THE PROTEIN REQUIREMENTS OF THE SOUTH AFRICAN ABALONE, *HALIOTIS MIDAE*

Submitted in fulfilment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Rhodes University

by

Thomas A. Shipton

December 1999

CONTENTS

ACKNOWLEDGEMENTS…… IV

ABSTRACT…… V

CHAPTER 1. INTRODUCTION…… 1

CHAPTER 2. AN ASSESSMENT OF THE USE OF CHROMIC OXIDE IN DIGESTIBILITY TRIALS FOR *HALIOTIS MIDAE*…….. 9

CHAPTER 3. EVALUATION OF AN *IN VITRO* DIGESTIBILITY TECHNIQUE FOR THE PREDICTION OF PROTEIN DIGESTIBILITY IN FORMULATED FEEDS FOR *HALIOTIS MIDAE*……..…… 32

CHAPTER 4. EFFECTS OF ANIMAL SIZE ON THE ABILITY OF *HALIOTIS MIDAE* TO UTILIZE SELECTED DIETARY PROTEIN SOURCES……. 49

CHAPTER 5. REPLACEMENT OF FISHMEAL WITH SELECTED PLANT PROTEIN SOURCES IN FORMULATED FEEDS FOR JUVENILE *HALIOTIS MIDAE*……. 74

CHAPTER 6. INVESTIGATIONS INTO THE QUANTITATIVE AMINO ACID REQUIREMENTS OF *HALIOTIS MIDAE*…… 103

CHAPTER 7. CONCLUDING DISCUSSION…… 120

REFERENCES……. 125

ACKNOWLEDGMENTS

Many people have played a role in this work – peers, sponsors, friends. Without my peers I wouldn't have learnt how to approach the problems, without the sponsors I couldn't have afforded to address the problems, and without my friends, well, I'd have packed up long ago and gone home……. so while I am grateful to them all, there are a number that I must mention individually.

Firstly, my supervisor Pete Britz. As a student, supervisors come one-a-penny, and while in my time I seem to have crossed a few of their paths, Pete has been the exception. Whether it has been persuading a non-surfing shark scared, pale pasty Englishman to jump off Port Alfreds West pier into barreling six foot surf; letting me wander off with my ideas about nutrition – and then, sometimes having to pick up the pieces; suggesting I take this or that into account; encouraging me through the darkest hours; the unenviable re-reading of scripts; what can I say Pete – thanks.

Much of the experimental work was carried out at the Port Alfred Marine Laboratory – endearingly known by those who work there as Legoland. The work would not have been possible without both a sense of humour and the help from Bucs and Jerri. What with Bucs and his experimental diving techniques, Jerri losing his beard, me, my refrigeration plant – I had a lot of fun, and can't thank you enough for being there for my abalone.

The departmental statistical gurus Horst Kaiser and Tony Booth are thanked for their help deciphering the numbers. Their courage in the face of continual barrages of daft questions will not go unforgotten. Thanks to dear Madam Anu, may you rest in peace now, finally write up, and stop sending me microbiological papers through internal mail that complicate an already complicated life……. and of course Abby for the arguments.

I am indebted to my sponsors : Sea Plant Products ltd., Hermanus Abalone ltd., The National Research Foundation, Rhodes University Joint Research Committee and THRIP. In particular, I'd like to thank Craig Clayden (Sea Plant Products Ltd.) for his infectious enthusiasm for the project, preparation of diets and the constant supply of experimental animals. Marrianne Hundley for the University of Natal for her input with the amino acid work. Mr Morley (Pharmacy Dept.) for advice, and allowing me access to his facilities.

Protein sources for the experimental diets were kindly donated by : EPOL SA, Esbjerg Fiskeindustri, Fiskernes Fiskeindustri, National Sorghum Breweries, Illova Sugars Ltd., Abakor Ltd., Sopropêche.

To Nico, KJ and Mr Aart – through the kaleidoscope of their sanity, they became the guardians of mine. Indeed rare people that walk the earth. Thank you for your love and support. Candice – for whom words are a little difficult now, thank you for everything. Rob – so the joke continues !

And finally to my parents, who have always held an unflinching belief in my education. Back in the autumn of 1974 they packed a pink blazered little boy off to the Hall School, Hampstead. Cap shorts and a cheesy grin – little did they know that his formal education would come to a close 25 years later ! For them mere gratitude is not enough.

ABSTRACT

The potential to reduce feed formulation costs by the replacement of existing protein sources in artificial feeds for *Haliotis midae* was assessed. A comparison between the efficacy of the direct method and chromic oxide marker techniques in determining apparent protein digestibility coefficients, revealed that while the former was not a suitable methodology for use with this species, the latter produced replicable and reliable results. It was established that the chromic oxide marker is inert, is not absorbed by the abalone, does not interfere with the digestive processes and moves through the intestine at the same rate to the protein. However, as this method was time consuming and expensive to implement, a multienzyme pH-stat *in vitro* protein digestibility technique using a three enzyme system was employed to rapidly assess the protein quality of 34 protein sources. The efficacy of the technique was established by correlating the *in vitro* digestibility estimates with *in vivo* digestibility coefficients obtained using the chromic oxide marker technique. The highest degree of *in vivo* predictability was attained when protein sources were separated according to origin, and significant correlations between either animal (r^2=0.89, P<0.004) or plant (r^2=0.79, P<0.04) protein sources were found. The effect of animal size on the qualitative protein requirements of two size classes of *H.midae* was assessed by feeding 12 isonitrogenous and isoenergetic single protein diets to juvenile and young adult animals (10-20 and 40-50mm shell length). The criteria for protein source selection were their bioavailability as determined using the pH-stat *in vitro* digestibility technique, and their cost and availability within South Africa. The protein sources identified for the trial comprised four fishmeals, casein, spirulina, abalone viscera silage, brewery waste, torula yeast, carcass, sunflower and cotton meals. The results indicated that in terms of growth and feed efficiency, the fishmeals and spirulina were the most suitable candidates as primary protein sources in formulated feeds for *H.midae*, and with the exceptions of the carcass meal and brewery waste, the remaining protein sources demonstrated promise as partial primary protein source replacements. The smaller size class of abalone displayed significantly reduced growth, feed and protein efficiency than their larger counterparts. In terms of feed conversion and growth response, two-way analysis of variance revealed significant interactions between protein source and animal size, suggesting that qualitative differences exist between the dietary protein requirements of the juvenile and young adult abalone. The commercial implication of this finding was discussed. An evaluation was undertaken to determine the effects of the partial and total replacement of dietary fishmeal with selected plant protein sources on growth and nutritional indices of juvenile *H.midae*. A commercial “Abfeed” formulation in which 100% of the protein component comprised LT-fishmeal was employed as a control. Fifteen isonitrogenous experimental diets were formulated in which the LT-fishmeal was substituted at either 10, 15, 30, 50, 75 or 100% with either spirulina, semolina, ground maize, torula yeast, soya, sunflower or corn gluten meals or combinations thereof. No significant differences were found in the growth rates between the control diet and those diets in which 30% of the fishmeal component had been replaced by either soya, sunflower meal, or torula yeast. In addition, 50% of the fishmeal component could be substituted with either soya meal or spirulina without affecting growth. Replacement of either 75 or 100% of the fishmeal had a significant negative

affect on growth. Pearson product moment correlations between dietary lysine levels and either growth rates or protein efficiency ratios revealed positive correlations ($r=0.77$, $P=0.0005$; $r=0.52$, $P=0.04$ respectively) suggesting that lysine may have been the first limiting amino acid in these diets. Carcass analysis revealed that dietary protein source had no significant effect on body composition. An assessment of the dietary arginine requirement of juvenile *H.midae* using whole proteins to supply graded levels of dietary arginine did not promote a growth response. It was concluded that arginine is probably not the first limiting amino acid in formulated feeds for *H.midae.* An assessment of the dietary lysine requirement of juvenile *H.midae* using two micro-encapsulation techniques (gelatin/acacia colloid or cellulose acetate phthalate) was not successful. The efficacy of the encapsulation techniques were established and an assessment of the degree of lysine supplementation undertaken. Failure of the crystalline lysine enriched diets to promote growth suggests that the prospects for using crystalline amino acids in essential amino acid requirement studies in *H.midae* is low. The results of the present study indicate that the prospects for replacing fishmeal with cheaper alternative protein sources in artificial feeds for *H.midae* is promising. Furthermore, while the technical difficulties such as the determination of the essential amino acid requirements of the abalone precluded the application of “least cost” programming, the prospects for its future application are promising.

CHAPTER 1

INTRODUCTION

Abalone are herbivorous marine gastropods belonging to the genus *Haliotis*. The genus contains approximately 100 species of which only 22 are of commercial importance (Hahn, 1989a). Of the six species found in South African waters, only *H.midae* is of commercial importance. The remaining five species either occur in insufficient numbers to be commercially exploitable, or are too small or difficult to harvest (Cook, 1998).

Abalone are sedentary animals typically inhabiting the rocky intertidal and shallow littoral zones to about 20 meters. Physical ease of access, combined with their sedentary nature make them easily accessible to both legal and illegal exploitation, which in combination with the high value of the animal – typically US$20-40/kg live weight (Rudd, 1994) – naturally makes the resource vulnerable to overexploitation. Indeed, abalone fisheries throughout the world have declined over the past 20 years, and in the cases of Mexico, Central and Southern California, culminated in a state of collapse (Guzm<n del Próo, 1992; Daniels and Floren, 1998; Davis *et al*., 1998). In South Africa, catches have reduced from a peak of 2800 tons in 1965 (Tarr, 1992) to little more than 600 tons post 1971 when a quota system was introduced. Recent developments, including increased recreational and poaching pressures, have renewed incentives to further reduce the total allowable catch, which in 1997 stood at 550 tons (Stuttaford, 1997). With catch quotas unlikely to improve in the foreseeable future, a number of fishing companies

involved in the abalone fishing industry have identified abalone aquaculture as an expansion alternative (Cook, 1998).

Pioneering work by the Japanese over 50 years ago (Murayama,1935 (in Hahn 1989b); Ino, 1952) set the stage for abalone aquaculture. While the development of culture and feed technologies that originated there have now been established in a number of countries including USA and Mexico (McBride, 1998), Ireland (Hahn, 1989c), Chile (Godoy and Jerez, 1998), China (Nie, 1992), South Africa (Cook, 1998), Taiwan (Chen, 1989), New Zealand and Australia (Hahn, 1989c). The development of abalone aquaculture in South Africa has to date lead to a total investment in excess of R80 million in the industry, and the establishment of twelve farms that are registered with the Abalone Farmers Association of South Africa (Cook, 1998).

Central to the success of any farmed animal, be it terrestrial or aquatic, is the provision of a nutritionally complete diet that satisfies both the basic physiological requirements and those necessary to support somatic growth. As marine herbivores, the diet of post-larval juvenile Haliotids consists primarily of diatoms and flagellate microalgae (Garland *et al*., 1985; Sakata, 1989) which on weaning (5-10mm shell length), changes to macroalgaes (Hahn, 1989d). Despite the widespread use of natural feeds in commercial abalone farms, a number of authors have suggested that the development of nutritionally complete artificial feeds are fundamental to the growth of the industry (Hahn, 1989b; Fallu, 1991; Britz *et al*., 1994). While Britz (1995) highlighted the advantages of artificial feeds over their natural macroalgal counterparts, Fleming *et al*. (1996) reviewed the current status of artificial feed development. In summary, while research is currently being undertaken in

eleven countries, fourteen diets are currently either in commercial or pilot production worldwide. In South Africa, the development of artificial feeds was instigated in 1989 at Rhodes University, and culminated in the development of a commercial feed, ABFEED®, manufactured by Sea Plant Products Ltd. in Hermanus.

Initial investigations into artificial feeds for abalone have demonstrated that animal feed science models can be successfully applied to Haliotid nutrition (Britz, 1995). Furthermore, while issues pertaining to the gross requirements for nutrients such as protein (Uki *et al*., 1986; Taylor, 1992; Mai *et al*., 1995a; Britz, 1996a), lipid (Uki and Watanabe, 1992; Mai *et al*., 1995b), vitamins and minerals (Uki *et al*., 1985b; Coote *et al*., 1996) have been identified and resolved, there is as yet a paucity of information concerning the more specific dietary requirements such as those for individual vitamins, minerals, amino acids and essential fatty acids. Nevertheless, as protein is not only the single most expensive dietary component, but is in addition pivotal to somatic growth, it is naturally one of the first nutrients to be investigated when attempts are made to design diets that maximize growth at least cost. Thus, protein requirements of abalone were chosen as the focus of this study, and only those issues pertaining to the protein and amino acid requirements of abalone will be discussed further.

