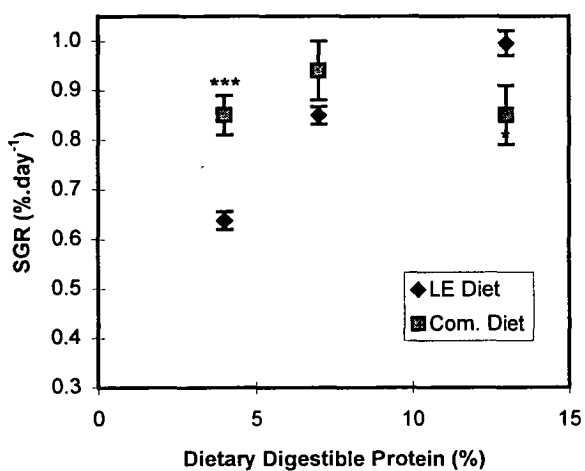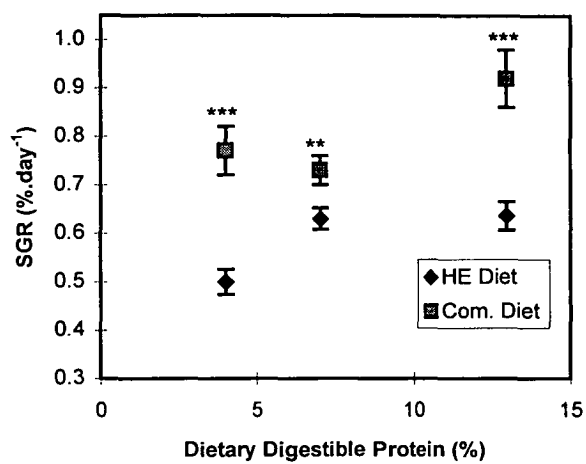
5.18 (a)   5.18 (b)

Fig. 5.18: Change in specific growth rate of abalone with change in diet (mean ± sem, n = 4).
(a) From LE diet to commercial diet. (b) From HE diet to commercial diet.
, * = Change significant at 0.05 and 0.001 level, respectively.

digestibility of 80% (Section 5.3) is assumed for protein sources in this diet, then these results equate to a dietary crude protein requirement of approximately 21.6%. This is similar to the estimate of 20% found for *H. discus* (Ogino and Kato, 1964) and falls at the lower end of the range (20-30%) recommended for *H. discus hannai* (Uki *et al.*, 1986b). It is below the range estimated by Mai *et al.* (1995b) for *H. tuberculata* (22.3-32.3%) and *H. discus hannai* (23.3-36.6%) and much lower than the minimum 30% reported by Taylor (1992) for *H. kamtschatkana* and 47% for *H midae* by Britz (1996). A DP requirement of 17.3% is relatively low for an aquaculture species but a precedent exists for *Penaeus vannamei* when appropriate protein/energy/amino acid balances have been achieved (Aranyakananda and Lawrence, 1993).

It is clear from the results presented here that *H. laevigata* grow significantly less well at a dietary energy level of 12.6 MJDE.kg^{-1}. In contrast, they approached a SGR of 1.0 at a dietary energy level of 10.6 MJDE.kg^{-1}. In the three diets that produced the fastest growth the DP:DE ratio ranged from 12.3-17.9 gDP.MJDE^{-1}. A previous estimate (Chapter 3) of 14.4 gDP.MJDE^{-1} falls well within this range. The mechanism by which the HE diet resulted in severe growth rate reduction could be explained if abalone eat to satisfy energy requirements and not protein. Abalone will reach energy satiation sooner consuming a high energy diet than a low energy diet. Thus, protein intake of abalone on a high energy diet may be much less than that of those on a low energy diet. This may result in faster growth of animals on lower energy diets. This hypothesis is difficult to reconcile with the statement by Fleming (1995), however, that abalone select foods that maximise the intake of nitrogen and that energy does not appear to play a major role in setting food preference. Another explanation for the reduction in growth rate noted in this experiment may be related to dietary lipid level. Almost all of the energy difference between LE and HE diets was due

to increased lipid content. Excess dietary lipid may reduce nutrient availability to the abalone by reducing protein digestibility. One group of researchers (Britz *et al.*, in press) have noted very low levels of lipase in the gut of *H. midae*, while van Barneveld *et al.* (1998) found that inclusion of fish oil above 60 g.kg^{-1} decreased the digestion of dietary N and amino acids, while inclusion of fish oil above 30 g.kg^{-1} decreased gross energy digestion. Van Barneveld *et al.* (1998) also concluded that oil type can influence the apparent faecal digestibility of N, amino acids and gross energy.

The relationship of muscle, viscera and shell to total weight was greatly affected by the protein content of the diet and, to a lesser extent, by the energy content. It is of commercial interest to note that the diets that produced fastest growth rate also yielded the highest percentage of foot muscle, were mid-range in viscera content, low in moisture and lowest in shell as a proportion of whole weight. Comparison of the protein content of the foot muscle of *H. laevigata* to that of *H. tuberculata* and *H. discus hannai* reported by Mai *et al.*, (1995b) is difficult as those workers did not separate foot muscle and viscera. The CP content of the foot muscle of our control animals was 67.1% (dw). At the conclusion of the experiment, the CP of those on the 3.8 g.MJDE^{-1} LE diet had declined to 44.2%, while those on the 29.2 g.MJDE^{-1} LE diet had increased to 70.6%. Similarly, those on the HE diets had decreased to 45.2% for those on the 3.2 g.MJDE^{-1} diet, and had increased to 72.3% for those on the 24.6 g.MJDE^{-1} diet. From this it could be deduced that our controls had been maintained on a diet of about 25% DP. This, in fact, was the case.

The carcass composition of the experimental animals was greatly affected by diet (Figs. 5.5-5.16). In general, foot muscle protein, moisture, and GE increased linearly as DP increased, while glycogen decreased linearly (except at very low dietary DP levels) and ash increased in curvilinear fashion for LE diets and linearly for HE diets. For abalone

viscera the trends were not as clear, except in the case of glycogen, that decreased in a curvilinear fashion as dietary DP increased. Gut contents are unlikely to have affected visceral composition as abalone were sacrificed three days after their final feeding.

The GE content of the foot muscle of animals fed diets of 100 and 130g DP.kg^{-1} for both LE and HE (18.0 and 18.1 MJ.kg^{-1}, respectively) is close to the value obtained for similar tissue from *H. rubra* by Thomas and Day (1995) (18.04± 0.25 MJ. Kg^{-1}). Unlike Thomas and Day (1995), however, this study showed that the viscera contained more energy than the foot muscle at all but the lowest protein levels. This may be a result of the abalone in this study being fed relatively high energy diets, compared to the animals investigated by Thomas and Day (1995) that had been fed macroalgae. Similarly, Fleming (1991) reported a GE content of 15.8 MJ.kg^{-1} for flesh of *H. rubra*, but these animals were maintained on a macroalgal diet.

We have found that the glycogen content of the foot muscle and viscera is heavily dependent on the diet of the abalone. It is therefore of little value to compare tissue glycogen levels measured here to those in the literature, unless the recent nutritional history of the animal is known. Greenlip abalone appear to be adept at converting excess dietary carbohydrate of manufactured feeds to glycogen and storing it in the foot and viscera. The findings in relation to foot muscle may be of special interest to seafood processors and growers alike, as glycogen has been shown by Olaechea *et al.* (1993) to be correlated with toughness in abalone meat. However, Carefoot *et al.* (1993) found no evidence that taste-testers could detect a difference between high- and low- glycogen abalone meat. Glycogen levels in abalone meat have previously been shown to vary greatly with species, location and time of year (Olley and Thrower, 1977).

Focusing on the composition of the fastest growing animals, it can be seen that the

animals fed diets containing 12.3 - 17.9 $gDP.MJDE^{-1}$ (LE) had mid-range levels of foot protein, glycogen and foot GE, low moisture and low ash. It is apparent that the diets that promote fast growth also avoid undesirable traits such as high moisture and high ash.

It is difficult to compare the protein retention results obtained here with those of Uki *et al.* (1986b) due to differences, for example, in species studied, diet formulation and dietary lipid levels. However, if one estimates the CP digestibility of the diets used by Uki *et al.* (1986b) as 85%, then the retention rate on the casein based diets reached a peak of 70% when abalone were fed the 95 $g.kg^{-1}$ CP diet (80 $g.kg^{-1}$ DP). This is close to the value obtained in this study for the diet containing 70 g $DP.kg^{-1}$ diet (LE). Values obtained by Uki *et al.* (1986b) then declined as dietary CP increased, to a low of 280 $g.kg^{-1}$ for the diet with 554 $g.kg^{-1}$ CP. Values obtained in the current experiment were below 30% for DP levels ≥ 220 g $DP.kg^{-1}$. Protein deposited in the shell was not included in this study.

Experiment II: digestibility

The digestibility of the dietary protein was estimated for diets with low (70), medium (190) and high (280 g $DP.kg^{-1}$) levels for both high and low energy diets. In the case of the 70 and 190 $gDP.kg^{-1}$ LE diets, and the 70 $gDP.kg^{-1}$ HE diet, digestibility of the protein of the whole diet was higher than the calculated value (80%) based on digestibility values of individual ingredients. This may indicate that some synergy or additive effect occurs in certain circumstances to enhance digestibility. The values obtained for the 280 $g.kg^{-1}$ LE and HE diets are very close to the calculated value and to each other. It would seem unlikely that the difference in SGR observed between abalone fed these diets is due to a difference in protein digestibility. More work is needed in this area, however, to clarify the mechanism of growth retardation.

Experiment III: diet change

Abalone from treatments 40, 70 and 130 g.kg^{-1} DP at both energy levels were returned to aquaria and fed a commercial diet. Animals previously fed HE diets showed a significant improvement in growth rate (P < 0.05). This demonstrates that the slowing of growth by high energy diets did not produce permanently stunted animals in this case. If the animals fed the 40 and 70 gDP.kg^{-1} HE diets were restricted in growth due to limited protein intake, then their growth should have improved when changed to the commercial diet, which in fact is what happened. The animals fed the 130 gDP.kg^{-1} HE may have been consuming feed until energy (not protein) satiation, leaving their protein intake inadequate to sustain optimal growth. On changing to the lower energy commercial diet, their intake rate may have increased, resulting in higher protein ingestion and faster growth, as observed.

Conversely, the animals fed the 40 gDP.kg^{-1} LE diet exhibited a significant increase (P < 0.001) in growth, when switched to the commercial feed (that contained higher GE than the LE diets). Assuming intake rates of the commercial diet were somewhat less than those of the LE diets, animals from the 40 g.kg^{-1} DPLE diet may have been over-compensated by the increased level of dietary protein, resulting in a net increase in growth rate. Animals from the 70 gDP.kg^{-1} LE diet would have been less affected, (they experienced a slight, non-significant increase in growth) and animals from the 130 gDP.kg^{-1} LE diet may have been ingesting less protein on the commercial diet than during the experiment, resulting in a slight reduction in growth rate. This is exactly what was observed. Unfortunately, feed consumption was not recorded for this experiment.

5.5 Conclusions

Relatively low protein, low energy diets promote fast growth in *H. laevigata*. Excess dietary protein and energy (as lipid) significantly reduces growth. The mechanism underlying this diet-related growth reduction is the subject of ongoing investigation, but preliminary work in this experiment (Experiment II) showed that the digestibility of dietary protein in the HE diets decreased as dietary protein level increased. This may help explain some of the growth reduction observed. The hypothesis that abalone eat to satisfy energy and may thus ingest less protein on high energy diets cannot be ruled out. More research on this topic is needed to explain these observations. Growth retardation is not permanent and abalone return to normal growth rates if suitable feed is provided. Carcass partitioning revealed that diets promoting the fastest growth resulted in a higher proportion of foot muscle, lower viscera and less shell than other diets. Analyses of meat and viscera showed that diet significantly affected proximate composition. Low protein diets particularly promoted glycogen deposition. These findings suggest that specialised diets, aimed at manipulating certain characteristics of abalone flesh, may have a future in abalone aquaculture. Further research is required on the potential of this strategy.

CHAPTER 6

THE DIETARY REQUIREMENT FOR LYSINE OF GREENLIP ABALONE (*Haliotis laevigata*) DETERMINED USING COVALENTLY BOUND AND FREE AMINO ACID SOURCES

6.1 Introduction

There are no published estimates of the quantitative requirements of abalone (*Haliotis* spp.) for any of the essential amino acids (EAA's) as defined by Allen & Kilgore (1975). Estimates of lysine requirement available for finfish range from 3.7-5.7 (% of dietary protein) with most values close to 5%. These reports are summarised in D'Mello (1994). Reports concerning invertebrates are far fewer, one such being Fox *et al.* (1995,a) who estimated the lysine requirement of *Penaeus vannamei* at 4.67% of dietary protein when the diet contained 45% crude protein and 4.49% at a crude protein content of 35%, when the lysine supplement was covalently bound to the protein. When L-lysine-HCl was used as a supplement in diets of 35% crude protein, the dietary requirement increased to 5.19%.

Most studies evaluating EAA requirements use the dose-response method, whereby a series of diets are formulated to contain increasing levels of the EAA under scrutiny. Diets commonly contain the EAA in the range from 0% to 10% of dietary protein, and all other EAA's are present in the same proportions as in the soft tissue of the animal. Studies involving finfish routinely use crystalline EAA's both to increase the level of the EAA under investigation and to supplement others to slight excess (Tibaldi and Lanari, 1991). Experiments of this design rely on growth as an indication of response, and typically result

in a "broken stick" plot, in which the growth rate of the fish increases until the optimal dietary EAA level is attained. Ingestion of diets containing higher levels of the EAA do not result in increased growth.