Synthesis of protein and amino acid nutrition in Haliotids

In a review of the current status of artificial feed development, Fleming *et al*. (1996) noted that while a number of protein sources are employed at low inclusion levels, the most commonly used protein sources incorporated into artificial feed formulations are fishmeal, casein and soya meal. In the event of negative fluctuations in commodity

prices, the dependence on only three protein sources to supply the majority of protein in formulations has obvious commercial implications. Indeed, with world fish stocks used for fishmeal production in decline (Harvey, 1991; New, 1991), the future supply of fishmeal is not likely to increase in the foreseeable future (Rumsey, 1993) which, in the context of increased competition for a limited resource will inevitably promote price increases. In addition, the commercial prospects for casein as a source of protein are probably restricted to exceptional cases such as New Zealand where cheap supplies can be sourced from the dairy industry (Fleming *et al*., 1996). In contrast, while soya meal has been shown to be an effective partial fishmeal replacement in fin fish diets (Dabrowski *et al*., 1989; Pongmaneerat and Watanabe, 1992; Viyakran *et al.,* 1992), and is likely to become a major protein in future aquaculture feeds (Crowder, 1990), its incorporation into commercial abalone feeds is promising. Furthermore, it has been well documented that abalone have the ability to utilize a large number of protein sources of both terrestrial and aquatic origin (Table 1.1), and thus the prospect for reducing the feed industry's reliance on only three major protein sources is promising.

Early attempts to determine abalone's dietary protein requirements have tended to be growth - response trials (Ogino and Kato, 1964; Uki *et al*., 1986; Taylor, 1992; Britz, 1996a). Protein sources under investigation are fed to animals at incremental levels, and the requirement calculated as the point at which the maximal yield is attained at the minimum level of dietary protein. Although this approach is easy to implement, it is too simplistic as not only is it difficult to define the point of maximal yield (Fleming *et al*., 1996), but in addition, it fails to take into account a number of factors affecting protein utilisation. While lack of consideration of protein quality in terms of available energy, amino acid composition

Table 1.1. Protein sources used in experimental formulations for abalone.

Protein Source	Abalone Species	Reference
Abalone viscera silage	*H.fulgens, H.midae*	Viana *et al*., 1994, 1996; present study
Alcoholic active sludge	*H.asinina*	Sagara and Sakai, 1974
Barley	*H.laevigata*	Fleming *et al*., 1998
Brewery waste	*H.midae*	Present study
Casein	*H.discus hannai, H.tuberculata H.iris, H.midae, H.fulgens, H.kamtschatakana, H.laevigata*	Uki and Watanabe 1992; Britz, 1996b; Mai *et al*., 1995a; Viana *et al*., 1993a; Taylor, 1992; Fleming *et al*., 1998; Van Barneveld *et al*., 1998; López *et al*., 1998; present study
Chlorella	*H.discus hannai*	Sagara and Sakai, 1974
Corn gluten meal	*H.discus hannai, H.midae*	Uki *et al*., 1985a, present study
Cotton meal	*H.midae*	Present study
Egg albumin, whole egg	*H.discus hannai*	Uki *et al*., 1985a
Faba beans	*H.laevigata*	Vandepeer *et al*., 1999
Field peas	*H.laevigata*	Vandepeer *et al*., 1999
Fishmeal	*H.discus hannai, H.midae, H.fulgens, H.laevigata H.tuberculata*	Uki and Watanabe, 1992; Britz, 1996b; Fleming *et al*.,1998; Viana *et al*., 1993a, 1996; López *et al*., 1998; present study
Fish silage	*H.fulgens*	Viana *et al*., 1994, 1996.
Gelatine	*H.midae, H.discus hannai, H.fulgens*	Uki *et al*., 1985a; Knauer *et al*., 1993; Viana *et al*., 1994; present study
Lupin Kernel meal	*H.laevigata*	Fleming *et al*., 1998 Vandepeer *et al*., 1999
Maize meal	*H.midae*	Present study
Meat meal / Carcass meal	*H.rubra, H.midae*	Maguire *et al*., 1993; present study
Rye grass protein concentrate	*H.discus hannai*	Uki *et al*., 1985a
Semolina	*H.rubra, H.laevigata*	Fleming, 1994; Fleming *et al*., 1998; Van Barneveld *et al*., 1998; Present study
Soya meal	*H.discus hannai, H.fulgens, H.laevigata, H.midae*	Uki *et al*., 1985a; Viana *et al*., 1994, 1996; Van Barneveld *et al*., 1998; Vandepeer *et al*., 1999; Britz, 1996b; present study
Spirulina	*H.midae*	Britz, 1996b; present study
Squid meal	*H.rubra*	Maguire *et al*., 1993
Sunflower meal	*H.midae*	Present study
Vetch	*H.laevigata*	Vandepeer *et al*., 1999
Yeast	*H.discus, H.midae*	Ogino and Kato, 1964; Britz, 1996b; present study

and digestibility may lead to inaccurate requirement estimations (Wilson, 1989), other factors such as temperature (Windell *et al*., 1978), animal size, sex and genotype (ARC, 1981) may also affect protein utilisation. In light of the lack of attention that has been

focused on these parameters in past experimental design, it follows that many of the trials have not been investigating the protein requirements *per se,* but have inadvertently incorporated other parameters into their experimental design. Thus, although the results are of use as general guides to the gross protein requirements, a more rigorous approach is needed to fully understand the protein and amino acid requirements of the abalone.

While it is generally accepted that animals eat to satisfy their energetic requirements (Smith, 1989), they do not have a protein requirement *per se*. Rather, they have a requirement for amino acids - both essential (EAA) and non-essential (NEAA) - that supplied in the correct ratios with the appropriate level of digestible energy will maximise somatic growth. Non-essential amino acids are those that can be synthesised by the animal, however, essential amino acids cannot be synthesised and must therefore be supplied as a part of the diet (Steffens, 1989a). Furthermore, as it is also generally accepted that the amino acid profile of an animal gives an indication of the relative proportions of the EAAs required in the diet (Cowey and Tacon, 1982; Benitez, 1989); initial investigations into the amino acid requirements of a novel group of species should determine both their essentiality and the levels at which they are found in the body of the animal. Allen and Kilgore (1975) demonstrated that *H.rufescens* requires the same 10 essential amino acids as most other animals (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine), and the amino acid profiles of a number of Haliotids have also been determined (Allen and Kilgore, 1975; Knauer *et al*., 1995a; King *et al*., 1996). However, it must be noted that while the amino acid profile gives an indication of the pattern of dietary EAAs required to promote optimal growth, it gives no indication of the absolute dietary requirements. In order to determine these requirements, the inclusion rate

of at least one of the EAAs must be determined empirically and the inclusion rates of the others calculated relative to the known. Britz *et al*. (1997) failed to determine the arginine requirement of *H.midae*, and there is currently no published information concerning either arginine or any of the other essential amino acid requirements, and as such, this is clearly an area of Haliotid nutrition that requires further investigation.

While quantitative determination of the protein, amino acid and energy requirements is a prerequisite to the development of diets that maximize feed utilization, the determination of nutrient availability from feed sources is also required if diets are to be designed that satisfy the nutritional requirements of the animals. Digestibility trials that provide an indication of nutrient bioavailability are therefore essential to the formulation of nutritionally complete diets. Trials with *H.laevigata* have met with considerable success (Fleming *et al*., 1998; Van Barneveld *et al.*, 1998; Vandepeer *et al*., 1999), and attained a level of sophistication such that reliable digestible crude protein, amino acid and energy estimations can be undertaken. Digestibility trials using *H.midae* have met with little success (Britz, 1995), and clearly this is an area of research that requires further investigation.

Aims and objectives of the current study

The current study should be viewed as a continuation of a broader research project initiated by Rhodes University into the development of artificial feeds for abalone. The aim of this study was to investigate the protein and amino acid requirements of *H.midae* with a view to providing information for the formulation of "least cost" feeds for the abalone farming industry.

The objectives were :

1. To identify and evaluate a suitable digestibility technique for use with *H.midae*.
2. To evaluate an *in vitro* protein digestibility technique, and use it to predict the apparent protein digestibility coefficients of potential protein sources for inclusion in artificial feeds.
3. To investigate the effects of animal size on the protein requirements of *H.midae*.
4. To investigate the effects of partial and total replacement of the fishmeal component with plant protein sources in formulations for *H.midae*.
5. To assess whether *H.midae* has the ability to utilize crystalline lysine and if so, use it to determine the essential amino acid requirements of the animal.

CHAPTER 2

AN ASSESSMENT OF THE USE OF CHROMIC OXIDE AS A MARKER IN DIGESTIBILITY TRIALS WITH *HALIOTIS MIDAE*.

INTRODUCTION

The determination of nutrient digestibilities of dietary components is a necessary prerequisite to the formulation of biologically and economically optimized diets. Digestibility is difficult to measure directly in Haliotids due to problems associated with the accurate measurements of feed ingested and faeces voided (Wee *et al.,* 1992). As a consequence, it is necessary to investigate indirect methods using inert markers which pass through the digestive system without being absorbed by the abalone. Digestibility of a nutrient is thus indirectly based on the ratios of marker to nutrient in the feed, and marker to nutrient in the faeces, circumventing the necessity to measure quantitatively the total amount of feed ingested and faeces voided. A reliable marker must not be absorbed by the animal, it must be inert and not interfere with the digestive processes, and must move along the gut at a similar rate as the component under study (DeSilva, 1988).

Attempts to determine nutrient digestibility coefficients in Haliotids are relatively recent. While Maguire *et al.* (1993) identified possible markers for use in digestibility trials, Wee *et al.* (1994) undertook a comparative study to determine the best marker for use with *H.rubra* and *H.laevigata*. It was concluded that internal markers such as acid insoluble ash, hydrolysis resistant organic matter (HROM) and crude fiber were unsuitable due to the

abalone's ability to digest complex carbohydrates, which form a constituent of these markers. The most promising results were obtained with acid insoluble ash and chromic oxide.

Although attempts to use chromic oxide as a marker in digestibility trials for *H.midae* have failed due to problems attaining replicable and reliable results (Britz, 1995), it has been successfully applied in studies with *H.laevigata* (Fleming *et al*., 1998; Van Barneveld *et al.*, 1998; Vandepeer *et al*., 1999) and other aquatic invertebrates such as penaeid shrimps (Fenucci *et al*., 1982; Merican and Shim, 1995; Ishikawa *et al*., 1996; Deering *et al*., 1996). Nevertheless, reports have suggested that it is an unsuitable maker for use with a number of invertebrate species. For example, Leavitt (1985) observed differential passage of the marker through the intestine of the American lobster, *Homarus americanus*, a result previously observed in caridean prawns (Forster and Gabbott, 1971). Unfortunately, these studies investigated the movement of chromic oxide with respect to total organic matter and not individual nutrients. Ishikawa *et al*. (1996) correlated the movement of chromic oxide with protein, carbohydrate and cholesterol in *Penaeus japonicus*. Although a low correlation between the movement of the marker with respect to carbohydrate and cholesterol was found, a strong correlation was observed between the marker and protein concentrations. They concluded that chromic oxide could be used as a marker to determine protein digestibility, but was unsuitable for carbohydrate or cholesterol digestibility determinations.

The aim of the present study was to assess the suitability of chromic oxide as an external marker in the determination of apparent protein digestibility in *H.midae*. This was achieved by determining whether the chromic oxide was absorbed by the abalone, determining the effect of chromic oxide on digestibility coefficients, and the

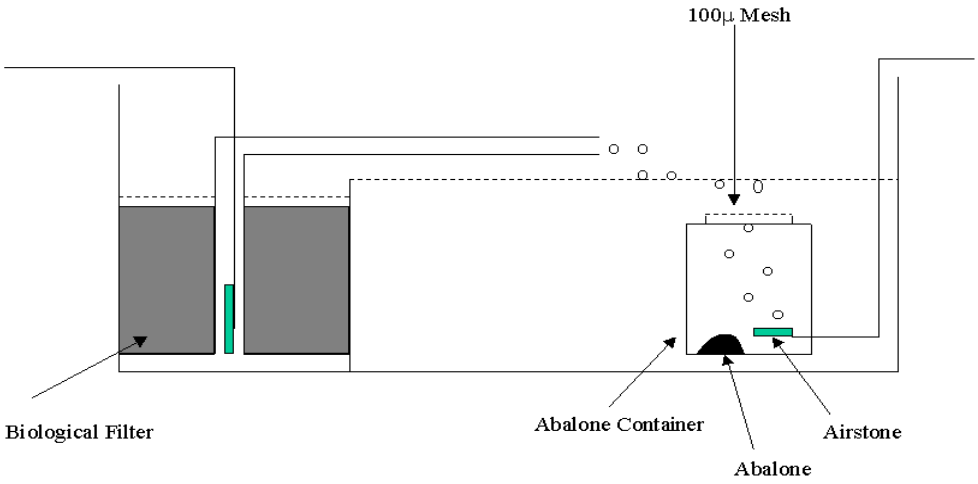
characterization of the movement of chromic oxide and protein along the intestinal tract of the abalone.

METHODS

Experimental system, diets and animals

Experimental Trials 1 and 3 that were undertaken to observe the types of faecal material produced, and to determine the chromic oxide and protein distribution in the faeces, were carried out in a constant environment room. Three replicate recirculating 106 litre glass aquaria, comprising a 36 litre biological filter and an open 70 litre compartment were used throughout the trials (Figure 2.1). Water flow through the filter was maintained by an airlift pump. Each animal was housed in a separate perforated 1 litre plastic jar with a screw top lid. A 40mm hole was drilled in each lid and a piece of 100μm mesh attached so as to cover the hole. This enabled feed and faecal material to be collected by lifting the jar and draining the water through the mesh. Half the surface area of the jar was painted black to encourage the photophobic abalone to move away from the mesh during the day when faecal collections took place. Water flow through the plastic jars was maintained by means of an air-stone. Water temperature was maintained at 18°C by means of a thermostatically controlled aquarium heater, and salinity at 35$\pm$1 p.p.t. The photoperiod was set at 10L:14D with the dark period starting at 18:00. Two thirds of the water in each aquarium was replaced each week with sand filtered seawater. Prior to experimentation the abalone had been reared exclusively on a fishmeal based commercial diet ("Abfeed", manufactured by Sea Plant Products Ltd).

Figure 2.1 Experimental System in which Trials 1 and 3 were undertaken.



In experimental Trials 2, 4 and 5 quantitative collection of faeces was undertaken in four litre glass jars that were placed in a water bath that was maintained at 18°C. As in Trials 1 and 3, a piece of 100μm mesh was attached to the lid of the jars to enable faecal collections to be undertaken. As it was necessary to collect all the faecal material produced during these trials, there was no movement of water between the jars containing the abalone and the water bath; and in addition, to prevent the loss of faecal material via filtration, there was no water filtration within the jars. Oxygenation and water quality was maintained by bubbling air into each jar, and depending upon the trial, replacing the water every four or six hours with sand filtered seawater. Faecal material was collected in the same manner as previously discussed for Trials 1 and 3, and the photoperiod set at 12L:12D with the dark period starting at 18:00.

Two extruded fishmeal based diets containing 20% protein were formulated. The control diet contained no chromic oxide, and the experimental diet, 0.5% chromic oxide (Table 2.1). Correction factors for total solids leaching from the diets were 0.91 and 0.92

for the experimental and chromic oxide diets respectively. These figures were determined by placing 4g of feed in a control container, placing it in an aquaria for a 12h period and calculating the dry weight loss over this period. Correction factors were used where appropriate during the quantitative trials.

Table 2.1 Composition of experimental diets (g $100g^{-1}$ dry weight).

Ingredient	Diets	
	Chromic Oxide	Control
Fishmeal [a]	31.4	31.4
Starch	21.5	21.5
Lipid [b]	3.8	3.8
Vitamin / Mineral mix [c]	1.0	1.0
Chromic Oxide	0.5	-
α-cellulose [d]	41.8	42.3

Dietary components supplied by : [a.] Caldera Ltd. Chile. [b.] Marine Oil Refiners, SA. [d.] Sigma Chemicals, USA.
[c.] Composition from Uki *et al*. (1985b)

Trial 1 - Preliminary observations into the types of faeces produced

Preliminary observations into the types of faeces produced were carried out over a seven day acclimation period to the experimental diet containing 0.5% chromic oxide. Three replicate groups of six abalone (69.9±3.5mm shell length, 74.8±5.3g) were fed the experimental diet *ad libitum* at 18:00 daily. The following morning at 08:00, unconsumed feed and faecal material that had accumulated throughout the night was removed. The types of faeces produced during the night and over the following 10 hour daylight period were observed, and the production of three distinct types of faeces found.

Trial 2 - Characterization of the diurnal and nocturnal pattern of faeces production over a 96h period

Three replicate groups of four abalone (65.4 ± 4.2mm shell length, 72.4 ± 6.7g) were used to characterize and quantify the pattern of production of the three faecal types, and to determine the optimal time to collect faecal samples. As abalone are erratic feeders and do not necessarily feed every night (Knauer, 1994), the animals were starved for a seven day period prior to experimentation. This was undertaken so as to ensure that on presentation of the feed, there would be a rapid feed response in which all the abalone would feed to satiation. The animals were presented with the chromic oxide labeled diet at the beginning of the 12h dark period at 18:00. Six hours later at 24:00, the uneaten feed was removed. Faecal samples were subsequently obtained every 6hrs for the following 36hrs, and subsequently every 12hrs until 96hrs had elapsed. Faecal samples were collected by lifting the glass jar that housed the abalone from the water and allowing the water to drain through the mesh which retained the faecal material. The abalone were then washed twice in a small amount of seawater to remove any faecal material that may have become attached to the shell of the animal. The washings were filtered through the mesh, thus ensuring that all the faecal material was collected. The mesh was placed on a piece of paper toweling to absorb excess water. Dissecting forceps were used to sort the faeces *in situ* into the three faecal types which were then lifted from the mesh and placed on aluminum foil. The faeces were oven dried at 60°C for 24 hrs, stored at -20°C, and the weights of each faecal type produced over the 96h period recorded.

Trial 3 - Determination of chromic oxide and protein distribution in the faeces, and the calculation of the apparent protein digestibility

Following the seven day acclimation period to the test diet in Trial 1, feeding was continued at 18:00 daily, as was the removal of unconsumed feed and faecal material the following morning at 08:00. Two faecal collections were made daily at 12:00 and 16:00. Faecal collections, processing and storage were undertaken in a similar manner to that previously discussed in Trial 2. The three types of faeces outlined in Trial 1 were collected individually as well as a random sample of pooled faecal material.

In order determine the chromic oxide and protein concentrations in the faeces, duplicate 20mg samples from each replicate were digested in 2.5ml H_2SO_4 (98% AR grade, Sigma chemicals) and 1.5g Kjeldahl-pak catalyst (Sigma Chemicals) at 360°C for 70 minutes. The digest was diluted to 50ml with deinonized water. A 5 ml aliquot was removed and analyzed for chromium according to Athanasopoulous (1993) using a GBC-909 atomic absorption spectrophotometer. The remaining 45mls was distilled in a Kjeldahl Vapourdest and analyzed for protein content (Kjeldahl N x 6.25).

Apparent digestibility coefficients were calculated according to the formula from Maynard and Loosli (1956):

$$\text{Apparent Protein Digestibility (\%)} = 100 - \left[100x \frac{\%Cr_2O_3 \text{ in feed}}{\%Cr_2O_3 \text{ in feces}} \text{ x } \frac{\text{\% protein in feces}}{\text{\% protein in feed}} \right]$$

Trial 4 - An assessment of the inertness of the chromic oxide marker and a comparison between the direct and marker methodologies of determining apparent protein digestibility coefficients

Due to variation in the apparent protein digestibility coefficients between the three fecal types, a comparison was made between the chromic oxide and total collection methodologies. In order to achieve this, a similar methodology to that described in Trial 2 was employed. In summary, following a 7 day starvation period, four replicate groups of four animals (67.4 ± 5.2mm shell length, 73.3 ± 7.7g) were presented with a known amount of chromic oxide labeled feed for a six hour period at the beginning of the dark period. Following this period the uneaten feed was removed, oven dried to constant weight at 60°C, and the total feed ingested calculated by subtraction. The faecal material was collected at 4 hourly intervals for the subsequent 96h period. Within replicates, faecal material was pooled and oven dried at 60°C to constant weight. The quantity of protein voided in the faeces was determined using Kjeldahl analysis (N x 6.25); and in combination with the known amount of protein ingested, the apparent protein digestibility coefficients calculated. In addition, to determine whether the presence of dietary chromic oxide was inert or affected the apparent protein digestibility coefficients, a similar trial was undertaken with the control diet containing no chromic oxide. It was hypothesized that a quantitative difference in apparent protein digestibility coefficients between the experimental and control diets, would indicate that the presence of the chromic oxide had an effect on digestibility coefficients.

Trial 5 - Determination of the absorption of the chromic oxide marker

Two approaches were used to determine whether the abalone had the ability to absorb the chromic oxide marker. In the first approach, abalone were fed a measured dose of the chromic oxide experimental diet, and the recovery of the chromic oxide in the seawater and faecal material was determined. The experimental conditions and regime were similar to those previously described in Trial 2. In summary, three replicate groups of four animals (65.4 ± 4.2mm shell length, 72.4 ± 6.7g) were starved for a 7 day period prior to experimentation. At the start of the trial, each replicate group was fed a measured dose of chromic oxide diet over a six hour period. At the end of the feeding period, uneaten feed was removed, oven dried to constant weight at 60°C, and the amount of chromic oxide ingested calculated by subtraction. The faecal material produced during the subsequent 96h period was collected at four hourly intervals. In addition, to account for chromic oxide that may have leached from the faeces during the four hour period between collections, a 10ml aliquot of seawater was collected from all the filtrates following the removal of the faecal material. The chromium contents in the faecal and water samples were analyzed according to the methodology outlined in Trial 3, and the respective percentage of chromic oxide recovered calculated for each replicate.

Due to difficulties associated with the collection of all the faecal material produced during total collection studies in an aquatic environment, an alternative methodology was developed to substantiate the results obtained from the chromic oxide recovery study. Thus, a study was undertaken to determine whether animals fed a chromic oxide diet retained chromic oxide in their body tissues. The tissues of wild caught animals that had not been exposed to the chromic oxide diet were used as a control. Five animals

(73.6±2.0mm shell length, 64.2±6.6g) were fed for a 60 day period on the experimental chromic oxide diet. After the 60 day period, the animals were purged for 96 hrs to evacuate faeces from the gut. Five wild animals were collected from Fish River Point, Eastern Cape (61.2±1.0mm shell length, 43.8±2.4g), and also purged for 96 hrs. The animals were shucked, oven dried at 105°C for 24 hrs and stored at -20°C until analyzed. To obtain a representative sample of shell deposited while the animals had been fed the chromic oxide diet, a 2-3mm band of shell nacre was scraped from the anterior growing edge of the shell using a scalpel. This was repeated on the wild-caught abalone. The shell and shucked material was analyzed for chromium using the method outlined in Trial 3. Analyses were performed in duplicate.

Statistical analysis

One way analysis of variance (ANOVA) followed where appropriate by Student-Newman-Keuls multiple comparison procedures were used to determine variations in the chromic oxide and protein concentrations between faecal types, the associated digestibility coefficients, the quantification of the types of faeces produced from a given meal, the associated estimation of chromic oxide and protein excretion, and the evacuation times of the three faecal types. Pearson product moment correlations were used to determine the strengths of association between faecal chromium and protein concentrations. Student t-tests were used to compare the effects of the chromic oxide marker on digestibility coefficients, to compare the absorption of chromium between wild and experimental animals, and within faecal types, to compare diurnal and nocturnal faeces production. To model the production of the three faecal types over a 96h period,

and to estimate the 5 and 50% evacuation times for each of the three faecal types, non-linear regressions using the following formula were employed:

$$\%P_t = 1 - e^{-kt}$$

Where k is the rate, and %Pt is the production of faeces at time t.

RESULTS

The faecal observations undertaken in Trial 1 indicated that the abalone produced three distinct forms of faeces that could be separated into faeces types (Figure 2.2) :

Type 1 – Dark green discrete faecal pellets and strings. Faecal pellets less than 4mm long, no visible mucous present. Strings up to 20mm long, string integrity maintained by mucosal coating.

Type 2 - Medium green faeces, faecal pellets less than 4mm long, often broken up to form clumps, mucous visibly present.

Type 3 – Light green amorphous faeces, no discernable pellet, faecal material homogeneously mixed with mucous.

The results from Trial 2, characterizing the production of the three faecal types from a single meal over a 96h period are presented in Figure 2.3. No significant differences were found between the quantities of the three types of faeces produced ($P>0.05$, Table 2.2). No significant differences were found in the time periods in which either 5 or 50%

Figure 2.2. Trial 1 - Types of faeces produced by *H.midae* fed a semi-purified diet containing 0.5% chromic oxide.