Studies that rely on the use of crystalline EAA's may encounter methodological problems such as reduced availability of EAA's due to leaching and differential uptake kinetics. For example, Deshimaru (1981) reported that prawns were able to deposit less than 1% of free amino acids, indicating the seriousness of these problems. One possible solution is to microencapsulate the EAA in lipid to impede its tendency to dissolve. The usual composition of such microencapsulated AA is 20% AA and 80% lipid. Unfortunately, recent results (see Chapter 5 and van Barneveld et al., 1998) have indicated that the growth rates of abalone are depressed when dietary lipid levels exceed about 5%. This effectively precludes the use of lipid microencapsulated AA, as supplementation of the EAA would exceed 5% dietary lipid and depress the growth rate. Another strategy is to covalently enrich a protein source with the EAA under investigation, using the methods of Fox et al. (1995a,b). Covalent enrichment of intact protein reduces leaching of the EAA under investigation to a minimal level. In addition, absorption of the EAA across the gut wall is assumed to be at a similar rate to other intact EAA's.

The aim of this experiment was to investigate the requirement of abalone for lysine because it is commonly the most-limiting EAA in diets based largely on wheat protein. Rather than relying entirely on the results of one method, in this experiment the lysine requirement of abalone was estimated using diets supplemented with free lysine and also diets enriched with covalently bound lysine. This allowed a comparison of the effectiveness of both methods.

6.2 Materials and Methods

Abalone

Juvenile greenlip abalone (*Haliotis laevigata*) were obtained from a local farm (South Australian Abalone Developments, Pty. Ltd.) for this experiment. These animals had been raised on diatoms until about 10 mm in length, when they were weaned onto a commercially available growout diet. The animals were held for one week in a flow-through holding tank and fed their usual commercial diet. Abalone were size-graded prior to selection for the experiment to reduce the variance as much as possible. Details of the animals prior to the experiment can be seen in Table 6.1.

Feed formulation and manufacture

Free-lysine diets ("FL" Diets)

A basal diet (Table 6.2) was formulated using wheat gluten and semolina (Allied. Milling Co., Adelaide, South Australia) as the only protein sources. Lysine was presumed to be limiting in the basal diet. Feed-grade lysine HCl (Vedan Enterprises Co., Dông Nai Province, Vietnam) was added in increasing amounts to create a series of diets with lysine making up an increasing proportion of the protein (Table 6.2). Diatomaceous earth was removed as lysine-HCl was added. Diets contained 0.5% chromic oxide as a digestibility marker, although, due to time constraints, no digestibility work was carried out. Diets were produced in the form of ribbon-like strips using a pasta machine. The ratio of each EAA to lysine was greater in the feed than in the soft body tissue of abalone (Table 6.3), based on earlier analyses (see Table 2.1).

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Table 6.1: Initial body weight data of abalone

Series	N (mortalities excluded)	Mean Body Weight ± se (g)
Lysine-HCl + Controls	358	0.98 ± 0.01
Covalently-Enriched	313	1.01 ± 0.01

Table 6.2: Formulation of free-lysine (FL) diets (g.kg^{-1})

Ingredient	Basal Diet	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8
Pre-gelled starch	405	405	405	405	405	405	405	405	405
Wheat gluten	300	300	300	300	300	300	300	300	300
Semolina	160	160	160	160	160	160	160	160	160
Diatomaceous earth (filler)	105	102.7	100.6	98.2	96.2	93.8	91.7	89.6	87.2
Fish oil	20	20	20	20	20	20	20	20	20
Chromic oxide	5	5	5	5	5	5	5	5	5
Vitamin premix	3	3	3	3	3	3	3	3	3
Mineral premix	2	2	2	2	2	2	2	2	2
Lysine-HCl	0	2.3	4.4	6.8	8.8	11.2	13.3	15.4	17.8
Total	1000	-	-	-	-	-	-	-	-

Covalently enriched gluten diets ("CEG" Diets)

A representative quantity of wheat gluten was sent to the Department of Food Science, The University of British Columbia, Canada, where the gluten was covalently enriched with lysine using the following method:

Wheat gluten was solubilized by the acid hydrolysis method of Wu *et al.* (1976) with slight variations. Either 0.1 or 0.2 M HCl was added to wheat gluten while stirring to make a 5% solution which was autoclaved at 124°C for 15 min. The suspension was cooled, the pH adjusted to 4.5 using 10M NaOH, and the supernatant decanted after standing. The precipitate was washed twice with ice cooled distilled water (adjusted to pH 4.5 with HCl) by centrifuging at 1100G and 7°C for 10 min. The pellet was resuspended in water (90% of the original volume) and adjusted to pH 6.0 using 10M NaOH.

Lysine enrichment of the wheat gluten was carried out according to Fox *et al.* (1995a) with minor variations (the amounts shown in brackets represent those for the trials / large-scale). To a slowly stirred solution of acid solubilized wheat gluten (10 mL / 4.7L) was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma, E-7750) (0.095g / 49.6g), *N*-hydroxysuccinimide (Sigma, H-7377) (0.025g /13.2g) and feed grade lysine or *L*-lysine HCl (Sigma, L-5626) (0.35g / 182.8g). The mixture was adjusted to pH 6.0 at room temperature and gently stirred for 4 hr. The reaction was terminated with glacial acetic acid to pH 2.5. The precipitates at pH 2.5, 4.5 and 8.5 were collected by centrifugation at 1100G and 7°C for 10 min following stepwise adjustment of the pH using 10 M NaOH. Pellets were resuspended in distilled water (<20% solution),

Table 6.3: Amino acid content of normal and covalently-enriched wheat gluten (g.kg^{-1})

and the ratio to lysine (RTL) of amino acids in abalone soft tissue and in the basal

diet.

Amino Acid	Gluten	Enriched Gluten	Abalone RTL	Basal Diet RTL
lys*	11.4 ± 0.3	49.0 ± 0.42	1.00	1.00
arg*	26.0	25.2 ± 0.04	1.46	2.28
his*	14.4	14.1 ± 0.64	0.27	1.26
ile*	22.5 ± 1.8	27.0 ± 0.03	0.75	1.97
leu*	46.4 ± 1.4	51.7 ± 0.25	1.11	4.07
met*	12.1	ND	0.29	1.06
phe*	35.4	33.7 ± 0.06	0.62	3.11
thr*	19.7 ± 1.0	18.9 ± 0.13	0.80	1.73
trp*	ND	ND	0.32	0.53
val*	24.2 ± 1.3	25.5 ± 0.42	0.86	2.12
ala	11.0 ± 0.6	13.1 ± 0.06	0.94	0.96
asp	24.5 ± 0.9	30.5 ± 0.10	1.59	2.15
cys	16.8	ND	0.26	1.47
glu	272.9	244.0 ± 0.18	2.60	23.94
gly	25.3 ± 3.8	19.5 ± 0.14	1.46	2.22
pro	79.7	63.0 ± 0.31	0.92	6.99
ser	35.7 ± 3.5	30.9 ± 0.01	0.89	3.13
tyr	25.1	18.1 ± 0.06	0.52	2.20

* = EAA. ND = not determined. Values are means (± se). Values for gluten were derived from one pre-oxidised and one normal acid hydrolysis sample (n=2). N = 1 for Met and Cys. Values without errors are from one sample only either oxidised or normal hydrolysis. Trp values estimated from literature. Values for enriched gluten are from two samples subjected to normal hydrolysis.

Table 6.4: Percentage lysine in original, acid hydrolysed and trial lysine enriched wheat gluten, determined from single analysis.

Wheat Gluten	Lysine (%)
Original	1.15

Acid Hydrolysed	
0.1 M HCl	1.16
0.2 M HCl	1.45

Lysine enriched gluten prepared from 0.1 M HCl hydrolysed gluten using:

	Feed Grade Lysine	L-lysine HCl
pH 2.5 fraction	5.36	5.37
pH 4.5 fraction	5.77	5.40
pH 8.5 fraction	7.16	7.32

Lysine enriched gluten prepared from 0.2 M HCl hydrolysed gluten using:

	Feed Grade Lysine	L-lysine HCl
pH 2.5 fraction	5.93	7.12
pH 4.5 fraction	-	6.48
pH 8.5 fraction	7.14	-

dialyzed (6000 - 8000 MW) at 4°C against running water for 24 hr and distilled water for

12 h, and then freeze dried. The protein fraction precipitated at pH = 2.5 (Table 6.4) was

blended with unenriched gluten and used to make a series of diets of increasing lysine level,

but with essentially unchanged levels of other EAA's. The series of diets ranged from 0%

enriched gluten (the basal diet) to 80% enriched gluten.

The two gluten fractions were sifted three times together, then added to a mixture

of all other dietary ingredients that had been previously prepared. The resulting dry mix

was thoroughly blended, then water was added and further mixing took place. The resultant

dough was rolled into a cylinder by hand and sliced into thin sections with a razor blade.

The sections were dried at 55 °C in a forced-draught oven overnight. All feeds were kept

refrigerated until used. Samples of each feed were analysed for crude protein and amino

acids as described in Chapter 3, with the exception that a coefficient of 5.75 was used to

convert "%N" values to crude protein instead of 6.25. The former value was used as it

more closely reflects the relationship between the nitrogen content of the feedstuff and the

protein content, as gluten is high in glutamic acid. The relatively lower molecular weight

of glutamic acid results in a lower coefficient (H. Bremner, Pers. Comm.). Moisture

content was calculated by freeze-drying feed samples.

Treatments

The 17 treatments (two series of eight diets, plus control) were each fed to four

replicate tanks of 10 abalone. Abalone were tagged and weighed as described in Chapter

3 and randomly assigned to treatments. There was no significant difference in weight

between replicates of each series at the beginning of the experiment (P > 0.05). Tanks were

randomly distributed.

Aquarium system

The aquarium system is described in Chapter 3. In this experiment the flow rate was 0.2 L.min^{-1} and air was supplied via a single airstone at 0.5 L.min^{-1}.

Feed Consumption

On six occasions during the experiment, uneaten feed was carefully removed from the CEG aquaria when new feed was due to be given, oven-dried overnight at 55 °C and weighed. These data were used to estimate feed consumption. Uneaten feed was collected in the same manner on seven occasions for the FL diet treatments.

Carcass Partitioning

This was carried out as described in Chapter 5.

Leaching

Feed from the basal diet and each FL diet treatment was weighed and placed into six replicate aquaria with similar water and air flow rates to those containing abalone. After six hours, the feed was removed, oven-dried, and dry matter loss was recorded. (Six hours was chosen as abalone are assumed to have completed feeding within this time period.) The feed samples were then pooled, milled, and a representative sample analysed for amino acid content using the methodology described in Chapter 3.

Statistical analysis

Gain in body weight was expressed as specific growth rate (SGR) calculated using the formula given in Chapter 3. Lysine requirements of the abalone were estimated from

weight gain using the second-order polynomial regression analysis model (Lovell, 1989). All statistics were calculated using Systat® (1992).

6.3 Results

Gluten enrichment

Amino acid analysis of the trial lysine enriched wheat gluten using HPLC revealed little difference in lysine content when either 0.1 or 0.2 M HCl were used in acid solubilization or when either feed grade lysine or *L*-lysine HCl were used in the lysine enrichment reaction (Table 6.4). (Statistical analysis of these results was not possible as only single samples were analysed.) Hence, the large-scale reaction was carried out using feed grade lysine and wheat gluten (261 g) solubilized with the milder 0.1 M HCl, yielding 99.24, 33.18 and 29.10 g of the pH 2.5, 4.5 and 8.5 fractions, respectively. Overall yield of the reaction was 61.9%.

The lysine content of the large-scale lysine enriched wheat gluten was increased 4.6-, 4.3- and 5.9-fold over that of the original gluten, for the pH 2.5, 4.5 and 8.5 fractions, respectively (Table 6.5). These were similar to or lower than the 6.2-fold increase achieved in the pH 8.5 fraction of the initial trial using 0.1 M HCl and *L*-lysine HCl (Table 6.4). Examination of Table 6.5 reveals that the amino acid composition of the enriched gluten fractions did vary when compared to the unenriched gluten. However, these changes (eg, proline, glycine and tyrosine) were greatest for the pH 8.5 fraction, which was not used to make experimental diets. Comparison of the unenriched gluten to the pH 2.5 fraction reveals few changes, perhaps the most noteworthy are isoleucine and leucine. Data of the dry matter, crude protein and amino acid content of each CEG diet can be seen in Table 6.6. Dry matter content of feed was typically 96-98%, with the basal diet having the lowest dry

matter content of 93.9%. The lysine content of CEG diets ranged from 4.75 g.kg^{-1} for feed containing 10% enriched gluten to 12.25 g.kg^{-1} for feed with 80% enriched gluten. Diets containing 50% and 60% enriched gluten contained a lower lysine content, as a percentage of protein, when analysed, than anticipated. In contrast, diet 40% CEG returned a higher-than anticipated lysine content. Generally, the (non-lysine) amino acid content of the CEG diet series did not change appreciably across the series, with the exception that the arginine content of CEG 10% diet (5.58 g.kg^{-1}) was considerably lower than that for other enriched diets (mean ± sem = 9.01 g.kg^{-1} ± 0.39) and that the tyrosine content of CEG diet 60% was considerably lower (4.44 g.kg^{-1})than that for other diets in the series (Table 6.6). ·

The lysine content of the FL diets ranged from 3.23 g.kg^{-1} for the basal diet to 10.22 g.kg^{-1} for diet 8 (Table 6.7).

Table 6.5: Average amino acid composition (%) of the unenriched wheat gluten and the pH 2.5, 4.5 and 8.5 fractions of the large scale lysine enriched wheat gluten, determined from duplicate analyses.

Amino Acid	Unenriched Wheat Gluten	pH 2.5 Fraction	pH 4.5 Fraction	pH 8.5 Fraction
Cys	1.68	NR	NR	NR
Asp	2.45	3.28	2.00	2.84
Met	1.21	NR	NR	NR
Thr	1.97	2.03	1.65	1.52
Ser	3.57	3.33	3.49	2.35
Glu	27.29	26.23	22.33	25.61
Pro	7.97	6.78	5.68	4.22
Gly	2.53	2.09	1.55	1.59
Ala	1.10	1.41	1.12	1.02
Val	2.42	2.74	2.42	2.13
Ile	2.25	2.90	2.36	2.25
Leu	4.64	5.56	4.36	4.28
Tyr	2.51	1.95	1.38	1.50
Phe	3.54	3.63	2.87	3.34
Lys	**1.14**	**5.26**	**4.95**	**6.75**
His	1.44	1.51	1.13	1.28
Arg	2.60	2.71	1.97	1.32

NR = Values for these amino acids not reported as the samples were not pre-oxidised. Tryptophan values were not reported.

Table 6.6: Amino acid profile (g.kg^{-1}), crude protein (%), lysine as a percentage of crude protein and dry matter content of CEG diets reported from duplicate analyses

AA	10%	20%	30%	40%	50%	60%	70%	80%
Cys	ND	ND	ND	ND	5.43	ND	3.94	5.37
Asp	7.01	7.06	6.80	6.70	7.27	5.79	6.29	7.73
Met	ND	ND	ND	ND	4.23	ND	3.89	3.76
Thr	6.28	6.49	6.64	5.45	6.58	5.21	6.61	6.34
Ser	10.63	10.59	10.60	10.05	10.13	9.56	8.93	10.73
Glu	86.72	84.29	84.33	86.12	80.65	70.01	74.59	82.03
Pro	23.66	23.26	23.47	25.23	22.80	18.98	17.77	19.16
Gly	7.41	8.02	7.31	7.65	6.89	5.27	5.54	6.42
Ala	3.63	4.02	4.12	3.80	3.82	3.91	3.52	4.01
Val	8.56	9.09	8.84	9.20	9.56	8.19	7.44	9.62
Ile	8.45	9.51	10.00	9.26	9.27	9.01	9.48	9.29
Leu	14.22	15.14	14.50	16.10	15.29	13.62	15.30	16.36
Tyr	6.74	6.47	7.36	7.75	6.81	4.44	6.74	5.92
Phe	11.74	11.81	11.19	12.46	11.92	7.30	11.43	9.13
His	4.87	5.18	5.32	5.97	5.95	3.85	5.01	3.97
Arg	5.58	8.75	8.62	9.70	10.28	8.81	9.62	7.32
Lys	**4.75**	**5.94**	**7.18**	**8.91**	**8.78**	**9.28**	**11.03**	**12.25**
CP (%)	21.94	22.71	23.05	23.46	23.92	23.60	23.92	24.47
% CP	2.16	2.62	3.11	3.80	3.67	3.93	4.61	5.00
% DM	96.0	96.6	96.9	97.0	96.9	96.9	96.3	95.9

Survival and growth

Mortality was low; a total of eight animals died during the course of the

experiment. These mortalities, all during the first week of the experiment, were presumed due to the stress of tagging, measurement and transfer. Growth rate was significantly affected by L-lysine-HCl supplementation ($P < 0.001$) and increased linearly (Fig. 6.1). The lowest growth rate (SGR = 0.384 ± 0.039) was recorded for abalone fed the basal diet with no lysine supplementation. The fastest growth rate was recorded for abalone fed the diet with the highest level of lysine (SGR = 0.879 ± 0.087; Fig. 6.1). There was no significant difference in growth rates between diet FL1 and FL7 (Table 6.8).

Growth rate was also significantly affected by supplementation with covalently-bound lysine ($P < 0.001$) in a curvilinear fashion (Fig. 6.2). The highest growth rate (1.134 ± 0.052) was recorded for abalone fed the diet containing 50% lysine-enriched gluten (lysine = 3.67% of dietary protein; Fig. 6.2),), although growth rates did not differ significantly between diets CEG 30% and 80% (Table 6.8).

Fig. 6.1

$y = 0.1546x + 0.1912$

$R^2 = 0.6225$

$y_{opt} = 0.89$

$y = -0.1206x^2 + 0.942x - 0.784$

$R^2 = 0.815$

$x_{opt} = 2.7\%$

$x_{max} = 3.9\%$
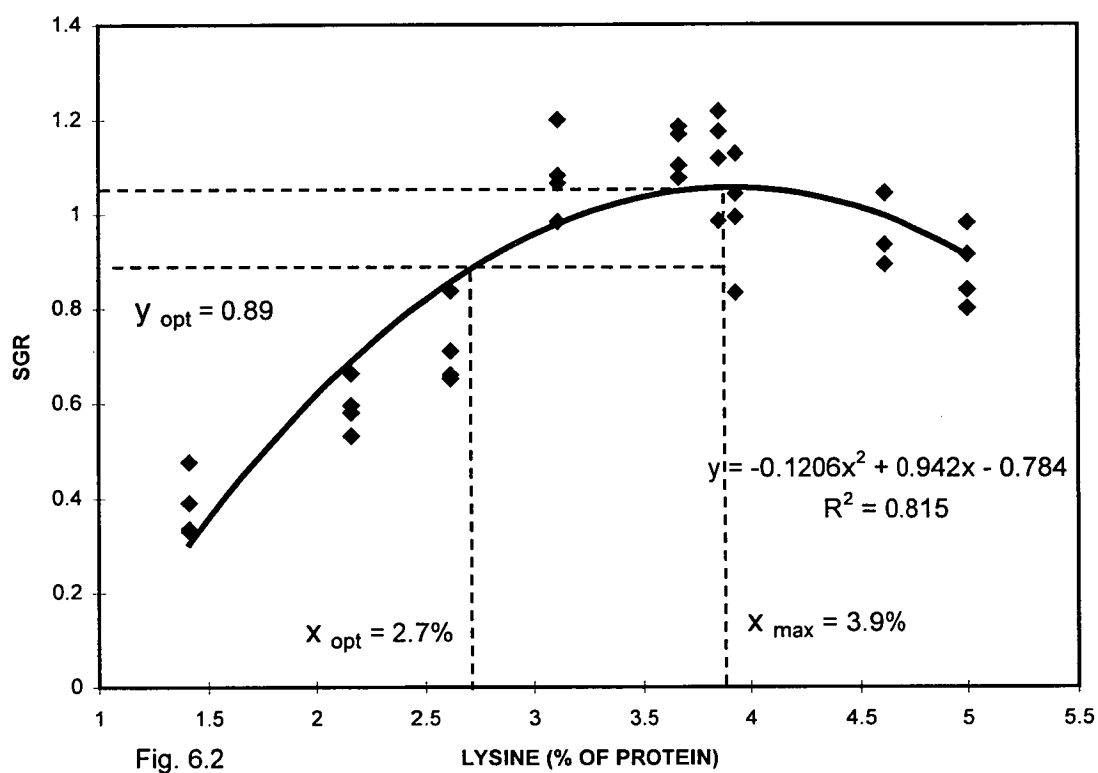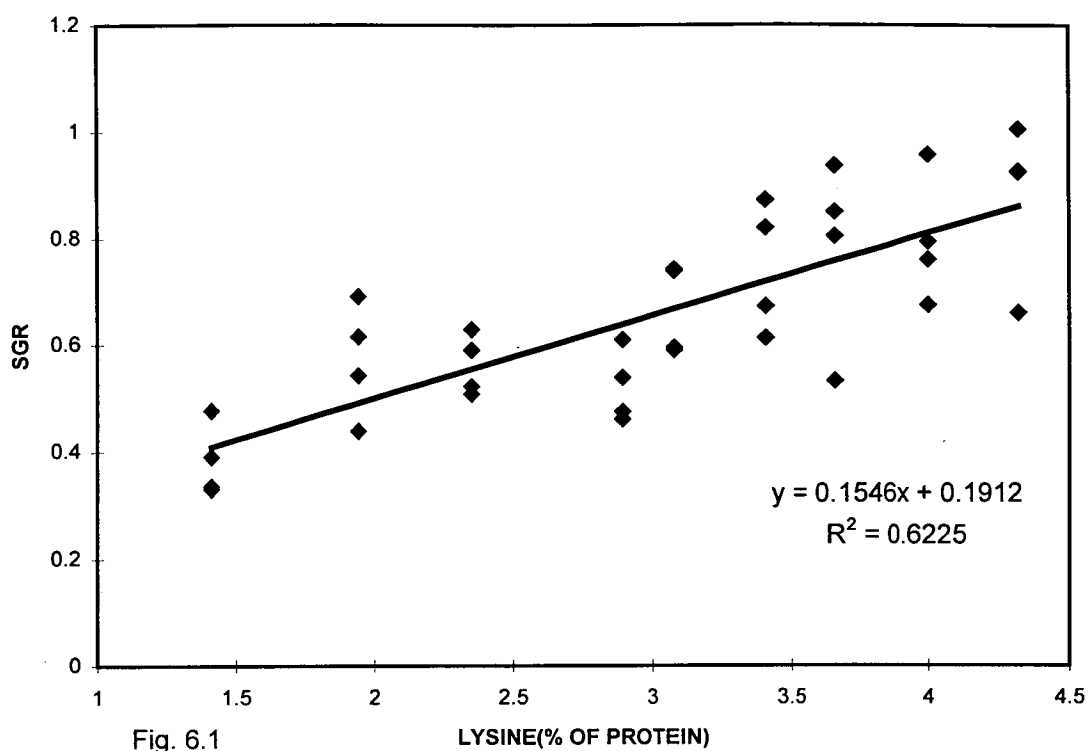
Fig. 6.2

Fig. 6.1: SGR of abalone fed diets supplemented with lysine-HCl.

Fig. 6.2: SGR of abalone fed diets supplemented with covalently-bound lysine.

Table 6.7: Amino acid profile (g.kg⁻¹), crude protein (%), lysine (percentage of crude protein) and dry matter content of FL diets

AA	Basal Diet	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8
Cys	5.11	ND	ND	ND	ND	ND	ND	ND	ND
Asp	8.74	6.60	6.32	6.21	6.93	6.21	6.12	5.89	5.88
Met	3.24	ND	ND	ND	ND	ND	ND	ND	ND
Thr	5.87	5.51	5.77	5.14	6.14	5.52	5.31	5.19	5.11
Ser	10.40	10.06	9.74	8.86	9.93	10.16	9.40	9.34	9.27
Glu	82.61	77.83	78.59	68.72	81.07	77.48	75.26	73.07	72.26
Pro	22.00	21.49	21.19	20.04	20.25	22.02	21.60	20.05	18.87
Gly	7.25	6.88	6.82	5.88	7.20	6.54	6.32	5.67	6.03
Ala	3.84	4.19	3.97	3.52	4.15	3.65	3.83	3.44	3.48
Val	8.68	8.86	8.65	7.77	9.31	8.69	8.08	7.59	7.93
Ile	8.59	8.84	8.76	8.31	8.89	8.17	8.03	7.49	7.64
Leu	14.54	13.43	13.52	12.19	14.26	14.02	13.03	12.48	12.27
Tyr	3.00	4.95	5.63	4.62	5.69	5.61	5.07	5.16	4.58
Phe	7.29	8.62	8.60	7.95	9.06	9.25	8.03	7.82	7.59
His	3.49	3.60	3.65	3.49	3.93	3.51	3.52	3.20	3.31
Arg	5.70	6.61	6.84	7.00	7.72	6.81	5.88	6.00	5.97
Lys	3.23	4.20	5.24	6.53	6.97	7.81	8.56	9.25	10.22
CP (%)	22.86	21.66	22.26	22.61	22.62	22.89	23.36	23.11	23.64
% CP	1.41	1.94	2.35	2.89	3.08	3.41	3.66	4.00	4.32
% DM	93.9	98.4	96.5	98.6	95.0	96.5	96.1	96.6	96.8

AA = Amino acid; ND = Not determined (as the sample had not been pre-oxidised); CP = Crude protein
FL1-8 = Free lysine diets 1 – 8; DM = Dry matter

Table 6.8: Specific growth rate (mean ± se, n = 4) of abalone fed diets supplemented with free-lysine or with covalently-bound lysine

| | SUPPLEMENT | | | |
| | FL | | CEG | |
DIET	SGR	SEM	SGR	SEM
Basal	0.384^a	0.039	0.384^a	0.039
1	0.572^{ab}	0.062	0.593^b	0.054
2	0.562^{ab}	0.033	0.715^b	0.086
3	0.521^{ab}	0.039	1.084^c	0.089
4	0.666^{bc}	0.049	1.124^c	0.101
5	0.744^{bc}	0.070	1.134^c	0.052
6	0.781^{bc}	0.101	1.000^c	0.124
7	0.796^{bc}	0.068	0.979^c	0.077
8	0.879^c	0.087	0.885^{bc}	0.080

FL = Free lysine; CEG = Covalently enriched gluten; SGR = Specific growth rate;

SEM = Standard error of mean for 4 observations

Values with the same superscript are not significantly different ($P > 0.05$)

Carcass Partitioning

Meat:shell ratios (MSR) were calculated for all treatments. There was no relationship between MSR and dietary lysine level for animals fed FL diets (Fig. 6.3). In contrast, the relationship between MSR and dietary lysine level for animals fed CEG diets were described by a quadratic function (Fig. 6.4).