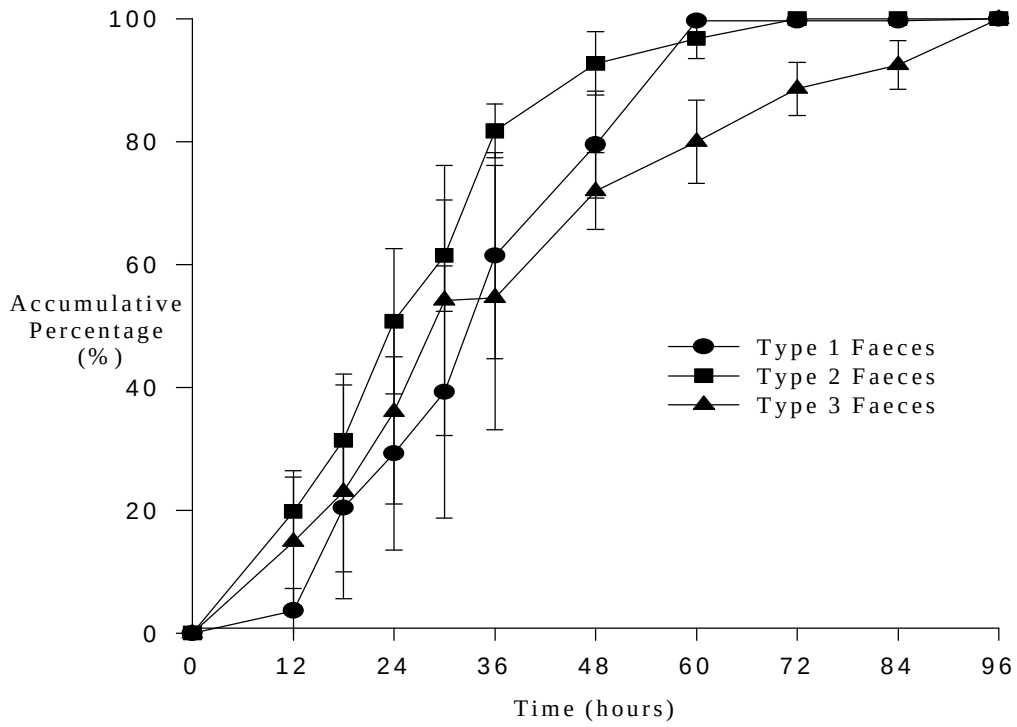
Type 1 Type 2 Type 3

of the three faecal types were produced over the 96h period (P>0.05, Table 2.3). Diurnal and nocturnal production of each faecal type did not differ significantly (P>0.05, Table 2.3).

The chromium and protein contents of the three faecal types determined during Trial 3 are presented in Table 2.4. One way analysis of variance revealed that between faecal types, there were significant differences in the both chromium and protein concentrations (chromium : P<0.0001, F=156.3; protein : P=0.006, F=13.4). While no significant difference was found between the protein contents of Types 2 and 3 faeces, Type 1 faeces were found to contain a significantly greater protein concentration than either Types 2 or 3 (P<0.05). In all faecal types, significant differences were found between the

chromium concentrations ($P<0.05$). The lowest chromium concentrations were observed in Type 3 faeces and the greatest in the Type 1 faeces.

Figure 2.3. Trial 2 - Production of the three faecal types over 96 hours following the cessation of feeding at time zero (Mean ± S.E.).



A Pearson Product Moment Correlation across all the faecal types indicated a significant positive correlation between faecal chromium and faecal protein contents (Figure 2.4, $r=0.89$, $P<0.001$, $n=9$). In confirmation of these results, a Pearson Product Moment Correlation between faecal chromic oxide and faecal protein levels obtained during Trial 5 again revealed a positive correlation ($r=0.76$, $P=0.0296$, $n=24$), and therefore suggest similarity in the rate of movement of chromic oxide and protein along the digestive tract.

Table 2.2. Trial 2 - Quantification of faecal production (dry weight). Values are presented as means and standard errors (n=3). Different alphabetic superscripts indicate significant differences (P<0.05).

Faecal Type	Total faeces produced (%)
Type 1	26.9 ± 2.4^{m}
Type 2	33.1 ± 5.4^{m}
Type 3	40.5 ± 7.8^{m}

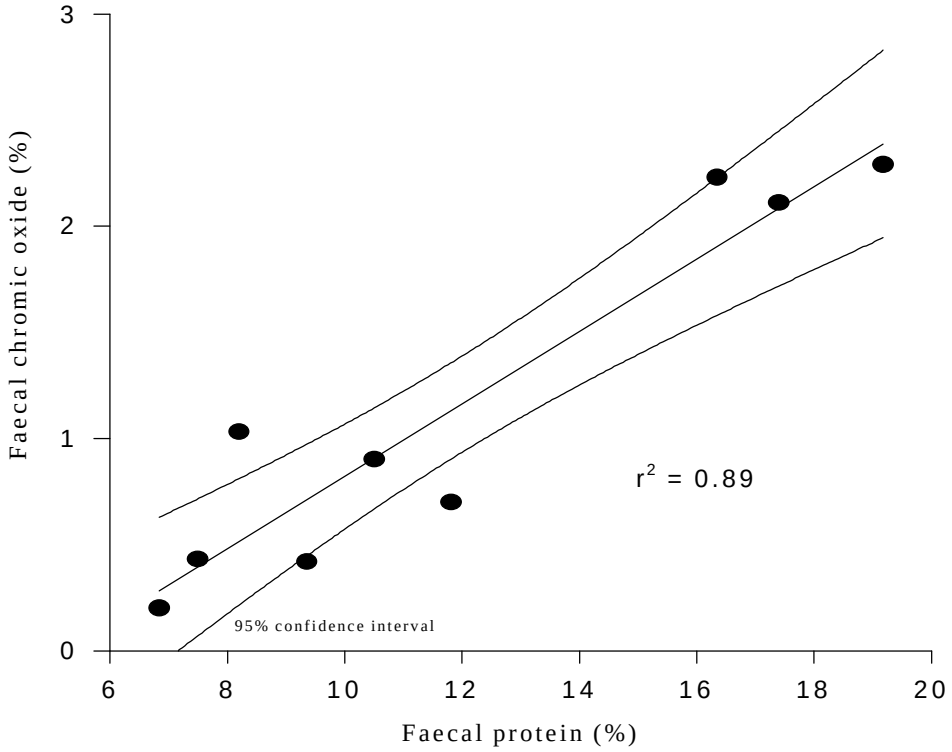
Table 2.3. Trial 2 - Non-linear regression coefficients describing the production of the faecal types over a 96h period, the estimated time of production of 5, 50 and 95% of each of the fecal types, and the diurnal and nocturnal pattern of feces production. Values expressed as means and standard errors. Within evacuation time periods and production periods, different alphabetic superscripts indicate significant differences (P<0.05).

Faeces Type	Regression coefficients k	r^2	5% Evacuation time (hrs)	50% Evacuation time (hrs)	Diurnal Production (%)	Nocturnal Production (%)
Type 1	0.024	0.95	13.01 ± 3.6^{a}	30.2 ± 8.3^{b}	45.3 ± 13.7^{d}	54.6 ± 13.8^{e}
Type 2	0.035	0.93	8.83 ± 1.2^{a}	20.5 ± 2.9^{b}	54.8 ± 10.8^{d}	45.2 ± 10.9^{e}
Type 3	0.025	0.86	14.4 ± 3.1^{a}	33.0 ± 6.8^{b}	44.0 ± 10.0^{d}	55.9 ± 10.0^{e}

Table 2.4. Trial 3 - Chromium and protein contents of the different faecal types. Values presented as means with standard errors. Different alphabetic superscripts indicate significant differences (P<0.05, n=3).

Faecal Type and Form	Chromium content (%)	Protein content (%)
Type 1 – Dark green discrete faecal pellets	2.21 ± 0.05^{c}	17.64 ± 0.83^{b}
Type 2 – Medium green amorphous faeces	0.89 ± 0.09^{b}	10.18 ± 1.05^{a}
Type 3 – Light green amorphous faeces	0.35 ± 0.08^{a}	7.90 ± 0.75^{a}

Figure 2.4. Trial 3 - Correlation between faecal protein and chromic oxide concentrations.



The calculated apparent protein digestibility coefficients using the three faecal types, and a pooled combination of the three faecal types are presented in Table 2.5. The highest digestibility coefficient and lowest standard error of the estimations was found in the tightly bound discrete Type 1 faecal strings. Digestibility coefficients were lower and associated with higher standard errors in the loosely bound amorphous Types 2 and 3 faeces. Although Type 3 faeces produced significantly lower digestibility coefficients than either Types 1 or 2 faeces ($P<0.05$), no significant difference was found between Type 3 faeces and the pooled combination of all faeces types ($P>0.05$). In addition, while no significant difference was found between the digestibility coefficients obtained using

Table 2.5. Trial 3 - Comparison between the apparent protein digestibility coefficients of different faecal types and pooled faecal collections. Values are presented as means and standard errors. Different alphabetic superscripts indicate significant differences (P<0.05, n=3).

Fecal Type	Apparent Protein Digestibility (%)
Type 1 - Dark green discrete faecal pellets	74.7 ± 1.0^{a}
Type 2 - Medium green amorphous faeces	61.3 ± 8.3^{ab}
Type 3 - Light green amorphous faeces	14.2 ± 15.4^{c}
Pooled collection - All faecal types	30.4 ± 10.6^{bc}

Types 1 and 2 faeces, the pooled combination of faeces produced significantly lower coefficients than Type 1 faeces (P<0.05). No significant difference was found between Type 2 faeces and the pooled sample of all faecal types (P>0.05). The standard error of the means – which provide an indication of the consistency and replicability of the results - ranged between 1.0 and 15.4, and were found to increase from the lowest value in Type1 faeces to the highest in Type 3.

The comparison between the digestibility coefficients obtained using the total faecal collection method, combined with feeding either the chromic oxide labeled or control diet (Trial 4, Table 2.6), indicated that the presence of dietary chromic oxide did not significantly affect apparent digestibility coefficients (P>0.05). It may therefore be assumed that the chromic oxide is inert and does not affect digestibility coefficients in *H.midae*. Furthermore, although analysis of variance between the digestibility coefficients obtained using the total collection (Table 2.6) and the chromic oxide methodologies (Table 2.5) revealed significant differences in digestibility estimations (F=12.1, P=0.004). Only the digestibility coefficient obtained using chromic oxide and

Table 2.6. Trial 4 - Comparison between apparent protein digestibility coefficients obtained using the total faecal collection methodology for the chromic oxide and control diets. Values are presented as means and standard errors (n=4). Different alphabetic superscripts indicate significant differences (P<0.05).

Diet	Apparent Protein Digestibility (%)
Total collection – chromic oxide diet (0.5% Cr_2O_3)	82.5 ± 4.9^{a}
Total collection – control diet (no Cr_2O_3)	79.6 ± 5.5^{a}

Type 3 faeces were found to be significantly different (P<0.05) to those obtained using the total collection method. In addition, with respect to the chromic oxide technique and Type 1 faeces, the larger standard errors of the means observed using the total collection method reveal a high degree of variability in the results, thus indicating problems obtaining replicable and reliable results using this methodology.

The assessment of the ability of *H.midae* to absorb the chromic oxide marker (Trial 5), revealed that chromic oxide recovery from a measured dose of chromic oxide labeled feed was 94.2±6.8% (Mean ± S.E., n=3). One way analysis of variance revealed no significant difference between the chromic oxide concentrations of the seawater in which the abalone were maintained during the 96h experimental period and the control (F=1.78, P>0.05). Thus, it may be assumed that faecal leaching of the chromic oxide did not significantly affect chromic oxide levels in the faeces. As the chromium contents of the shell and shucked material from the animals fed the chromic oxide labeled diet and the control group did not differ significantly (P>0.05, Table 2.7), it may be concluded that *H.midae* does not have the ability to absorb the chromic oxide marker.

Table 2.7. Trial 5 - Comparison of chromium contents of shell and body tissues of *H.midae* fed a diet containing a chromic oxide marker and a wild control group. Values are expressed as means and standard errors (n=5). Different alphabetic superscripts indicate significant differences for the parameter concerned.