Feed Consumption

Control abalone consumed significantly less feed than all other treatments ($P <$ 0.001). Feed consumption increased significantly in linear fashion across the series of FL diets ($P > 0.05$) (Fig. 6.5). In contrast, feed consumption for abalone fed CEG diets (Fig. 6.6) did not differ significantly across the series ($P > 0.05$).

Leaching Losses

Lysine loss from the basal diet after immersion for six hours was moderate (13.3%), but losses from the diets supplemented with lysine HCl ranged from 33.7-54.5% of total lysine (Table 6.9). Lysine loss from the CEG diets was not investigated, but could be expected to be similar to that of the basal diet, as the lysine in both cases is an integral part of the protein.

Fig. 6.3

LYSINE AS % OF DIETARY PROTEIN

$$y = -0.0189x^2 + 0.1319x + 0.5507$$
$$R^2 = 0.6569$$

Fig. 6.4

LYSINE AS % OF DIETARY PROTEIN
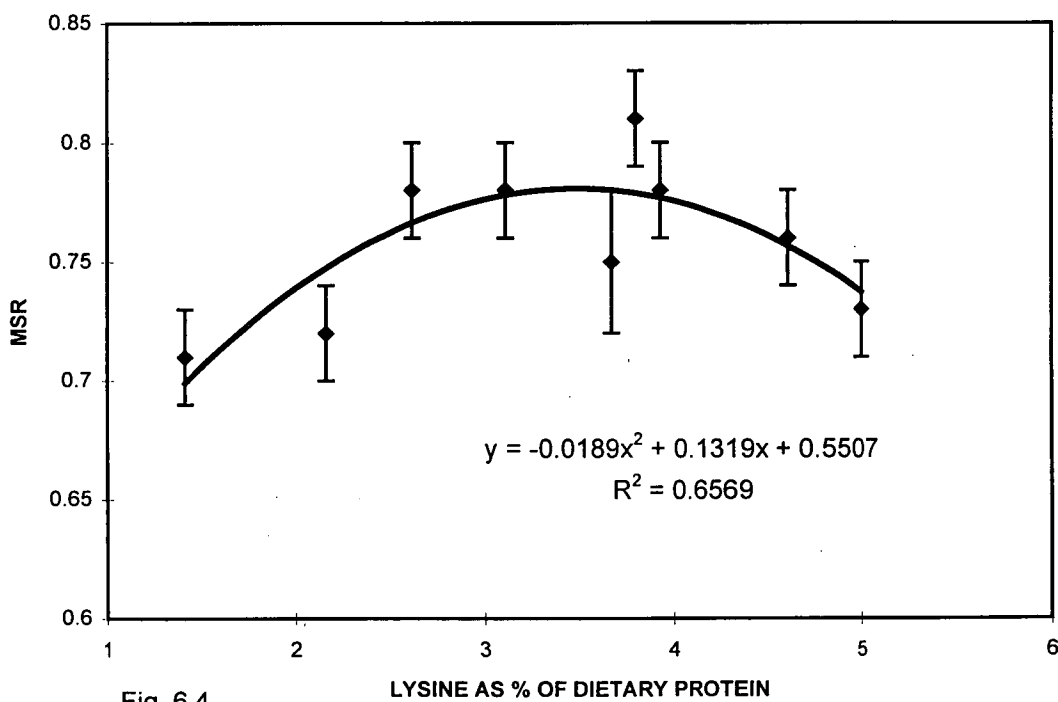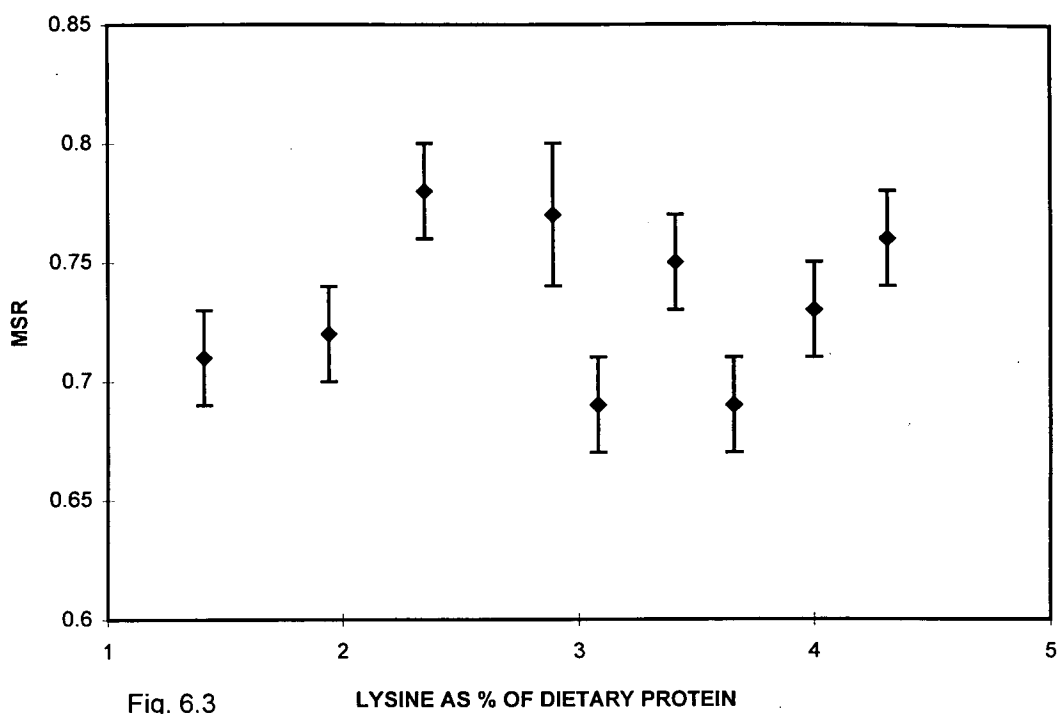
Fig. 6.3: Relationship between Meat:Shell Ratio (MSR) and dietary supplementation with lysine.HCl. (Mean ± sem, n = 20)

Fig. 6.4: Relationship between MSR and dietary supplementation with covalently-bound lysine. (Mean ± sem, n = 20)

Fig. 6.5

$$y = -5.6184x^2 + 38.259x - 18.263$$
$$R^2 = 0.7959$$
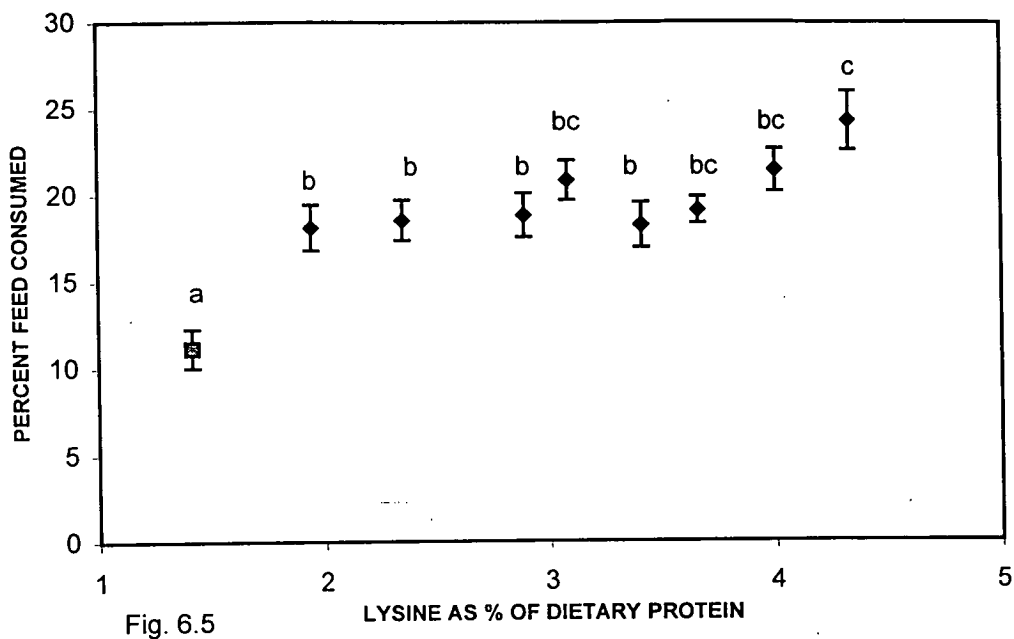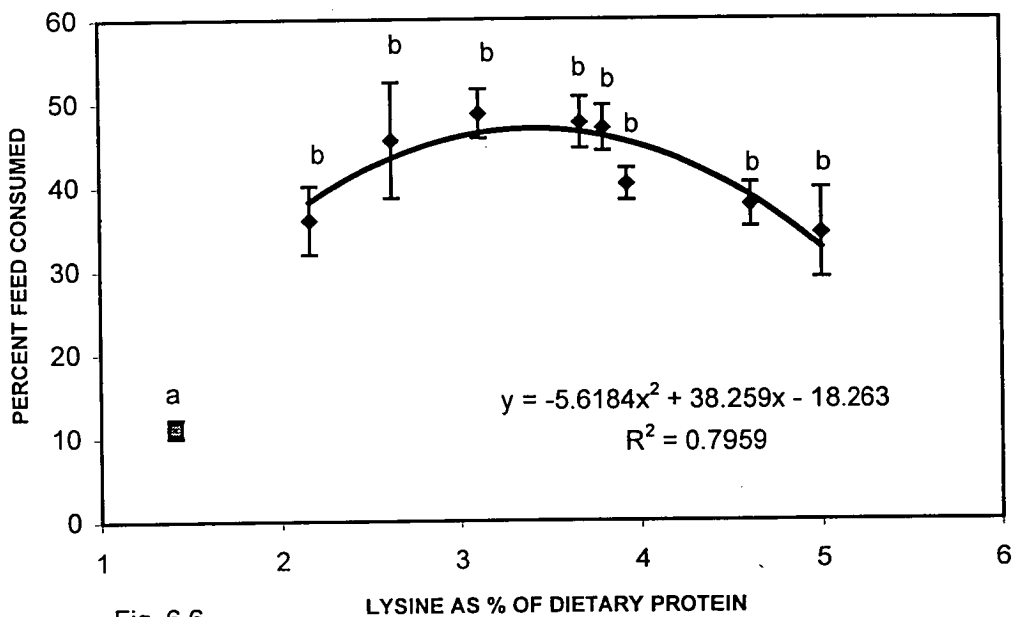
Fig. 6.6

Fig. 6.5: Feed consumption by abalone of diets supplemented with free lysine HCl (mean ± se, n = 6).

Fig. 6.6: Feed consumption by abalone of diets supplemented with covalently enriched gluten (mean ± se, n = 6).

Data point at left of both plots represents consumption of the basal diet.

Means with the same superscript are not significantly different (P > 0.05).

Table 6.9: The effect of leaching (6 h) on a dietary supplement of lysine HCl in FL diets.

Diet	Lysine Content (Intact Feed; g.kg^{-1})	Lysine Content (Leached Feed; g.kg^{-1})	Lysine Leached (% of original amount)
Basal	3.23	2.80	13.3
FL1	4.20	2.55	39.3
FL2	4.33	2.87	33.7
FL3	6.53	3.32	49.2
FL4	6.97	3.27	53.1
FL5	7.81	4.70	39.8
FL6	8.56	4.44	48.1
FL7	9.25	4.21	54.5
FL8	10.22	5.31	48.0

FL1-8 = Free lysine diets 1-8

6.4 Discussion

Gluten enrichment

In a series of small-scale experiments carried out at the University of British Columbia, it was concluded that neither hydrolysis of gluten with 0.2 M HCl nor use of reagent-grade L-lysine HCl improved lysine content of modified gluten over the use of 0.1 M HCl or use of feed-grade lysine HCl. Consequently, the large-scale reaction used hydrolysis at 0.1 M HCl and feed-grade lysine HCl to produce enough lysine-enriched gluten for use in the growth experiment. The lysine-enriched gluten was precipitated at pH = 2.5, 4.5 and 8.5. Although the pH = 8.5 fraction was richer in lysine than the pH = 2.5 fraction, the latter was used in all CEG diet blends as there was 96 g available, compared to only 28 g of the pH = 8.5 fraction. This avoided having to blend different gluten fractions. Unfortunately, the lysine content of CEG diets that included 40%, 50% and 60% enriched gluten was higher, lower, and lower, respectively, than the anticipated content (Table 6.6). When analysed values were converted to lysine content as a percentage of crude dietary protein, they shifted the diet along the X-axis of plots, such as Fig. 6.2, resulting in "crowding" in the range of 3.5-4.0%. Other than formulation errors or inconsistencies in analyses, no explanation for these deviations can be suggested. In general, however, their overall effect on the end result could be expected to be small.

Growth Experiment

The growth response of abalone to dietary supplementation with lysine HCl was significant ($P < 0.001$), but linear within the range of the experiment. Two confounding factors may be responsible for this result. The first is differential uptake kinetics between free lysine and covalently-bound lysine, which may be explained as follows: following the

ingestion of feed, free lysine molecules are thought to be rapidly absorbed across the gut wall, resulting in a rapid increase in lysine concentration in the haemolymph. In a relatively short time this excess lysine is thought to be excreted, leaving the body in a temporary state of lysine deficiency. Protein synthesis that occurs during this time will be retarded by a shortage of lysine.

The second factor is feed consumption. Comparison of Figs. 6.1 and 6.5 shows that they are very similar. If free lysine was an attractant to abalone, they would be expected to increase their feed consumption as dietary lysine supplementation increased, and show increased growth as a result. This explanation would not hold for the CEG diets, as lysine loss across the series would not be expected to increase with supplementation rate, as the lysine was an integral part of the dietary protein. Lysine loss from CEG diets is assumed to be similar to that of the basal diet. The conclusion is that the growth response to free-lysine supplementation reflects the sum of at least two confounding factors, while the growth response to covalently-bound lysine reflects more accurately the effects of dietary supplementation of a deficient diet with the nutrient under investigation.

The linear response of SGR to lysine HCl supplementation did not allow a prediction of optimal dietary lysine requirement to be made. Although it is clear that losses of lysine HCl supplements from the FL diets due to immersion for six hours were high (Table 6.8), it is difficult to quantify the effect this had on the growth of the abalone. An assumption was made that abalone completed feeding within six hours of feed being added to the tanks, but this does not permit an estimate of the average lysine content of ingested feed to be made. The observations made here permitted the quantification of the loss of a lysine supplement from abalone diets, and reinforced the candidate's view that free amino acid supplements are unlikely to be cost-effective in commercial abalone diets and are unlikely to be of use in experimental diets.

The growth response of abalone to dietary supplementation of CEG diets was also significant ($P < 0.001$), but curvilinear. The fitting of a second-order quadratic model to the data predicted maximal growth to occur with a lysine content of 3.9% of dietary protein. The lower 95% confidence interval about this point was 0.89, corresponding to a dietary lysine content of 2.74 g.kg^{-1}. Lysine content could therefore be reduced to 2.74 g.kg^{-1} with only a 5% likelihood of a reduction in growth rate (Fig. 6.2). This could be termed the optimal dietary lysine level, and is lower than any of the published finfish lysine requirements (D'Mello, 1994). The maximum growth rates achieved by abalone fed CEG diets were greater than those of abalone fed FL diets, indicating that the covalently-bound lysine supplement may be available for protein synthesis for a greater length of time than free lysine. At levels above about 4% (CEG Diets 60%, 70% and 80%), growth began to decline, although not significantly. High systemic levels of lysine (indeed, any amino acid), may inhibit protein synthesis by competitive inhibition with other amino acids at binding sites of t-RNA.

6.5 Conclusions

The lysine content of wheat gluten was increased at least 4.6-fold when the gluten was hydrolysed with 0.1M HCl and precipitated at pH = 2.5. This fraction yielded 61.4% of the final yield of enriched gluten. Enrichment was greater (up to 5.9-fold) for the fraction precipitated at pH = 8.5, but yield was lower (18%). Abalone exhibited a classic growth response to diets supplemented with covalently-bound lysine. This response enabled dietary lysine requirement to be estimated at 3.9% of dietary protein. The lysine content of the diet could theoretically be reduced to 2.7% with a 5% likelihood of growth reduction. Meat to shell ratio was also affected by dietary lysine content, when the lysine

supplement was covalently bound. The dietary lysine level recommended by this study should be met in commercial feeds by the inclusion of intact protein sources, rather than by supplementation of low-lysine diets with free lysine. Assuming that the bulk ingredients of the diet are cereal-based, the recommended lysine level may be achieved by inclusion of relatively high-lysine feed ingredients, such as fish- or meat-meal, ideally, on a digestible lysine basis. Covalent enrichment of low-lysine feedstuffs with lysine would be uneconomical.

The growth response of abalone to a dietary supplement of lysine HCl was linear and did not allow a dietary requirement to be estimated. The growth response was also confounded by feed consumption and by the different uptake kinetics between free- and covalently-bound lysine. Meat to shell ratio was not affected by dietary lysine HCl supplementation. The loss of lysine, when supplemented as lysine HCl, ranged from 33.7-54.5% and may partially explain the lesser growth response of abalone to this supplement.

This experiment has clearly demonstrated that, to obtain meaningful estimates of dietary amino acid requirements for abalone requires a different strategy to that usually employed in finfish nutrition research.

CHAPTER 7

GENERAL SUMMARY

The nutritional requirements of abalone are generally poorly understood. The paucity of information in this area has hampered the production of a cost-effective manufactured feed for the abalone culture industry in Australia.

A series of experiments were conducted to evaluate the protein, energy and lysine requirements of *H. laevigata*. A preliminary experiment on mineral requirements produced valuable results on calcium and phosphorus requirements and provided the candidate experience in abalone handling and husbandry (see Appendix 2). The next experiment was a "range-finding" experiment investigating dietary crude protein requirements of abalone. Following this, the model successfully used in swine nutrition research was applied. This model involved estimating the ability of abalone to digest protein and energy from selected feedstuffs. This information was then used to formulate experimental diets to investigate the effects of different digestible protein:digestible energy ratios on growth and body composition. The final experiment compared two different methods of dietary lysine supplementation and estimated the dietary lysine requirement of greenlip abalone.

The preliminary experiment (see Appendix 2) demonstrated that abalone require no more than 0.5% calcium in a manufactured feed and that a phosphate supplement significantly increased growth rate. These dietary findings have been incorporated by South Australian abalone feed makers, resulting in the addition of a phosphate supplement and the deletion of a relatively large $CaCO_3$ supplement (30 g.kg^{-1}), therefore increasing the scope for varying the content of other feedstuffs.

The next experiment, aimed at estimating the optimal dietary protein level, demonstrated that *H. laevigata* grow remarkably well at relatively low protein levels (close to 20%). This estimate was considerably lower than almost all previously published estimates and may be due to the use of a balanced amino acid profile in the diets. An estimate was made of the optimal DP:DE ratio (14.4 g DP.MJDE^{-1}) but it was necessary to confirm this with a more elaborate, statistically robust experiment.

Digestibility studies revealed that defatted soybean flour was the most cost-effective protein and energy source among feedstuffs tested. Digestibility results were used in the formulation of diets with ten different levels of protein, each at two different energy levels in order to estimate the optimal DP:DE ratio. The abalone fed the high-energy diets grew poorly compared to those on the low-energy diets. The extra energy in the high-energy diets was supplied almost entirely by lipid and it is thought that this adversely affected the growth of the abalone. The exact mechanism of growth suppression remains unknown, but two hypotheses have been advanced. The first suggests that if abalone eat to energy satiation, their protein intake may be too low to support fast growth on high-energy diets. The second hypothesis is that excess dietary lipid may interfere with the digestion of protein and hence retard growth. Recent research has tended to support this hypothesis (van Barneveld *et al.*, 1998). The effect is not permanent and stunted animals return to normal growth rates when returned to low-energy diets. The optimal DP:DE ratio was found to be in the range 12.3-17.9 g DP.MJDE^{-1}, confirming the earlier estimate. This study also showed that abalone fed diets within the above range had better meat yield than those fed diets with excess protein. Meat from these animals also contained less moisture and ash than that from animals fed high-protein diets. In conclusion, these results suggest a commercial diet containing about 15 g DP.MJDE^{-1} would promote fast growth and

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produce good quality meat in abalone.

The final experiment sought to estimate the dietary requirement of *H. laevigata* for lysine. No quantitative estimates for the requirements of any of the essential amino acids are found in the literature for abalone. The ability of abalone to utilise free amino acid supplements (the traditional way of supplementing amino acids in experimental diets for finfish) in diets effectively, was unknown. Fortunately, recent work in the area of penaeid nutrition enabled a new method of amino acid enrichment (covalent attachment to intact protein) to be compared to the traditional free amino acid method. Although abalone responded to the free lysine supplements in an otherwise lysine-poor diet, the response was linear and did not provide enough information to calculate an optimal dietary level. Fortunately, the growth response to the covalently-bound lysine supplement was not only greater than that to free-lysine, but was curvilinear allowing a optimal dietary lysine requirement to be estimated. *H. laevigata* appear to require 3.9% of dietary protein as lysine for optimal growth, in diets with a crude protein content of 22-24%. Future work aimed at estimating essential amino acid requirements of abalone should employ the covalent-enrichment methodology.

The relatively broad "response plateau" exhibited by greenlip abalone in relation to both dietary protein and dietary (covalently bound) lysine (Figs. 3.1 & 3.2, and Fig. 6.2, respectively) suggests that there is considerable scope for manipulation of dietary formulations to supply these requirements. However, the response to *Dunaliella salina* supplementation (Appendix 1) indicates that other aspects of abalone nutrition require further attention.

In general, findings of this thesis are encouraging in relation to the attributes of greenlip abalone for aquaculture. Survival rates were excellent and growth rates of about

$1.0\%.day^{-1}$ could be achieved even with experimental diets. Most importantly, abalone can be grown on diets with low ingredient costs in relation to DP, DE and lysine. However, preparation of diets with extended water stability is a challenge and may adversely affect feed costs.

To conclude, there is a need for further studies in the field of abalone nutrition, particularly in the areas of amino acid requirements and diet processing, particularly aimed at improving water stability of the diet. Improved understanding of the nutrition of these animals will allow cheaper, more environmentally-benign feeds to be manufactured, aiding the viability of the Australian abalone culture industry.

APPENDIX 1

THE EFFECT OF DIETARY SUPPLEMENTATION WITH *Dunaliella salina* AND A COMMERCIAL ENZYME MIX.

A 1.1 Introduction

Anecdotal evidence from abalone farmers (D. Morrison, Pers. Comm.) suggested that inclusion of a spray-dried algal meal of *Dunaliella salina* to diets acted as a phagostimulant and/or attractant to abalone and may result in faster growth through improved feed intake. An experiment was planned to test the efficacy of the algal meal. Commercial enzyme preparations are often routinely added to livestock rations to increase the digestibility of feeds. Although the true digestibility of some feedstuffs was very high (eg soyflour), the digestibility of others (eg fishmeal) were quite poor. It was possible that a commercial enzyme preparation could be beneficial to overall digestibility of a diet. A decision was made to also test the effect of such a supplement on an abalone diet.

A 1.2 Materials and Methods

Abalone

Juvenile greenlip abalone (*Haliotis laevigata*) held at the South Australian Research & Development Aquatic Sciences Centre were used for this experiment. The animals were held until needed in a flow-through holding tank and fed a commercial diet.

Feed formulation and manufacture

A semi-purified diet (Diet 1) was formulated (Table A1.1) containing 1% kaolin. Two other diets (Diets 2 and 3) were formulated to contain spray-dried microalgal meal ("Algro", Betatene, Ltd., Cheltenham, Victoria, Australia) and a commercial enzyme mixture containing protease, lipase and amylase ("Ebizyme", CSR Ltd., Pyrmont, New South Wales, Australia), respectively, at the expense of kaolin. Diets contained 0.5% chromic oxide as an indigestible marker and were produced in the form of flat chips using a pasta machine.

Aquarium system

This was is described in Chapter 3. In this experiment the flow rate was 0.2 $L.min^{-1}$ and air was supplied via a single airstone at 0.5 $L.min^{-1}$.

Treatments

Abalone were tagged, weighed and measured as described in Chapter 3 and randomly assigned to treatments. The three treatments were each fed to four replicate tanks of 10 abalone. Feed was initially supplied at 2% of initial body weight per day thrice weekly,

Table A1.1: Experimental diet formulation

Diet Ingredient	Inclusion Rate (g.kg^{-1})
Soyflour	400
Wheat fraction	372.4
Fishmeal	90
Casein	80
Oil	20
Sodium alginate	12
Kaolin/microalga/enzyme*	10
Phosphate supplement	5
Chromic oxide	5
Vitamin premix	3
Mineral premix	2
Vitamin C	0.5
Vitamin E	0.1

* Diet 1 contained kaolin. In Diets 2 and 3, the kaolin was replaced by microalga and enzyme, respectively.

and adjusted as necessary. Aquaria were randomly distributed. The experiment ran for 80 days, with measurements and sampling at day 41, and final measurements at day 80. Due to reductions in animal numbers due to sampling, during the second phase of the experiment, animal numbers were reduced to 28 (control), 26 (algal meal treatment) and 27 (enzyme treatment). These were distributed over four replicate aquaria per treatment, so the stocking density was different for the second period of the experiment. It was, however, equal for all three treatments.

Digestibility

Faecal samples were collected, dried and analysed as described in Chapter 4. Digestibility coefficients for both protein and energy were estimated for each diet.

Feed intake

On five occasions during the first 41 days, uneaten feed was removed from the aquaria by siphon hose, oven dried and weighed. Observations made toward the end of the experiment revealed that the abalone fed Diets 2 and 3 were consuming their ration on the first night. Their ration was increased for the last two feedings.

Statistical analysis

Gain in body weight was expressed as specific growth rate (SGRBW) and was calculated using the formula given in Chapter 3. Gain in shell length was expressed as SGRSL, and was calculated similarly. All statistics were calculated using Systat® (1992).