	Cr μg g^{-1} dry weight	
Animals	Shucked material	Shell material
Cultured animals fed Cr_2O_3 diet	22.6 ± 11.9 [a]	20.8 ± 8.6 [b]
Wild caught animals	23.6 ± 10.3 [a]	15.2 ± 8.1 [b]

DISCUSSION

The results of this study indicate that as an external marker in apparent protein digestibility studies in *H.midae*, chromic oxide satisfies the essential requirements necessary to produce replicable and reliable digestibility estimations. Specifically these requirements, as outlined by DeSilva (1988), are that the marker is not absorbed by the animal, is inert and does not interfere with the digestive processes, and moves along the gut at the same rate as the component under study.

Although the retrieval of measured doses of chromic oxide fed to the abalone was found to be 94.2±6.8%, and therefore indicates that a portion of the chromic oxide was unaccounted for; the comparison of shell and body tissues from animals fed a chromic oxide labeled diet suggest that *H.midae* does not absorb chromic oxide. The apparent failure to account for all the chromic oxide fed as a measured dose illustrates the problems associated with the direct method of digestibility determination in a slow feeding aquatic animal such as a Haliotid. In particular, the feeding action presents problems when accurate measurements of feed ingestion are required. The rasping action

of the radula during feeding physically breaks up the feed, which not only increases nutrient leaching, but in addition disperses small particles of uneaten feed into the environment. As this particulate matter is difficult to collect, feed ingestion measurements are prone to over-estimations. In addition, collections of all the faeces produced from a given meal are intrinsically problematic in an aquatic environment.

In comparison to the control diet, the inclusion of chromic oxide in the test diet did not significantly affect apparent protein digestibility coefficients, and therefore suggests that chromic oxide is inert and does not affect protein digestibility in *H.midae*.

The initial observations of the faeces produced by the *H.midae* indicate that types of faeces produced are similar to those previously described by Wee *et al*. (1992) in *H.rubra*. It is therefore probable that the two species have similar digestive and defecatory processes. However in this study, and in contrast to *H.rubra,* significant differences were found between the protein concentrations in the different faecal types. Furthermore, while Wee *et al*. (1992) failed to report chromic oxide concentrations between faecal types, the current results indicate that there were also significant differences between the chromic oxide concentrations of the different faecal types. Similar partitioning of the chromic oxide component has been reported in other aquatic animals such as caridean prawns (Forster and Gabbott, 1971), lobsters (Bordner *et al*., 1983; Leavitt, 1985) and crayfish (Brown *et al*., 1986). Ishikawa *et al*. (1996) demonstrated that despite variations in protein and chromium concentrations in the faecal material of *Penaeus japonicus*, there was a strong correlation between the evacuation of the two components, thus as a similar rate of movement of the nutrient and marker was

established, chromic oxide could be used to accurately determine apparent protein digestibility in the species. As the results in the present study demonstrate a strong positive correlation between both faecal chromic oxide and protein concentrations in the three faecal types, and in addition the emergence of faecal protein and chromic oxide over a 96h period; it can be concluded that the chromic oxide is evacuated at a similar rate to the protein, and may therefore be used to predict apparent protein digestibility in *H.midae*. However, it is interesting to note that although Ishikawa *et al*. (1996) found a significant correlation between faecal chromic oxide and protein in *Penaeus japonicus*, they found a weak correlation between chromic oxide and carbohydrate faecal concentrations, thus as differential movement of the marker and carbohydrate along the gastrointestinal tract was established, it was concluded that chromic oxide was an ineffectual marker when applied to carbohydrate digestibility studies. It must therefore be noted that while the current study demonstrates that chromic oxide can be used to reliably predict protein digestibility in *H.midae*, further research is required to demonstrate whether it can be used to predict the digestibility of other nutrients.

The high degree of variation in the apparent digestibility coefficients produced by the Type 2, 3 and pooled faecal samples is unacceptable. Examination of both the apparent digestibility coefficients and their associated standard errors, indicates that Type 1 faeces produce the highest apparent digestibility coefficients, and in addition, promote the lowest standard errors. Although, the low standard error suggests that analysis of Type 1 faeces provides the highest degree of replicability in digestibility determinations, it provides no indication as to whether this type of faeces provides an accurate estimation of protein digestibility. The standard method to determine whether an indirect technique

promotes a reliable estimation of apparent digestibility is to compare it with the total collection method. Comparison between the digestibility coefficients obtained using the two methods suggest that although the total collection method produced higher digestibility coefficients than those obtained using the chromic oxide marker, the Type 1 faecal material produced the closest approximation to the total collection method. The intrinsic problems of accurately measuring feed consumption and faecal production associated with the total collection method, suggest that the results obtained using this method may have overestimated the digestibility coefficients.

The quantification of the relative proportions of three types of faeces produced provides further evidence to suggest that Type 1 faeces produce the most accurate estimations of apparent protein digestibility. Although as a percentage of the total, the quantity of Type 1 faeces produced was similar to Type 2 or 3 faeces; the quantities of both protein and chromic oxide excreted as Type 1 faeces were found to be greater than those of either Types 2 or 3 faeces. Thus it may concluded that the main portion of a given meal is processed as Type 1 faeces, and a such provides a representative sample for digestibility determinations. Nevertheless, this being the case, it fails to account for the higher variability and general reduction in digestibility coefficients observed in the Type 2 and 3 faeces. As digestibility is calculated by observing the ratios of marker to nutrient in the feed and the faeces, at low chromic oxide concentrations, small errors in nutrient determinations produce large errors in the recorded digestibility coefficients. This was of particular significance in this study as the small faecal samples (approx. 20mg) made it difficult to accurately determine protein concentrations in samples containing the lower protein concentrations. Thus although a strong correlation between marker and protein

was found in this study, small errors in the protein determination in the faeces containing the lower chromic oxide concentrations would have produced the observed high variability in the apparent protein digestibility coefficients. Furthermore, differential leaching of the nutrients from the different faecal types may have produced the high variability in digestibility coefficients. Although an attempt to reduce the affects of leaching was made by collecting faecal samples at four hourly intervals, no attempt was made to determine the effect of leaching on the different faecal types. It is possible that leaching affected the loosely bound amorphous Types 2 and 3 faeces to a greater extent than the more tightly bound Type 1 faecal strings, and as such not only lowered but increased the variability in the digestibility coefficients obtained from these faeces. However, further research on the digestive physiology of *H.midae* is required to determine how the three faecal types are produced and why nutrient concentrations vary.

Although it is difficult to account for the mechanisms with which the three faecal types are produced, two factors may have played a role in their production. While it possible that Type 3 faeces may have been a product of Type 1 and 2 feces that had been physically broken up and mixed with pedal mucous as the abalone moved around the aquaria; the production of periotrophic mucosal membranes found around the fecal pellets of invertebrates (Forester, 1953; Lautenschlager *et al*., 1978) may have also played a role. Periotrophic membranes form around faecal pellets as they pass along the intestine, and it is possible that differential production of this membrane may account for production of the three faecal types. However, further investigations into both the physiology of faeces production and the effect of faecal disturbance by the animals needs to be undertaken before these issues can be resolved.

As no significant differences were found between the diurnal and nocturnal production of the three faecal types, it can be concluded that there is no 24h periodicity of faecal emergence in *H.midae*. Furthermore, as the evacuation times of the three fecal types did not differ significantly, it may also be concluded that it is not possible to collect a specific type of faeces at a specific time period. However, using the faecal emergence data, it may be concluded that faecal collections should be made between 12 and 60hrs post-feeding when the majority of the faecal material is produced.

In conclusion, the results of the study suggest that chromic oxide can be used to provide replicable and reliable apparent protein digestibility coefficients in *H.midae*. A result confirmed by the study undertaken in Chapter 3.

CHAPTER 3

EVALUATION OF AN *IN VITRO* DIGESTIBILITY TECHNIQUE FOR THE PREDICTION OF PROTEIN DIGESTIBILITY IN FORMULATED FEEDS FOR *H.MIDAE*.

INTRODUCTION

Feeding trials are the most dependable method for determining the nutritive value of novel protein sources (Eid and Matty, 1989). However, such experiments are not only time consuming but incur considerable expense, making this approach undesirable when assessing large numbers of protein sources. This is of particular relevance to Haliotid nutrition where inherent slow growth rates preclude the rapid assessment of novel protein sources. *In vitro* protein digestibility techniques provide a fast and cost effective alternative to feeding trials as large numbers of protein sources may be examined rapidly, and those showing the most promise in terms of digestibility isolated for longer term growth trials. Practical applications of *in vitro* digestibility techniques have demonstrated that the technology can be successfully applied to aquatic animals with *in vitro* systems developed for salmonids (Dimes *et al*., 1994; Bassompierre *et al*., 1998), tuna (Carter *et al*., 1999) and shrimp (Ezquerra *et al*., 1997; García-Carreño *et al*., 1997).

A number of *in vitro* methodologies have been developed. All are conducted in closed systems employing the addition of proteolytic enzymes to a protein suspension. Proteolytic enzymes are supplied either as a combination of individual proteolytic enzymes (Hsu *et al*., 1977; Saterlee *et al*., 1979) or as intestinal enzyme extracts obtained

from the animals under study (Grabner and Hofer, 1985; Eid and Matty, 1989; Dimes *et al*., 1994; Ezquerra *et al*., 1997). A number of parameters can be used to determine the degree of proteolysis. Grabner (1985) developed a physiologically orientated methodology that simulated the physical and chemical conditions of the alimentary tracts of the animals under study. Digestion was determined by the analysis of the soluble digestion products, and although acceptable digestibility estimates were achieved, the approach was not only time consuming but incurred considerable expense. An alternative is the pH-shift methodology (AOAC, 1990: sec. 982.30) in which the degree of proteolysis is measured by the drop in pH in the system over the digestion period. Unfortunately, the drop in pH during the digestion period may compromise enzyme function, leading to the incomplete proteolysis of the samples and therefore an underestimation of the digestibility coefficients. An alternative to this system is the pH-stat method (Pedersen and Eggum, 1983) in which the degree of digestion is determined by the volume of alkali titrated into the system to maintain a constant pH over the digestion period. Such a method has the obvious advantage over the pH-Shift technique as enzyme function will not be affected by fluctuations in pH; in addition it does not require the costly and lengthy analysis of the digestion products. Furthermore, recent research has demonstrated that the pH-stat method was an accurate means to estimate protein digestibility in both salmonids (Dimes and Haard, 1994) and penaeid shrimps (Ezquerra *et al*., 1997).

The aim of this study was therefore to determine whether an *in vitro* pH-stat protein digestibility technique, using either a hepatopancreatic enzyme extract from *H.midae* or a combined enzyme mixture could be used to accurately predict *in vivo* protein digestibility

in *H.midae*, and if so, use the technique to predict the *in vivo* protein digestibility of a number of novel protein sources.

METHODS

Enzymes and in vitro procedure

A three enzyme system described by Hsu *et al.* (1977) was used for the *in vitro* procedure. A mixture of porcine intestinal peptidase (50-100 units per g solid), bovine pancreatic chymotrypsin (Type II, 40-60 units per mg protein) and porcine pancreatic trypsin (Type IX, 13000-20000 BAEE units per mg protein) was prepared such that 1ml contained 1.61 mg trypsin, 3.96 mg chymotrypsin and 2.36 mg peptidase. The enzyme mixtures were prepared daily and stored on ice until required.

An abalone hepatopancreatic enzyme fraction was prepared according to Knauer *et al.* (1996). In summary, hepatopancreatic tissue from 10 abalone (Shell length : 72 ± 3.5mm) was removed, placed on ice and homogenised for three minutes using a Ika Ultra Turrax T25 homogeniser in an equivalent volume of 0.1M citric acid – 0.2M phosphate buffer (pH 6.5). The sample was centrifuged for 45 minutes at 18400 x g at 4°C. The supernatant dialysed (molecular cut off weight 12000 – 14000) against the buffer solution for 48 hrs and the extract stored at –30°C until required. The activities of all enzyme mixtures were determined by running pH-stat assays using sodium caseinate as a reference protein.

The protein sources under investigation were oven dried to constant weight at 70°C, ground to a fine powder with a pestle and mortar and passed through a 125 μm sieve to ensure uniformity of particle size.

A modified three enzyme pH-stat system described by Pedersen and Eggum (1983) was used for the *in vitro* determination. A 10 ml aqueous protein suspension (1 mg N per ml distilled water) was placed in a jacketed reaction vessel containing a magnetic stirrer and heated to 37°C. The pH of the suspension was equilibrated to pH 8.00 over a 10 minute period using a combination of 0.01 N NaOH and 0.01 N HCl. Over a two minute period, 1ml of enzyme mixture was equilibrated at 37°C to pH 8.00. It was added to the protein suspension and the auto-titration started (Autotitrator : Metrohm Herisau Potentiograph E436. Titrant : 0.02 N NaOH). The volume of titrant added to the system was recorded every 5 minutes for the 35 minute reaction period and used as an indication of digestibility. All protein sources were assayed in duplicate. The baseline uptake of titrant for each of the protein sources was determined by performing duplicate pH-stat assays in the absence of the enzyme mixture at pH 8.00.

A total of 34 protein sources were assessed for their *in vitro* digestibility; of these, 17 were locally available plant protein sources, and 17 animal protein sources. Thirteen of the 17 animal protein sources were commercially available fishmeals, 15 of which were produced outside South Africa. The remaining protein sources were all sourced within South Africa.

In vivo digestibility procedure

In vivo protein digestibility was determined according to the methodology described in Chapter 2. Eleven single protein test diets were formulated to contain 20% protein, 6% lipid and 0.5% chromic oxide. Three replicate groups of 6 animals (69.9±3.5mm shell length) were used for the digestibility determinations. Following an acclimation period of 7 days to each diet, faecal collections were made twice daily for a thirty day period. Protein was determined using micro-Kjeldahl analysis (Nitrogen conversion factors according to Tacon, 1990a), and the chromium by atomic absorption spectroscopy on a GBC-909 atomic absorption spectrophotometer (Nitrous oxide / acetylene carrier at 357.9µm). Apparent protein digestibilities were calculated according to Maynard and Loosli (1956).

Statistical analysis

The efficacy of the *in vitro* procedure was established by calculating linear regressions between the *in vitro* titrations and the *in vivo* apparent digestibility coefficients. The regression equations for the correlations were calculated, and *in vitro* digestibility coefficients calculated using the model : $y = a + b\ x_t$, where y is the *in vitro* digestibility coefficient, a and b are constants, and x is the amount of titrant added to the system during the *in vitro* procedure at time t.

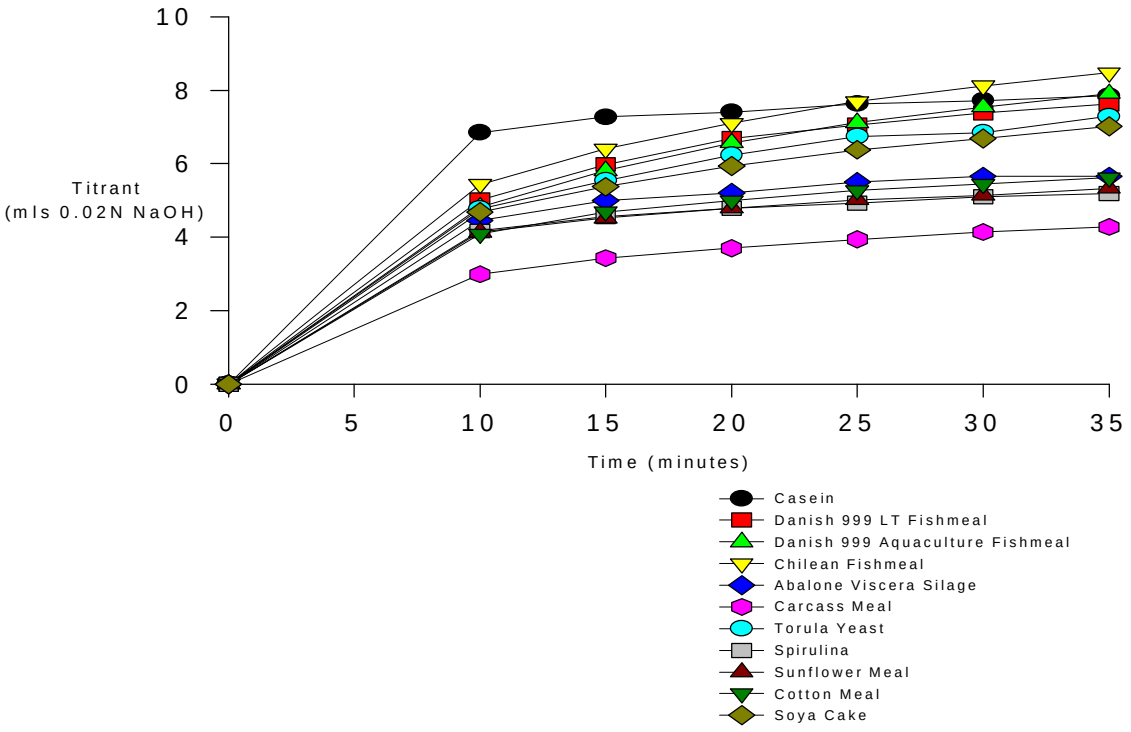
RESULTS

Titration curves for the 11 protein sources incubated with the three enzyme mixture over the 35 minute experimental period are shown in Figure 3.1. Within *in vitro*

measurements, replicable results were obtained, and a pooled standard deviation of 0.088 mls titrant representing an average error of 0.91% in the digestibility coefficients was recorded.

Titrations using the hepatopancreatic enzyme extract failed to produce a result with the reference protein sodium caseinate, and was therefore excluded from further experimentation.

Figure 3.1 Titration curves for the eleven protein sources used to verify the pH-stat procedure.



Linear regressions performed between the *in vivo* apparent digestibility coefficients and the volumes of titrant added to the *in vitro* procedure at five minute intervals during the 35 minute incubation period are presented in Table 3.1. The regression coefficients (r^2)

were used to determine the optimal incubation time to predict *in vivo* digestibility coefficients from the *in vitro* titrations. In order to determine whether the origin of the protein source (animal or plant) affected the predictability of the system, regression coefficients were calculated for all the protein sources under investigation and separately for proteins of either animal or plant origin. When all the protein sources were taken into account, an incubation period of 10 minutes (T_{10}) produced the best *in vivo* predictability ($r^2 = 0.71$, S.E. = 6.02, $P<0.001$). Successive incubations in excess of 10 minutes reduced the accuracy of the system reducing correlation coefficients and increasing the standard errors of the regressions. The lowest level of predictability was therefore observed after a 35 minute incubation period ($r^2 = 0.40$, S.E = 8.62, $P<0.04$).

Table 3.1. The effects of protein source and incubation time on the correlations between *in vitro* and *in vivo* results.

Protein		Incubation Time (minutes)					
		10	15	20	25	30	35
All proteins (n=11)	r^2	0.71	0.65	0.56	0.50	0.43	0.40
	s	6.02	6.61	7.39	7.91	8.42	8.62
	P<	0.001	0.002	0.008	0.01	0.03	0.04
Animal Proteins (n=6)	r^2	0.89	0.84	0.71	0.62	0.55	0.49
	s	5.14	6.30	8.35	9.51	10.47	11.19
	P<	0.004	0.01	0.03	0.06	0.09	0.12
Plant Proteins (n=5)	r^2	0.66	0.79	0.77	0.76	0.75	0.73
	s	3.84	3.03	3.16	3.24	3.27	3.39
	P<	0.09	0.04	0.05	0.06	0.06	0.06

r^2 is the regression coefficient
s is the standard error of the regression

The predictability of the system was improved when the proteins were analysed according to their origin. The highest correlation coefficient, and hence most accurate *in*

vivo prediction was observed when the animal proteins were analysed in isolation. A highly significant correlation (r^2 = 0.89, S.E. = 5.14, P<0.004) was observed after a ten minute digestion period (Table 3.1, Figure 3.2). In a similar trend to that found when all the protein sources were taken into account, increases in the ten minute digestion period reduced correlation coefficients and hence predictability of the system. The highest level of *in vitro* predictability with the plant protein sources was observed after a fifteen minute digestion period, and although a significant correlation was again found between the *in vitro* and *in vivo* digestibility coefficients (r^2 = 0.79, S.E. = 3.03, P<0.04. Table 3.1, Figure 3.3), the regression coefficient was reduced with respect to that observed with the animal proteins. It was therefore apparent that the estimation of *in vitro* digestibility was superior when animal proteins were assayed. In addition, as higher correlation coefficients were recorded when the protein sources were analysed according to their origin (animal or plant) as opposed to a combined group, predictability of the system was enhanced when the origin of the protein source under investigation was taken into account. This being the case, correlation coefficients and the respective regression equations used to predict *in vitro* digestibility coefficients in both animal and plant protein sources are presented in Table 3.2.

The calculated *in vitro* apparent digestibility coefficients and their corresponding *in vivo* coefficients are presented in Table 3.3. The close similarity between the respective *in vitro* and *in vivo* digestibility coefficients indicates that the system can effectively predict *in vivo* digestibility coefficients in *H.midae*. The procedure successfully ranked all the

Figure 3.2 Correlation between *in vivo* apparent digestibility coefficients and *in vitro* titrations – animal proteins.

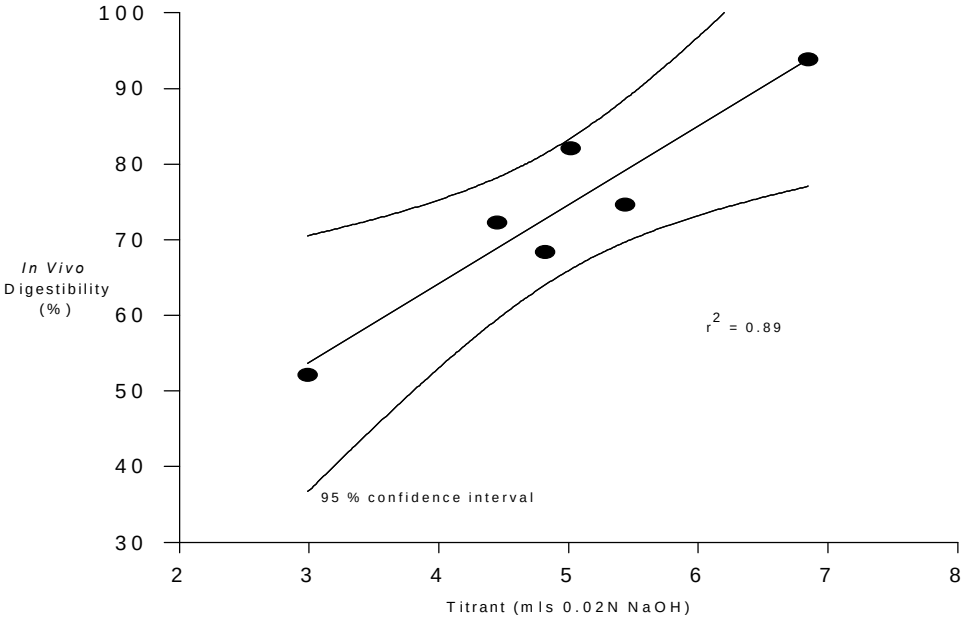


Figure 3.3 Correlation between *in vivo* apparent digestibility coefficients and *in vitro* titrations – plant proteins.

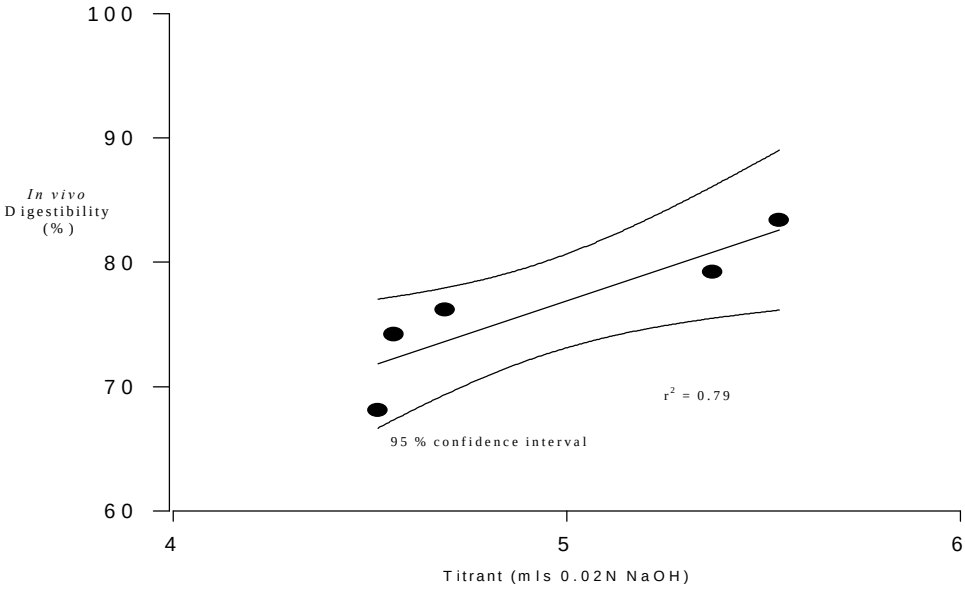


Table 3.2. Regression equations and correlation coefficients between *in vitro* and *in vivo* apparent protein digestibility.