A 1.3 Results

Survival and growth

Mortality was very low; a total of only three animals died during the first 14 days. These deaths were presumed to be due to the stress of tagging, handling and anaesthesia. After 41 days, growth, expressed as SGRBW, did not differ significantly between treatments ($P > 0.05$), but SGRSL was significantly different ($P < 0.05$) between diets 1 and 3 (Table A1.2). At the conclusion of 80 d, the SGRSL of the abalone from both the algal and enzyme treatments was not significantly different to that of the control group ($P > 0.05$). The SGRBW of the abalone from the algal treatment was significantly higher ($P < 0.05$) than that of the control group. The difference amounted to an increase of 19.7%. In the case of the enzyme supplement, the difference was not significant ($P > 0.05$).

Digestibility

Digestibility coefficients for both protein and energy for each diet can be seen in Table A1.3. There was no significant difference in digestibility of either protein or energy between diets ($P > 0.05$).

Feed intake

Abalone fed diets 2 and 3 consumed slightly more feed than those fed diet 1 during the first 41 day period, but the difference was not significant ($P > 0.05$). Near the end of the experiment abalone fed diets 2 and 3 were seen to consume all of their ration on the first night. Their rations were increased for the last two feeds.

Table A1.2: Growth rate of abalone after 41 and 80 days

Diet	SGRSL (41 days)	SGRSL (80 days)	SGRBW (41 days)	SGRBW (80 days)
1	$0.191^a \pm 0.023$	$0.209^a \pm 0.019$	$0.779^a \pm 0.083$	$0.692^a \pm 0.056$
2	$0.212^a \pm 0.019$	$0.237^a \pm 0.008$	$0.884^a \pm 0.032$	$0.828^{ab} \pm 0.050$
3	$0.228^{ab} \pm 0.011$	$0.230^a \pm 0.005$	$0.855^a \pm 0.028$	$0.766^a \pm 0.017$

All values are mean ± sem, n = 4.

Means with same superscript are not significantly different ($P > 0.05$).

SGRSL = Specific growth rate of shell

SGRBW = Specific growth rate of bodyweight

Diet 1 = Control diet

Diet 2 = Algal meal supplemented diet

Diet 3 = Enzyme supplemented diet

Table A1.3: Digestibility coefficients of dietary protein and energy of experimental diets

Diet	Protein Digestibility (%)	Energy Digestibility (%)
1	79.5[a] ± 0.7	72.2[a] ± 0.9
2	78.0[a] ± 1.0	71.4[a] ± 1.2
3	78.0[a] ± 1.1	71.5[a] ± 1.5

Values given as mean ± sem, n = 3, except Diet 1 where n = 2.

Means with the same superscript are not significantly different.

Diet 1 = Control diet

Diet 2 = Algal meal supplemented diet

Diet 3 = Enzyme supplemented diet

A 1.4 Discussion

Growth rates

The addition of a supplement of 1.0% algal meal (Diet 2) increased SGR of BW over that of the basal diet (Diet 1), after 80 days (Table A1.2). In contrast, the addition of a supplement of 1% enzyme mixture (Diet 3) resulted in no significant increase in growth of either shell or bodyweight over that of the basal diet ($P < 0.05$) after 80 days. The incorporation of the enzyme supplement in the feed resulted in a rapid loss of pellet integrity. The pellets took on a "fuzzy" appearance within a few hours of immersion and became fragile. As farmers report a desire for increased water stability of feed pellets, it would appear that an enzyme supplement would be unlikely to find an application in abalone feed.

Estimation of the digestibility coefficients for protein and energy for each diet revealed no significant differences. It is possible that the methods employed here were not sensitive enough to detect a difference, if one existed. It is unlikely that the observed increases in growth rates due to the algal supplement was due to increased digestibility of dietary components. The algal meal may have contributed a micronutrient that is lacking in the control diet. Possible candidates include vitamins or fatty acids.

By the end of the experiment, the rations of Diet 2 and 3 had to be increased as they were being consumed completely on the first night. It is possible that, by this stage, there was a significant difference in intake rates, although this may simply be due to the larger size of animals fed Diets 2 and 3.

A 1.5 Conclusions

Inclusion of a supplement (1% dw) of dried *Dunaliella salina* increased SGR of abalone by 19.7% over that of controls ($P < 0.05$) on a bodyweight basis after an 80 day period. Although the mechanism of action by which it increases growth rate is unknown, inclusion of this supplement in abalone diets would appear to have economic merit. Due to the loss of pellet integrity and failure to significantly improve growth rates, an enzyme supplement is unlikely to be used by abalone feed manufacturers in the near future.

APPENDIX 2

THE EFFECT OF DIFFERENT COMBINATIONS OF DIETARY CALCIUM AND PHOSPHORUS ON THE GROWTH OF JUVENILE *Haliotis laevigata*.
(Reprinted from Aquaculture, 145: 267-279.)

This Appendix is located in a pocket on the inside of the thesis back cover.

APPENDIX 3

LIST OF ABBREVIATED AMINO ACIDS

Abbreviation	Full Name
Cys	Cysteine
Asp	Asparagine
Thr	Threonine
Ser	Serine
Glu	Glutamine
Pro	Proline
Gly	Glycine
Ala	Alanine
Val	Valine
Met	Methionine
Ile	Isoleucine
Leu	Leucine
Tyr	Tyrosine
Phe	Phenylalanine
Trp	Tryptophan
Lys	Lysine
His	Histidine
Arg	Arginine

APPENDIX 4

CHEMICAL COMPOSITION OF FEEDSTUFFS AND EXPERIMENTAL DIETS.

Table A4.1: Crude protein (CP), gross energy (GE), ash, crude fibre (CF), crude fat

(CFat) and dry matter (DM) of feedstuffs used in experimental diets

	Sem.	Casein	Soy.	FM	Gel.	CF.	FO	WS
CP	116±2.8	860±8.5	470±5.7	630±14.1	971±4.2	2.0±0.3	0	2.2±0.2
GE	16.00	21.30	18.50	19.25	19.62	15.22	37.57	16.00
Ash	4.6±0.7	22.5±2.1	68.7±8.5	135±12.7	0	1.0±0.1	ND	1.0±0.2
CF	2.02 ±0.14	0	4.95 ±2.19	1.05 ±0.07	0	0.1 ±0.03	ND	0.1 ±0.02
CFat	17.6 ±1.4	5.3 ±0.7	21 ±3.5	68 ±5.7	1.0 ±0.1	0	991 ±0.4	0
DM	899 ±8.5	943 ±5.7	912 ±5.1	922 ±4.2	954 ±3.8	926 ±6.2	ND	931 ±5.9

Sem. (semolina), Soy. (defatted soyaflour), FM (fishmeal), Gel. (gelatine), CF (cornflour), FO (fish oil), WS (wheat starch). ND = Not determined. Values (mean ± se, n = 2) in g.kg^{-1}. N = 1 for GE.

Table A4.2: Crude protein (CP), gross energy (GE), calculated ash and crude fat (CFat)

of diets used in Chapter 3

Diet											
	11	10	9	8	7	6	5	4	3	2s	1s
CP	461±	418±	395±	374±	353±	323±	299±	274±	239±	188±	122±
GE	18.92	18.70	19.02	17.35	18.73	18.08	18.32	17.99	17.96	16.96	17.29
Ash	4.23	4.15	4.13	4.07	4.03	4.00	3.96	3.92	3.84	3.76	3.64
CFat	3.04	2.94	2.88	2.83	2.78	2.73	2.68	2.62	2.52	2.42	2.26

Values for CP are mean ± se, n = 2 in g.kg^{-1}. Crude fat and dry matter are given as percent. N = 1 for GE.

Table A4.3: Calculated crude protein (CP), fibre and crude fat (CFat) (all g.kg^{-1}) of four

base diets used in Chapter 5

	Diet 1	Diet 2	Diet 3	Diet4
CP	1.9	379.2	1.7	379.2
Fibre	0.8	13.1	0.8	13.1
Ash	101.5	34.6	205.5	139.6
CFat	55.0	103.7	6.0	53.7

Table A4.4: Calculated crude fibre (Cfib), crude fat (Cfat) and ash (all g.kg^{-1}) of free-

lysine (FL) diets used in Chapter 6.

					Diet				
	Basal	1	2	3	4	5	6	7	8
Cfib	10	10	10	10	10	10	10	10	10
Cfat	28	28	28	28	28	28	28	28	28
Ash	117	115	113	110	108	106	104	102	99

Crude protein and dry matter values were determined analytically and can be seen in Table

6.7.

REFERENCES

ABARE., 1996. Australian Bureau of Agricultural and Resource Economics. Canberra. 48 pp.

Abbas, J.A., Basson, P.W. and El-Din, A.Y., 1992. Protein content of benthic marine algae from Bahrain coastline. Ind. J. Mar. Sci., 21(1): 62-63.

ARC (Agricultural Research Council), 1981. Protein and amino acid requirements. In: The Nutrient Requirements of Pigs. Commonwealth Agricultural Bureaux, Farnham Royal, Slough, pp. 67-124.

Akiyama, T., Arai, S. and Murai, T., 1985. Threonine, histidine and lysine requirements of chum salmon fry. Bull. Jpn. Soc. Sci. Fish., 51: 635-639.

Allen, W.V. and Kilgore, J., 1975. The essential amino acid requirements of the red abalone, *Haliotis rufescens*. Comp. Biochem. Physiol., 50A: 771-775.

Arai, S., Nose, T. and Hashimoto, Y., 1972. Amino acids essential for the growth of eels, *Anguilla anguilla* and *A. japonica*. Bull. Jpn. Soc. Sci. Fish., 38: 753-759.

Arai, S., 1981. A purified test diet for coho salmon, *Oncorhynchus kisutch*, fry. Bull. Jpn. Soc. Sci. Fish., 47: 547-550.

Aranyakananda, P. and Lawrence, A.L., 1993. Dietary protein and energy requirements of the white-legged shrimp, *Penaeus vannamei* and the optimal protein to energy ratio. In: From Discovery To Commercialization. M. Carrillo, L. Dahle, J. Morales, P. Sorgeloos, N. Svennevig, J. Wyban (Eds.). European Aquaculture Society Special Publication No. 19, Oostende, Belgium.

AOAC, 1980. Official Methods of Analysis of the Association of Official Analytical Chemists. W. Horwitz (Ed.) Thirteenth Ed., Association of Official Analytical Chemists, PO Box 540, Benjamin Franklin Station, Washington DC 20044.

Barkai, R. and Griffiths, C.L., 1988. An energy budget for the South African abalone *Haliotis midae* Linnaeus. J. Moll. Stud., 54: 43-51.

Batterham, E.S., Andersen, L.M., Baignent, D.R. and White, E., 1990. Utilisation of ileal digestible amino acids by growing pigs: Effect of dietary lysine concentration on efficiency of lysine retention. Brit. J. Nutr., 64: 81.

Bennett, R. and Nakada, H.I., 1968. Comparative carbohydrate metabolism of marine Mollusca - I. The intermediary metabolism of Mytilus californianus and *Haliotis rufescens*. Comp. Biochem. Physiol., 24: 787-797.

Bergot, F., 1991. Digestibility of native starches of various botanical origins by rainbow

trout (*Oncorhynchus mykiss*) pp. 857-865. In: Fish Nutrition In Practice, S.J. Kaushik and P. Luquet (Editors) INRA, Paris, 1993.

Bikker, P., 1991. Protein and lipid accretion in body components of growing pigs. Dept. Of Animal Husbandry, Wageningen Agricultural University. PhD Thesis.

Black, J.L. and Griffiths, D.A., 1975. Effects of live weight and energy intake on nitrogen balance and total N requirement of lambs. Br. J. Nut., 33: 399.

Brafield, A.E., 1985. "Laboratory Studies of Energy Budgets". In: Fish Energetics: New Perspectives", P. Tytler and P. Calow (Editors). Croom Helm Ltd., Beckenham, Kent. 349 pp.

Britz, P., Hecht, T., Knauer, J. and Dixon, M., 1994. The development of an artificial feed for abalone farming. S.Afr. J. Sci., 90: 7-8.

Britz, P., 1996. Effect of dietary protein level on growth performance of South African abalone, *Haliotis midae*, fed fishmeal-based semi-purified diets. Aquaculture, 140: 55-61.

Britz, P., Hecht, T. and Knauer, J., 1997. Rates of digestion and enzyme activity in *Haliotis midae* fed a formulated diet. In press.

Brown, P.B., Robinson, E.H., Clark, A.E. and Lawrence, A.L., 1989. Apparent digestible energy coefficients and associative effects in practical diets for red swamp crayfish. J. World Aq. Soc. 20: 122-126.

Brown, L.D. and Murray, N.D., 1992. In: Abalone of the World. Biology, Fisheries and Culture. (Eds. S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo. Fishing News Books, Cambridge. 608pp.

Bush, M.S., 1988. The ultrastructure and function of the intestine of *Patella vulgata*. J. Zool. 215(4): 685-702.

Campbell, J.L., 1965. The structure and function of the alimentary canal of the black abalone, *Haliotis cracherodii* Leach. Amer. Micros. Soc. Trans., 84: 376-395.

Campbell, R.G., Taverner, M.R. and Curic, D.M., 1983. The influence of feeding level from 25-40 kg live weight on the performance and body composition of female and entire male pigs. Anim. Prod., 36: 193.

Campbell, R.G., Taverner, M.R. and Curic, D.M., 1985. The influence of feeding level on the protein requirement of pigs between 20 and 45 kg live weight. Anim. Prod., 40: 489.

Carefoot, T.H., Qian, P-Y., Taylor, B.E., West, T. and Osborne, J., 1993. Effect of starvation on energy reserves and metabolism in the northern abalone, *Haliotis kamtschatkana*. Aquaculture, 118: 315-325.