Protein	Regression equation
Animal Proteins (n = 6) $r^2 = 0.89$ s = 5.14 P < 0.004	$IV = 22.34 + 10.45\ T_{10}$
Plant Proteins (n = 5) $r^2 = 0.79$ s = 3.03 P < 0.04	$IV = 24.24 + 10.53\ T_{15}$

IV is the calculated *in vivo* apparent protein digestibility coefficient.
T_{10} and T_{15} is the amount of titrant added to the system during respective 10 and 15 minute incubation periods.

Table 3.3. Comparison of *in vivo* and *in vitro* apparent protein digestibilities.

Protein Source	Apparent *in vivo* digestibility (%)	Apparent *in vitro* digestibility (%)
Animal Proteins [a]		
Casein	93.8	93.9
Fishmeal – Danish 999 LT	82.0	74.8
Fishmeal – Chilean	74.6	79.2
Fishmeal – Danish 999 Aqua	68.3	72.7
Abalone Viscera Silage	72.2	68.8
Carcass Meal	52.1	53.6
Plant Proteins [b]		
Torula Yeast	83.4	82.5
Soya cake – solvent extracted	79.2	80.7
Cotton cake – solvent extracted	76.2	73.6
Spirulina	74.2	72.2
Sunflower meal – solvent extracted	68.1	71.8

[a] Equation : $IV = 22.34 + 10.45\ T_{10}$
[b] Equation : $IV = 24.24 + 10.53\ T_{15}$
Proteins supplied by : Casein - Sigma Chemicals, USA; Danish 999 - Esbjerg Fiskeindustri, Denmark; Chilean fishmeal - Caldera Ltd. Chile; Carcass meal - Abakor Ltd. SA; Torula yeast - Illova Sugar, SA; Sunflower, Soya cake, Cotton cake, EPOL, SA; Spirulina - Western Cape Tanneries, SA.

proteins correctly according to their *in vivo* digestibility coefficients; exceptions being the respective miss-ranks between the Danish 999 low temperature fishmeal and the Chilean fishmeal, and the abalone viscera silage and the Danish 999 aquaculture fishmeal.

The baseline uptake of titrant recorded over a 35 minute period, at pH 8.00 and in the absence of enzyme mixture, varied considerably between protein sources. Depending upon the protein source, between 7.8 – 38.8% of the total titrant added to the system during an incubation could be accounted for as baseline uptake. Nevertheless, the predictability of the procedure was not enhanced when *in vitro* values were corrected for baseline uptake. The corresponding correlation coefficients for the animal and plant protein sources at T_{10} and T_{15} reduced from 0.89 to 0.76 and 0.79 to 0.53 respectively. This being the case, the baseline uptake of titrant was not accounted for when calculating the regression equations.

In vitro apparent digestibility coefficients of 11 animal and 12 plant protein sources are presented in Table 3.4. In order to obtain *in vitro* digestibility predictions, the respective equations (Table 3.2) that describe digestibility in both animal and plant protein sources were employed. Taking all the fishmeals into account, apparent digestibility ranged from 79.2 to 65.3%. Bloodmeal and carcass meal both under-performed with respect to the fishmeals and recorded apparent digestibility coefficients of 51.7% and 53.6% respectively. Apparent digestibility coefficients in the plant materials ranged from 82.5% in torula yeast to 35.6% in mechanically extruded soya. In general, plant materials that had undergone heat treatments performed better than those that had not. This was particularly apparent with the solvent and mechanically extruded soya, where the

apparent digestibility coefficient increased by 45.1% in the heat treated solvent extruded sample.

Table 3.4. Calculated *in vitro* apparent digestibility coefficients of selected protein sources (mean ± s.e).

Protein Source	Apparent *in vitro* digestibility (%)
Animal Proteins[a]	
Fishmeal – Danish FF – FF	77.4 ± 0.3
Fishmeal – Danish 999 – Std	75.8 ± 2.0
Fishmeal – Argentinean	75.6 ± 1.8
Fishmeal – Danish FF - Sp. A	73.8 ± 0.9
Fishmeal – Danish FF - Sp. B	72.6 ± 0.4
Fishmeal – Danish 999 - Sp. B	71.9 ± 0.4
Fishmeal – South African 1	70.3 ± 0.5
Fishmeal – French Sp. G	67.3 ± 0.5
Fishmeal – French C.P.S.P. 90	65.8 ± 0.0
Fishmeal – South African 2	65.3 ± 0.0
Blood meal	51.7 ± 0.5
Plant Proteins[b]	
Peanut meal – mechanically extruded	77.3 ± 1.3
Linseed – ground	71.8 ± 0.0
Wheat bran	68.8 ± 0.0
Sunflower – mechanically extruded	64.8 ± 0.9
Corn gluten	57.0 ± 0.9
Maize – ground	54.2 ± 0.3
Brewery waste 1	51.1 ± 0.8
Wheat – ground	48.4 ± 0.4
Brewery waste 2	47.8 ± 0.9
Barley – ground	43.4 ± 0.4
Chick Pea – ground	36.9 ± 0.5
Soya – mechanically extruded	35.6 ± 0.2

[a] Equation : $IV = 22.34 + 10.45T_{10}$

[b] Equation : $IV = 24.24 + 10.53\ T_{15}$

Proteins supplied by : Danish 999 fishmeals - Esbjerg Fiskeindustri, Denmark; Danish FF fishmeals - Fiskernes Fiskeindustri, Denmark; French fishmeals - Sopropêche, France; South African fishmeals - 1. Concentra Ltd; 2. Eyethu Ltd., SA; Bloodmeal - Abakor Ltd., SA; Argentinean fishmeal, Soya meals, Cotton cake, Sunflower, Corn Gluten, Wheat Bran, EPOL, SA; Brewery wastes : 1. South African Breweries; 2. National Sorghum Breweries, SA.

DISCUSSION

The pH-stat *in vitro* assay employed in this study produced results that were, for the most part, comparable with *in vivo* digestibility estimates. Although the correlation coefficients between *in vitro* and *in vivo* digestibility reported in this study were lower than those reported by Pedersen and Eggum (1983) in rats, they are in general agreement with similar work carried out in aquatic animals (Anderson *et al.*, 1993; Dong *et al.*, 1993; Dimes *et al*., 1994; Ezquerra *et al*., 1997). While Ezquerra *et al.* (1997) successfully predicted *in vivo* protein digestibility in shrimp with a correlation coefficient of 0.77 for both animal and plant materials, Dimes *et al*. (1994) found a significant correlation (r^2 = 0.87) between *in vitro* and *in vivo* digestibility in salmonids fed fishmeal diets. These results compare favourably with the correlations of 0.89 and 0.79 found between the *in vitro* and *in vivo* digestibility coefficients for the respective animal and plant proteins in this study.

Corrections for baseline uptake of titrant reduced the accuracy of the *in vitro* digestibility estimations. It is not clear why this should have been the case; however, it can be assumed that the volume of titrant measured in assays run in the absence of the enzyme mixture did not adequately predict baseline consumption during digestion. This could be attributed to the fact that the addition of proteolytic enzymes may have inactivated endogenous enzymes in the protein samples which may have contributed significantly to the baseline uptake of alkali (Pedersen and Eggum, 1983). *In vitro* predictability improved if assays of either animal or plant origin were analysed separately. Differences between regression line intercepts and slopes combined with the different time frames

required to optimise predictability, probably indicate differences in the mechanisms as well as in the rates of hydrolysis between the two types of protein.

It is interesting to note that although the abalone hepatopancreatic enzyme extract failed to produce a positive result in this study, similar work carried out with white shrimp (Ezquerra *et al*., 1997) has shown that the replacement of a four enzyme mixture with a shrimp hepatopancreatic extract, improved correlation coefficients (r^2) from 0.71 to 0.77, and therefore the *in vivo* predictability of the system. Although protease activities in the enzyme preparations were controlled, the composition of the enzyme mixture in the hepatopancreatic extract was different to that in the four enzyme mixture. Clearly, digestive enzyme extracts are superior to synthetic enzyme mixtures as they provide a closer approximation to the *in vivo* digestive process. It is therefore not unrealistic to assume that *in vitro* digestibility predictions in this study would be improved if the three enzyme mixture was successfully replaced with a hepatopancreatic enzyme extract from *H.midae*. The failure of the hepatopancreatic enzyme extract to produce a result was probably due to enzyme inactivation in the alkaline conditions under which the procedure was carried out. Recent studies (Serviere-Zaragoza *et al.*, 1997; Hernández-Santoyo *et al*., 1998) with *H.fulgens* have shown that the highest proteolytic activity in the digestive system is found in the intestinal and rectal fluids of the animal, furthermore, proteolytic enzymes of the hepatopeancrease showed isoelectric points between 3.8 and 6.3. In addition, while Britz (1995) recorded the gut pH in *H.midae* to be 5.2, the gut pH in the animals in this study was found to be 6.5. It is therefore probable that enzyme fractions from the hepatopancreatic region should be acidic in nature and that the alkaline conditions used in the current *in vitro* procedure suppressed enzyme function; a positive

result may be achieved by running the pH-stat assay under acidic conditions. An alternative would be to isolate an alkaline enzyme fraction from the intestinal and rectal region of the digestive system where alkaline proteolytic activity was found to be maximal in *H.fulgens* (Seviere-Zaragoza *et al*., 1997). Nevertheless, a better understanding of both the digestive enzyme profile and the optimal conditions for enzyme function in *H.midae* is required if a viable enzyme fraction is to be isolated for use in the *in vitro* procedure.

The *in vitro* digestibility of the abalone viscera silage was found to be lower that that observed *in vivo,* thereby indicating that the digestibility of partially hydrolysed materials may be underestimated by the pH-stat procedure. Proteins in silages are liable to enzymatic hydrolysis during the production process due to the presence of endogenous proteolytic enzymes in the silage material (Viana *et al*., 1993b). As the pH-stat procedure is a kinetic method that directly measures the percentage of peptide bonds hydrolysed in the protein (Dimes and Haard, 1994), the partial digestion of the viscera silage prior to the *in vitro* assay would reduce the *in vitro* digestibility coefficient as a percentage of the peptide bonds will have already been cleaved. Furthermore, in this trial, the French CPSP 90 and Sp. G fishmeals performed poorly in terms of their *in vitro* digestibility. These are high quality fishmeals that, in order to improve their digestibility, are enzymatically hydrolysed during processing (Sopropêche, 1995a). Although verification with *in vivo* digestibility coefficients were not undertaken, *in vivo* digestibility comparisons with a low temperature fishmeal in trout have indicated that these products produced superior digestibility coefficients (Sopropêche, 1995b). It is therefore likely that enzymatic pre-treatment of the samples during processing lead to an underestimation of

the *in vitro* digestibility of these fishmeals. Dimes *et al.* (1994) obtained a similar result in salmonids, and found that a partially digested ocean perch fishmeal produced an underestimation in the *in vivo* digestibility coefficient. It was concluded that the pH-stat system was not suited to feed samples that have been partially hydrolysed during preparation.

The presence of digestive protease inhibitors must be investigated when a new ingredient is identified for feed production. Plant materials, and in particular the legumes such as soya beans are known to contain protease inhibitors that bind with chymotrypsin / trypsin rendering them inactive (De Silva and Anderson, 1995). High levels of mortality can be caused by protease inhibitors at concentrations as low as 0.1% in diets with low or moderate protein concentrations (Burgess *et al.*, 1994). However, this is not always the case, Olli *et al*. (1994) showed that Atlantic salmon could compensate with digestive proteases when challenged with inhibitors equivalent to 5mg/g feed. Nevertheless, the presence of protease inhibitors in feeds must be addressed if feed utilisation is to be optimised. Hsu *et al.* (1977) pointed out that processing conditions can improve protein digestibility by destroying protease inhibitors and opening the protein structure to enzyme action, an issue pertinent to the current experiment. Plant materials that had undergone heat treatment to inactivate protease inhibitors performed better in terms of their *in vitro* digestibility than those that had not undergone treatment. Digestibility coefficients in both the soya and sunflower meals improved by 45.1% and 7.0% respectively in those samples that, as opposed to cold mechanic pressing, had undergone a heat and solvent extraction process to remove the oil fraction. A result confirmed by García-Carreño *et al.* (1997) who, working with a shrimp hepatopancreatic extract, observed a similar

interaction between heat treatment and *in vitro* digestibility coefficients in plant seed materials. Nevertheless, as the details of the processing conditions under which the meals were prepared was not known, it is difficult to assess why the heat treatment of the soya produced such a marked improvement in the digestibility coefficient when compared to the sunflower. Furthermore, as there may be a number of protease inhibitors within a plant, differing in both structure and specific action (DeSilva and Anderson, 1995), comparisons between plant proteins are difficult. However, it is evident that the pH-stat procedure in its current configuration is sufficiently sensitive to determine the presence of enzyme inhibitors in the protein substrates.