Chiou, J.Y. and Ogino, C., 1975. Digestibility of starch in carp. Bull. Jpn. Soc. Sci. Fish. 41: 465-466.

Cho, C.Y. and Slinger, S.I., 1979. Apparent digestibility measurement in feedstuff for rainbow trout. In: J. Halver and K. Tiews (Editors). Proceedings of the World Symposium on Finfish Nutrition and Fishfeed Technology. Vol. II, Hamburg, 20-23 June 1978, Heeneman, Berlin, pp. 239-247.

Cho, D.M., Pyeun, J.H., Byeun, D.S. and Kim, C.Y., 1983. Proteolytic activity of the crude enzyme extracted from the digestive tract of marine gastropods. Bull. Korean Fish. Soc., 16: 216-224.

Clavier, J., 1992. The ormer (*Haliotis tuberculata*) fishery of France and the Channel Islands. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo (Editors). Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge. 608 pp.

Close, W.H., Berschauer, F. and Heavens, R.P., 1983. The influence of protein:energy value of the ration and level of feed intake on the energy and nitrogen metabolism of the growing pig. 1. Br. J. Nut., 49: 255

Colvin, P.M., 1976. Nutritional studies on penaeid prawns: protein requirements in compounded diets for juvenile *Penaeus indicus*. Aquaculture, 7: 315-326.

Coote, T.A., Hone, P.W., Kenyon, R. and Maguire, G.B., 1996. The effect of different combinations of dietary calcium and phosphorus on the growth of juvenile *Haliotis laevigata*. Aquaculture, 145: 267-279.

Cowey, C.B. and Tacon, A.G.J., 1983. Fish nutrition - relevance to invertebrates. In: G.D. Pruder, C.J. Langdon and D.E. Conklin (Eds.) Proceedings of the Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition. Louisiana State University, Division of Continuing Education, Baton Rouge, pp. 13-30.

Cox, K.W., 1960. Review of the abalone of California. Calif. Fish Game, 46: 381-406.

Crofts, D.R., 1929. *Haliotis*. Liverpool Mar. Biol. Comm. Mem., 29: 1-174.

de Lange, C.F.M., Sauer, W.C., Mosenthin, R. and Souffrant, W.B., 1989. The effect of feeding different protein-free diets on the recovery and amino acid composition of endogenous protein collected from the distal ileum and faeces in pigs. J. Anim. Sci., 67: 746-754.

de Lange, C.F.M., Souffrant, W.B. and Sauer, W.C., 1990. Real ileal protein and amino acid digestibilities in feedstuffs for growing pigs as determined by the ^{15}N-isotope dilution technique. J. Anim. Sci., 68: 409-418.

Deshimaru, D. and Shigeno, K., 1972. Introduction to the artificial diet for prawn (*Penaeus japonicus*). Aquaculture, 1: 115-133.

Deshimaru, O., 1981. Studies on nutrition and diet of prawn, *Penaeus japonicus*. Mem. Kagoshima Fish. Exp. Stat., 12: 1??-118.

De Silva, S.S. and Anderson, T.A., 1994. Fish Nutrition in Aquaculture. Chapman and Hall, London, 319 pp.

Ebert, E.E. and Houk, J.L., 1984. Elements and innovations in the cultivation of red abalone *Haliotis rufescens*. Aquaculture, 39: 375-392.

Eggum, B.O., 1973. A Study of certain factors influencing protein digestibility in rats and pigs. PhD Thesis, Institute of Animal Science, Copenhagen.

Ellis, N. 1995. Digestibility of prawn diets. CSIRO Institute of Plant Production & Processing. Biometrics Unit Report 95/15. Division of Fisheries, Cleveland, Queensland. 12 pp.

Elyakova, L.A., Schevchenko, N.M. and Avaeva, S.M., 1981. A comparative study of carbohydrase activities in marine invertebrates. Comp. Biochem. Physiol., 69B: 905-908.

EPA., 1993. Guidelines for Licensing Discharges to the Marine Environment. The Office of the Environment Protection Authority, Adelaide, South Australia. 53pp.

Erasmus, J.H., Covne, V.E.X. and Cooke, P.A. 1994. Characterisation of enteric bacteria in the abalone *Haliotis midae*. Third International Marine Biotechnology Conference. Tromsoe, Norway. Tromsoe University.

Fallu, R., 1991. Abalone farming, Oxford Fishing News Books, pp. 1-195.

FAO., 1973-1988. Yearbook of Fisheries Statistics. Catch and Landings. 45-60, UNFAO.

Fankboner, P., 1991. Pearl culture in abalone. Infofish Int., (4): 52-55.

Fankboner, P., 1993. Pearls and abalone. Aquaculture Magazine. Nov./Dec. pp. 28-38.

Farlinger, S. and Campbell, A., 1992. Fisheries management and biology of northern abalone, *Haliotis kamtschatkana*, in the northeast pacific. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo(Editors). Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge, 608pp.

Fleming, A.E., 1991. The nutritional biology of the blacklip abalone, *Haliotis rubra*. Ph.D. Dissertation, Melbourne University, Australia, 99pp.

Fleming, A.E., 1995. Digestive efficiency of the Australian abalone *Haliotis rubra* in relation to growth and feed preference. Aquaculture, 134: 279-293.

Fleming, A.E., van Barneveld, R.J. and Hone, P.W., 1996. The development of artificial diets for abalone: a review and future directions. Aquaculture, 140: 5-53.

Foale and Day., 1992. Recognisability of algae ingested by abalone. Aust. J. Mar. Fresh. Res. 43: 1331-1338.

Fong, W. and Mann, K.H., 1980. Role of gut flora in the transfer of amino acids through a marine food chain. Can. J. Fish. and Aquatic Sci., 37: 88-96.

Fox, J.M., Lawrence, A.L. and Li-Chan, E. 1995a. Dietary requirement for lysine by juvenile *Penaeus vannamei* using intact and free amino acid sources. Aquaculture, 131: 279-290.

Fox, J.M., Li-Chan, E., & Lawrence, A.L., 1995b. Carbodiimide-mediated covalent attachment of lysine to wheat gluten and its apparent digestibility by *Penaeid* shrimp. J. Agric. Food Chem. 43: 733-737.

Fretter, V. and Graham, A., 1976. A Functional Anatomy of Invertebrates. Academic Press, London.

Fuller, M.F. and Garthwaite, P., 1993. The form of response of body protein accretion to dietary amino acid supply. J. Nutr., 123: 957.

Garland, C.D., Cooke, S.L., Grant, J.F. and McMeekin, T.A., 1985. Ingestion of the bacteria on and the cuticle of crustose (non-articulated) coralline algae by post-larval and juvenile abalone (*Haliotis ruber* Leach) from Tasmanian waters. J. Exp. Mar. Biol. Ecol., 91: 137-149.

Genade, A.B., Hirst, A.L., and Smit, C.J., 1988. Observations of the spawning, development and rearing of the South African abalone *Haliotis midae* Linn. S. Afr. J. Mar. Sci., 6: 3-12.

Godoy, C., Jerez, G. and Ponce, F., 1992. The introduction of abalone into Chile. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo (Editors). Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge pp. 485-488.

Gorfine, H. and King, R., 1991. New food for abalone. Austasia Aquaculture, 5(11): 40.

Grant, J.F., 1981. Abalone culture in Japan: Development and current commercial practice. Tas. Fish. Dev. Auth., 23: 2-17.

Groppe, J.C. and Morse, D.E., 1993. Molluscan chymotrypsin-like protease: Structure, localisation and substrate specificity. Arch. Biochem. Biophys., 305(1): 159-169.

Guzmán del Próo, S.A., 1992. A review of the biology of abalone and its fishery in Mexico. In:S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo (Editors). Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge, 608 pp.

Hagemeister, H. and Erbersdobler, H., 1985. Chemical labelling of dietary protein by transformation of lysine to homoarginine: A new technique to follow intestinal digestion and absorption. Proc. Nutr. Soc., 44: 133a.

Hahn, K.O., 1989. K.O. Hahn (Editor). Handbook of Culture of Abalone and Other Marine Gastropods. CRC Press Inc., Boca Raton, Florida.

Harada, K., Eguchi, A. and Kurosaki, Y., 1987. Feeding attraction activities in the combinations of amino acids and other compounds for abalone, oriental weatherfish and yellowtail. Nippon Suisan Gakkaishi, 53: 1483-1489.

Harada, K., 1989. Feeding attraction activities of L-dipeptides for abalone, oriental weatherfish and yellowtail. Nippon Suisan Gakkaishi, 55: 1629-1634.

Harris, J.M., 1993. The presence, nature and role of gut microflora in aquatic invertebrates: a synthesis. Microb. Ecol., 25: 195-231.

Harris, J.O., 1994. Digestive physiology of the greenlip abalone *Haliotis laevigata* Donovan: A preliminary investigation of digestive anatomy microbial environment occurrence and algal digestion. B App. Sc. (Hons.) Thesis, University of Tasmania, Launceston, 113 pp.

Hooker, N. and Morse, D.E., 1985. Abalone: The emerging development of commercial cultivation in the United States. In: J.V. Huner and E.E. Brown. (Eds.) Crustacean and Mollusc Aquaculture in the United States. AVI Publishing Co., Inc., Westport, Connecticut pp. 365-413.

Hylleberg-Christiansen, J., 1972. Carbohydrates of some marine invertebrates with notes on their food and on the natural occurrence of the carbohydrates studied. Mar. Biol., 14: 130-142.

Inaba, D., Ogino, C., Takamatsu, C., Ueda, T. and Kurokawa, K., 1963. Digestibility of dietary components in fishes-II. Digestibility of dietary protein and starch in rainbow trout. Bull. Jpn. Soc. Sci. Fish. 29: 242-244.

Ino, T., 1952. Biological studies on the propagation of the Japanese abalone (genus *Haliotis*). Bull. Tokai. Reg. Fish. Res. Lab., 5:1-102.

Johnson, D.W., Al-Harassy, A. and Al-Harthy, M., 1992. The Sultanate of Oman abalone fishery. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo (Editors). Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge 608 pp.

Kideys, A.E. and Hartnoll, R.G., 1991. Energetics of mucus production in the common whelk *Buccinum undatum* L. J. Exp. Mar. Biol. Ecol. 150: 91-105.

King, R.H., Rayner, C.J., Kerr, M., Gorfine, H.K. and McShane, P.E., 1996. The tissue

protein and amino acid requirements of abalone (*Haliotis rubra*). Aquaculture, 140: 109-113.

Knauer, J., Britz, P. and Hecht, T., 1993. The effect of seven binding agents on 24-hour water stability of an artificial weaning diet for the South African abalone, *Haliotis midae* (Haliotidae, Gastropoda). Aquaculture, 115: 327-334.

Knauer, J., Hecht, T., and Duncan, J.R., 1993. Development of an artificial weaning diet for the South African abalone, *Haliotis midae*. Proceedings of the Aquaculture Association of Southern Africa, 1:161-165.

Knauer, J., 1994. Development of an artificial weaning diet for the South African abalone, *Haliotis midae*. M.Sc. Thesis, Rhodes University.

Knauer, J., Hecht, T., and Duncan, J.R., 1994. Proximate composition of the South African abalone, *Haliotis midae* (Haliotidae: Gastropoda). Aquaculture and Fisheries Management 25: 351-354.

Knauer, J., Hecht, T., and Britz, P., 1995. A note on the feeding behaviour and growth of juvenile South African abalone *Haliotis midae*, fed on an artificial weaning diet. South African J. Sci., 91: 91-93.

Koike, Y., Flassch, J.P., and Mazurier, J., 1979. Biological and ecological studies on the propagation of the ormer, *Haliotis tuberculata* Linnaeus. II. Influence of food and density on the growth of juveniles. La Mer (Bulletin de la Societe franco-japonaise d'oceanographie) 17(1): 43-52.

Konosu, S. and Mori, T., 1959. Amino acid composition of shell fish proteins. Bull. Jpn. Soc. Sci. Fish., 25: 153-155.

Lleonart, M. and Handlinger, J., 1997. "Mudworms" in abalone. Proc. 4th Ann. Abalone Aquaculture Workshop, 4-6 July, Port Fairy, Victoria, Australia. Fisheries Research and Development Corporation.

Lindberg, D.R., 1992. Evolution, distribution and systematics of Haliotidae. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo. Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge pp. 3-18.

López-Alvarado, J., Langdon, C.J., Teshima, S-I. and Kanazawa, A., 1994. Effect of coating and encapsulation of crystalline amino acids on leaching in larval feeds. Aquaculture 122: 335-346.

Lovell, T., 1989. Nutrition and Feeding of Fish. Van Nostrand Reinhold, New York, 260 pp.

Low, A.G., 1982. Digestibility and availability of amino acids from feedstuffs for pigs: A review. Livestock Prod. Sci., 9: 511-520.

Ma, T., Lou, M. and Zhang, Q., 1989. Utilisation of giant kelp in food engineering. J. Mar. Drugs Haiyang Yaowu, 8(1): 46-48.

Mai, K., Mercer, J.P. and Donlon, J., 1995a. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. III. Response of abalone to various levels of dietary lipid. Aquaculture, 134: 65-80.

Mai, K., Mercer, J.P. and Donlon, J., 1995b. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. IV. Optimum dietary protein level for growth. Aquaculture, 136: 165-180.

Mai, K., Mercer, J.P. and Donlon, J., 1996. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. V. The role of polyunsaturated fatty acids of macroalgae in abalone nutrition. Aquaculture, 139: 77-89.

McCormick, T.B. and Hahn, K.O., 1983. Japanese abalone culture practices and estimated costs of juvenile production in the USA. J. World Maricult. Soc., 14: 149-161.

McLean, N., 1970. Digestion in *Haliotis rufescens* Swainson (Gastropoda: Prosobranchia). J. Exp. Zool., 173: 303-318.

Mason, V.C., Bech-Andersen, S., and Rudemo, M., 1978. Hydrolysate preparation for amino acid determinations in feed constituents. Z. Tierphysiol., Tierernahrg. u. Futtermittelkde. 41:226-235.

Maynard, L.A. and Loosli, J.K., 1969. Animal Nutrition, 6th Edition. McGraw-Hill, New York, 613 pp.

Mercer, J.P., Mai, K. and Donlon, J., 1993. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* Linnaeus and *H. discus hannai* Ino. I. Effects of algal diets on growth and biochemical composition. Invertebr. Reprod. Dev., 23: 75-88.

Miller, D.S. and Payne, P.R., 1963. A theory of protein metabolism. J. Theor. Biol., 5: 398-414.

Morrison, J. and Whittington, J., 1991. Development of an artificial food for abalone. Austasia Aquaculture, 5(9): 53-54.

Moughan, P.J. and Rutherfurd, S.M., 1990. Endogenous flow of total lysine and other amino acids at the distal ileum of the protein- or peptide-fed rat: The chemical labelling of gelatin protein by transformation of lysine to homoarginine. J. Sci. Food Agric. 52: 179-192.

Mozquiera, A., 1992. Abalone growth rates on an artificial diet. Austasia. Aquaculture, 6(1): 50-51.

Murat, J.C. and Serfaty, A., 1974. Simple enzymatic determination of polysaccharide (glycogen) content of animal tissues. Clin. Chem. 20: 1576-1577.

Nakagawa, T. and Nagayama, F., 1988. Distribution of glycosidase activities in marine invertebrates. J. Tokyo Univ. Fish., 75: 239-245.

Nash, C.E., 1991. The production of abalone. In: C.E. Nash (Editor) Production of Aquatic Animals. Elsevier, Amsterdam pp. 173-181.

Nie, Z.Q., 1992. A review of abalone culture in China. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo (Editors). Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge 608 pp.

Nielsson, I., 1994. Abalone comes to Iceland. Infofish International, (2): 49-52.

Nose, T., Arai, S., Lee, D.L. and Hashimoto, Y., 1974. A note on amino acids essential for growth of young carp. Bull. Jpn. Soc. Sci. Fish., 40: 903-908.

Nose, T., 1979. Summary report on the requirements of essential amino acids for carp. In: J.E. Halver and K. Tiews (Eds.) Finfish Nutrition and Fishfeed Technology, Vol. 1. Heenemann, Berlin, pp. 145-156.

Ogino, C. and Ohta, E., 1963. Studies on the nutrition of abalone. I. Feeding trials of abalone, *Haliotis discus* Reeve, with artificial diets. Bull. Jpn. Soc. Sci. Fish., 29:691-694.

Ogino, C. and Kato, N., 1964. Studies on the nutrition of abalone. II. Protein requirements for growth of abalone, *Haliotis discus*. Bull. Jpn. Soc. Sci. Fish., 30: 523-526.

Olaechea, R.P., Ushio, H., Watabe, S., Takada, K. and Hatae, K., 1993. Toughness and collagen content of abalone muscles. Biosci. Biotech. Biochem., 57: 6-11.

Olley, J. And Thrower, S.J., 1977. Abalone-an esoteric food. Adv. Food Res., 23: 143-186.

Oliva-Teles, A., Gouveia, A.J., Gomes, E. And Rema, P., 1994. The effect of different processing treatments on soybean meal utilisation by rainbow trout, *Onchorhynchus mykiss*. Aquaculture 124: 343-349.

Peck, L.S., Culley, M.B. and Helm, M.M., 1987. A laboratory energy budget for the ormer *Haliotis tuberculata* L. J. Exp. Mar. Biol. Ecol. 106: 103-123.

Percival, E. and McDowell, R.H., 1967. Chemistry and Enzymology of Marine Algal Polysaccharides. Academic Press, London. pp. 219.

Prince, J.D. and Shepherd, S.A., 1992. Australian abalone fisheries and their management. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo (Editors). Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge 608 pp.

Ravi, J. and Devaraj, K.V., 1991. Quantitative essential amino acid requirements for growth of catla, *Catla catla*. Aquaculture, 96: 281-291.

Reigh, R.C., Braden, S.L. and Craig, R.J., 1990. Apparent digestibility coefficients for common feedstuffs in formulated diets for red swamp crayfish, *Procambarus clarkii*. Aquaculture 84: 321-334.

Robbins, K.R., Norton, H.W., and Baker, D.H., 1979. Estimation of nutrient requirements from growth data. J. Nutr., 109: 1710-1714.

Sagara, J. and Sakai, K., 1974. Feeding experiment of juvenile abalones with four artificial diets. Bull. Tokai Reg. Fish. Res. Lab., 77: 1-7.

Saito, K., 1984. Ocean ranching of abalones and scallops in northern Japan. Aquaculture, 39: 361-373.

Santiago, C.B. and Lovell, R.T., 1988. Amino acid requirements for farmed Nile tilapia. J. Nutr., 118: 1540-1546.

Sauer, W.C. and Ozimek, L., 1986. Digestibility of amino acids in swine: Results and their practical applications. A review. Livestock Prod. Sci., 15: 367-388.

Sauer, W.C., Dugan, M., de Lange, K., Imbeah, M. and Mosenthin, R., 1989. Considerations in methodology for the determination of amino acid digestibilities in foodstuffs for pigs. In: M. Friedman, (Ed.). Absorption and Utilisation of AA Vol. 3, CRC Press, Florida pp. 217-230.

Schiel, D.R., 1992. The paua (abalone) fishery of New Zealand. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo (Editors). Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge 608 pp.

Schwarz, F.J. and Kirchgessner, M., 1993. Influence of different carbohydrates on digestibility, growth and carcass composition of carp (*Cyprinus carpio* L.) pp. 475-478. In: Fish Nutrition In Practice, S.J. Kaushik and P. Luquet (Editors) INRA, Paris, 1993.

Shiau, S.Y. and Lung, C.Q., 1993. No dietary vitamin B_{12} required for juvenile tilapia *Oreochromis niloticus x O. Aureus*. Comp. Biochem. Physiol., 105A: 147-150.

Shimeno, S., Hosokawa, H., Hirata, H. and Takeda, M., 1977. Comparative studies on carbohydrate metabolism of yellowtail and carp. Bull. Jpn. Soc. Sci. Fish. 43: 213-217.

Sugahara, M., Baker, D.H., Harman, B.G. and Jensen, A.H., 1969. Effect of excess levels of dietary crude protein on carcass development in swine. J. Anim. Sci., 29: 598-601.

Suyama, M. and Sekine, Y., 1965. Studies on the amino acid composition of shell-fish proteins. Bull. Jpn. Soc. Sci. Fish., 31: 634-637.

SYSTAT, 1992.Statistics, version 5.2. SYSTAT, Evanston, ILL., 724 pp.

Tarr, R.J.Q., 1992. The abalone fishery of South Africa. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo (Editors). Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge 608 pp.

Taylor, B., 1992. Abalone nutrition: Optimum protein levels in artificial diets for *Haliotis kamtschatkana.* J. Shellfish Res., 11(2): 556.

Tegner, M.J. and Butler, R.A., 1985. The survival and mortality of seeded and native red abalones, *Haliotis rufescens,* on the Palos Verdes peninsula. Calif. Fish and Game, 71(3): 150-163.

Tegner, M.J., Demartini, J.D. and Karpov, K.A., 1992. The California red abalone fishery: a case study in complexity. In: S.A. Shepherd, M.J. Tegner and S.A. Guzmán del Próo (Editors). Abalone of the World. Biology, Fisheries and Culture. Fishing News Books, Cambridge, pp. 370-383.

Thomas, M. and Day, R. W., 1995. Site selection by a small drilling predator: why does the Gastropod *Haustrum baileyanum* drill over muscle tissue of the abalone *Haliotis rubra*? Mar. Freshwater Res., 46: 647-655.

Thomas, M.R., 1995. Design of Prawn Diet Component Digestibility Studies. CSIRO Institute of Plant Production & Processing. Biometrics Unit Report 95/17. Division of Fisheries, Cleveland, Queensland. 3 pp.

Tibaldi, E. and Lanari, D., 1991. Optimal dietary lysine levels for growth and protein utilisation of fingerling sea bass (*Dicentrarchus labrax* L.) Fed semi-purified diets. Aquaculture, 95: 297-304.

Uki, N., Kemuyama, A. and Watanabe, T., 1985(a). Nutritional evaluation of several protein sources in diets for abalone *Haliotis discus hannai.* Bull. Jpn. Soc. Sci. Fish., 51: 1835-1839.

Uki, N., Kemuyama, A. and Watanabe, T., 1985(b). Development of semipurified test diets for abalone. Bull. Jpn. Soc. Sci. Fish., 51: 1825-1833.

Uki, N., Sugiura, M. and Watanabe, T., 1986(a). Requirement of essential fatty acids in the abalone *Haliotis discus hannai.* Bull. Jpn. Soc. Sci. Fish., 52: 1013 - 1023.

Uki, N., Kemuyama, A. and Watanabe, T., 1986(b). Optimum protein level in diets for abalone. Bull. Jpn. Soc. Sci. Fish., 52: 1005-1012.

van Barneveld, R.J., 1993. Effect of heating proteins on the digestibility, availability and utilisation of lysine by growing pigs. PhD Thesis. Dept. of Agriculture, University

of Queensland, 221 pp.

van Barneveld, R.J., Fleming, A.E., Vandepeer, M.E., Kruk, J.A. and Hone, P.W., 1998. Influence of dietary oil type and oil inclusion level in manufactured feeds on the digestibility of nutrients by juvenile greenlip abalone (*Haliotis laevigata*). In preparation.

Varian, (1989). Chromium in Chrome Ores. In: Analytical Methods for Flame Atomic Absorption Spectroscopy. Varian Publication Rev. March 1989 pp. 116.

Viana, M.T., Lopez, L.M., and Salas, A., 1993. Diet development for juvenile abalone *Haliotis fulgens*: Evaluation of two artificial diets and macroalgae. Aquaculture, 117: 149-156.

Viana, M.T., Cervantes-Trujano, M. And Solana-Sansores, R., 1994. Attraction and palatability activities in juvenile abalone (*Haliotis fulgens*): nine ingredients used in artificial diets. Aquaculture, 127: 19-28.

Villamar, D.E. and Langdon, C.J., 1993. Delivery of dietary components to larval shrimp (*Penaeus vannamei*) by means of complex microcapsules. Marine Biol., 115: 635-642.

Walker, T., 1990. Abalone culture in Japan. Austasia Aquaculture, 5(1): 18-20.

Webber, H.H., 1970. Changes in metabolic composition during the reproductive cycle of the abalone *Haliotis cracherodii* (Gastropoda: Prosobranchiata). Physiol. Zool., 43: 213-231.

Wee, K.L., Maguire, G.B. and Hindrum, S.M., 1992. Methodology for digestibility studies with abalone. I. Preliminary studies on the feeding and defecatory behaviour of blacklip abalone, *Haliotis rubra*, fed natural and artificial diets. In: G.L. Allan and W. Dall (Editors), Proceedings of the Aquaculture Nutrition Workshop, 15-17 April, 1991, NSW Fisheries, Brackish Water Fish Culture Research Station, Salamander Bay, Australia, pp. 192-196.

Wee, K.L., Maguire, G.B. and Hindrum, S.M., 1994. Methodology for digestibility studies with abalone. 2. Comparison of markers in an artificial diet for blacklip abalone (*Haliotis rubra*) and greenlip abalone (*Haliotis laevigata*). In: Paper delivered at the Third Asian Fisheries Forum, 26-30 October, 1992, Singapore.

Whittlemore, C.T. and Fawcett, R.H., 1976. Theoretical aspects of a flexible model to simulate protein and lipid growth in pigs. Anim. Prod., 22: 87.

Wilson, R.P., Allen, O.W., Robinson, E.H. and Poe, W.E., 1978. Tryptophan and threonine requirements of fingerling channel catfish. J. Nutr., 108: 1595-1599.

Wilson, R.P., Poe, W.E. and Robinson, E.H., 1980. Leucine, isoleucine, valine and histidine requirements of fingerling channel catfish. J. Nutr., 110: 627-633.

Wilson, R.P. and Poe, W.E., 1985. Apparent digestible protein and energy coefficients of common feed ingredients for channel catfish. Prog. Fish-Cult., 47: 154-158.

Wilson, R.P., 1994. Amino acid requirements of finfish. In: J.P.F. D'Mello (Editor). Amino acids in farm animal nutrition. CAB International, Wallingford, 418 pp.

Wu, C.H., Nakai, S. and Powrie, W.D., 1976. Preparation and properties of acid-solubilised gluten. J. Agric. Food Chem. 24: 504-510.

Yamaguchi, K., Araki, T., Aoki, T., Tseng, C-H. and Kitamikado, M., 1989. Algal cell wall-degrading enzymes from viscera of marine animals. Bull. Jpn. Soc. Sci. Fish., 55: 105-110.

Zhang, Y., Partridge, I.D., Keal, H.D. and Mitchell, K.G. 1984. Dietary amino acid balance and requirements for pigs weaned at 3 weeks of age. Animal. Prod., 39: 441.