In conclusion, the pH-stat procedure using the three enzyme system proposed by Hsu *et al.* (1977) provides a reliable and rapid means to determine protein digestibility coefficients in *H.midae*. Improvements in the predictability would probably be achieved upon replacement of the three enzyme proteolytic mixture with a digestive enzyme extract from *H.midae.* However, before this can be achieved a better understanding of the digestive enzyme profile and optimal conditions for enzyme function in the species is required.

CHAPTER 4

EFFECTS OF ANIMAL SIZE ON THE ABILITY OF *H.MIDAE* TO UTILIZE SELECTED DIETARY PROTEIN SOURCES

INTRODUCTION

As outlined in Chapter 1, abalone have been shown to accept a wide variety of dietary protein sources. The first comparative studies that evaluated the effects of protein source on abalone growth isolated fishmeal and casein as good candidates for inclusion in artificial feeds (Uki *et al*., 1985a; Uki and Watanabe, 1986; Viana *et al*., 1993a). Although subsequent studies have isolated a number of other sources (Table 1.1), the most commonly used proteins in current experimental and commercial formulations are fishmeals, casein and defatted soya (Fleming *et al.*, 1996). A number of alternative proteins are utilised, however inclusion levels are low and generally do not contribute significantly to the gross protein content. Despite the acceptable growth rates achieved using fishmeal and casein based diets, the high raw material costs associated with these proteins make it economically desirable to isolate cheaper sources of high biological value for incorporation into feeds. In this respect, defatted soya appears to be an ideal candidate. The combination of the low raw material costs, availability and a favourable amino acid profile suggest that soya has potential to become a major protein source in commercial abalone feed formulations.

An initial investigation into the protein requirements of *H.midae* identified fishmeal and spirulina as suitable protein sources for inclusion in commercial formulations (Britz, 1996b). Although the growth rates achieved using these proteins are satisfactory, high raw material costs make it economically desirable to identify and evaluate cheaper alternative protein sources. The purpose of this study was therefore to broaden the scope of the initial study and evaluate a larger number of potential protein sources for inclusion in the commercial formulations.

In addition, the effect of animal size on the qualitative protein requirements of abalone was addressed. Britz and Hecht (1997) demonstrated that in order to promote maximal growth, young adult abalone required a higher dietary protein content than juvenile abalone. Thus, while quantitative differences in the protein requirements of different size classes of *H.midae* have been demonstrated, to date no work has investigated whether there are qualitative differences in the protein requirements between juvenile and young adult abalone. Two factors could account for such differences. Firstly, as juveniles develop and they move from a diatom based diet to a macro-algal diet, so their ability to digest various nutrients alters. There is some evidence to support this as Uki and Watanabe (1992) found differences in the ability of 13mm and 52mm shell length *H.discus hannai* to digest heat treated fishmeal. This they concluded may be due to a decrease in activity and specialisation of proteases with age. This being the case, protein digestibility and more importantly amino acid availability from a given protein source may differ with age and animal size. Thus although the amino acid requirements of the animals may be the same, their response to a given protein source will vary resulting in qualitative differences in their protein requirements. The second factor that may have an

effect on the requirement is quantitative differences in the amino acid requirements between juvenile and young adult abalone. It is generally accepted that the amino acid pattern of the lean tissue of an animal gives a good indication of the balance of amino acids required in the diet (Wilson, 1989), and although this has yet to be established empirically with abalone, it is likely to be the case with adult abalone as the majority of the protein synthesised will be lean tissue. This however may not be the case for juvenile abalone where developmental physiological processes as opposed to lean tissue synthesis may be instrumental in determining the amino acid requirements.

According to their performance in the *in vitro* digestibility trial (Chapter 3), twelve protein sources were chosen for evaluation. In addition, cost and commercial availability of the protein sources in South Africa were taken into account. Thus, of the twelve protein sources chosen, four were fishmeals which, with casein, sunflower and cotton meals, performed well in terms of their *in vitro* digestibility and are currently available in South Africa. Carcass meal, torula yeast, spirulina and brewery waste were chosen as again they performed well in terms of their *in vitro* digestibility, and in addition are industrial by-products currently produced in South Africa. Abalone viscera silage was also investigated as although not currently under commercial production, it is potentially an inexpensive abalone fishery by-product that has shown good potential as a protein source in diets for *H.fulgens* (Viana *et al*., 1996). In order to assess the affect of animal size on qualitative protein requirements of the abalone, the twelve protein sources were fed to two size classes of abalone, one comprising seven month, and the other thirty month old abalone.

METHODS

Experimental system

The experiment was conducted at the Port Alfred Laboratory using water drawn from the mouth of the Kowie River estuary on the Indian Ocean (33°45'S; 26°00'E). A 10,000 litre semi-recirculating system was employed. Water quality was maintained using biological filtration and the replacement of 25% of the system volume per day. A 12L:12D photoperiod was used throughout the experimental period. Temperature and salinity were maintained at 20±1°C and 35±1 p.p.t. respectively. Animals were housed in 24 fibreglass experimental containers (30x50x26cm, 39 litres volume, 4 water exchanges per hour). Within experimental containers, three replicate groups of animals were housed in perforated 2 litre plastic jars. Water flow through the jars was maintained by the use of airstones.

Experimental animals

Two size classes of hatchery-reared abalone (*H.midae*) were used in the experiment (15±1mm shell length, 0.57±0.2g; 45±2mm shell length, 23±3g). Three replicate groups, comprising eight or thirty individuals per replicate of the respective large and small size classes of animal were fed each of the test diets. Prior to experimentation, the animals were fed exclusively on a fishmeal based artificial diet and acclimated to the experimental system for a one month period.

The duration of the experiment was 316 days. Weight and shell length measurements were taken at monthly intervals throughout the experimental period. Weight was recorded

to the nearest 0.01g using an electronic balance and shell length to the nearest 0.01mm using vernier callipers. Prior to these measurements being taken, the animals were starved for a 24 hour period to ensure that the penultimate meal had been processed and did not affect the animal's weight. A 10% (w/w) solution of magnesium sulphate was used to anaesthetise the animals during the measurement process.

Animals were fed to satiation daily, and the feed and protein consumed over the experimental period recorded and corrected for leaching. Correction factors for total solids and protein leaching (Table 4.1) were determined for each diet by placing 4g of feed in a control container containing no abalone and placing it in the aquaria for a twelve hour period. Total solids and protein leached were calculated as dry weight loss over this period. The feed conversion ratios (grams dry feed consumed / grams wet weight gain) and protein efficiency ratios (grams wet weight gain / grams protein consumed) were calculated for all treatments.

Table 4.1. Correction factors for total solids and protein leaching over a twelve hour period at 20°C.

Protein	Correction Factor	
	Total Solids	Protein
Danish LT Fishmeal	0.93	0.76
Danish Standard Fishmeal	0.87	0.77
Danish Aquaculture Fishmeal	0.89	0.71
Chilean Fishmeal	0.91	0.93
Casein	0.96	0.83
Carcass Meal	0.80	0.79
Viscera Silage	0.65	0.66
Spirulina	0.63	0.57
Sunflower Meal	0.82	0.73
Torula Yeast	0.80	0.95
Cotton Meal	0.82	0.74
Brewery Waste	0.89	1.00

Daily feed consumption (% body weight per day) was calculated for the initial 90 days of the trial using the formula :

$$C_{\%\ b.\,wt} = C_g / W_t \times 100$$

where C_g is the mean daily feed consumption corrected for leaching and W_t is the mean abalone weight at time *t* (days). W_t was calculated using the formula :

$$W_t = W_o \times ((SGR/100) + 1)^{t-1}$$

where W_o is the mean initial abalone weight and SGR is the specific growth rate. The specific growth rates of the abalone were calculated using the formula :

$$SGR = ((\ln(W_f) - \ln(W_i)) / t\ ) 100$$

where SGR is the specific growth rate (% body weight increase per day), $\ln(W_f)$ is the natural log of the mean final weight of abalone, $\ln(W_i)$ is the natural log of the mean initial weight of abalone, and *t* is the time in days.

The condition factors of the animals on day 316 were calculated according to Britz (1995) using the equation :

$$\text{Condition Factor} = \text{weight (g)} / \text{length (mm)}^{2.99} \times 5575$$

The mean condition factors of the large and small abalone at the beginning of the experimental period were 1.22 and 1.07 respectively.

Dietary formulations

Twelve protein sources were selected for evaluation and formulated into twelve single protein diets containing 20% protein, 6% lipid (Table 4.2). The protein sources evaluated in this trial were chosen using the *in vitro* protein digestibility data. As discussed in Chapter 3 the *in vitro* protein digestibility coefficient gives an indication of the bioavailability of the protein to the animal, and can therefore be used to give an indication of which proteins are likely to promote good growth in the abalone. The proteins were sorted according to their origins, and the top performing 7 animal and 5 plant proteins chosen for the trial. It must be noted that at the time of formulation, the *in vitro* digestibility data had yet to be verified with the *in vivo* coefficients, thus although it was possible to rank the proteins in terms of their digestibility, it was only possible to formulate the diets on a gross protein basis as opposed to a digestible protein basis.

An attempt was made to balance the diets in terms of energy content and protein / energy ratios. In the absence of abalone digestible energy (DE) values for the dietary components used in the formulations, the digestible energy values were calculated using figures derived from fish. Although it is not entirely acceptable to apply digestible energy values across species (Jobling, 1983), Britz and Hecht (1997) justified their use as similar feed conversion and protein efficiency have been observed in both abalone and fish fed similar diets. Nevertheless, it must be noted that DE values of a given dietary component may vary between species and that in the absence of empirical figures for abalone, these values may be inaccurate. Thus, although the total DE and protein / energy ratios appear to be balanced across the dietary treatments, this may not have been the case. The energy supplied by the carbohydrate (dextrin) and lipid fractions of the diets were calculated

Table 4.2. Ingredient composition and nutrient analysis of experimental diets (g/100g, air-dry basis).

	Diet											
	Danish - LT Fishmeal	Danish - Std Fishmeal	Danish - Aqua Fishmeal	Chilean Fishmeal	Casein	Carcass Meal	Viscera Silage	Torula Yeast	Spirulina	Sunflower Meal	Cotton Meal	Brewery Waste
Ingredient												
LT Fishmeal [a]	28.23	-	-	-	-	-	-	-	-	-	-	-
Standard Fishmeal [a]	-	30.96	-	-	-	-	-	-	-	-	-	-
Aquaculture Fishmeal [a]	-	-	29.10	-	-	-	-	-	-	-	-	-
Chilean Fishmeal [b]	-	-	-	31.23	-	-	-	-	-	-	-	-
Casein [c]	-	-	-	-	22.56	-	-	-	-	-	-	-
Carcass Meal [d]	-	-	-	-	-	39.65	-	-	-	-	-	-
Abalone Viscera Silage [m]	-	-	-	-	-	-	56.08	-	-	-	-	-
Torula Yeast [e]	-	-	-	-	-	-	-	48.89	-	-	-	-
Spirulina [f]	-	-	-	-	-	-	-	-	52.60	-	-	-
Sunflower Meal [g]	-	-	-	-	-	-	-	-	-	63.45	-	-
Cotton Meal [g]	-	-	-	-	-	-	-	-	-	-	57.20	-
Brewery Waste [h]	-	-	-	-	-	-	-	-	-	-	-	55.85
Starch	21.50	21.50	21.50	21.50	20.00	25.00	25.00	23.50	22.00	25.50	23.50	32.50
Lipid [l]	3.65	3.80	3.58	3.78	5.89	0.00	0.36	3.46	3.48	4.79	1.20	5.15
Vitamin / Mineral mix [j]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cellulose [K]	45.62	42.74	44.82	42.49	50.55	34.35	17.56	23.15	20.92	5.26	17.10	5.50
Analysis (calculated)												
Protein	20	20	20	20	20	20	20	20	20	20	20	20
Lipid	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
P/E Ratio (mg/KJ)	24.6	24.6	24.6	24.6	24.8	24.7	24.7	24.7	24.7	24.7	24.6	24.7
Analysis (measured)												
Protein	21.9	20.0	21.1	20.8	19.2	20.7	19.7	22.2	18.7	20.6	20.8	19.3
Lipid	6.2	6.2	6.3	6.1	5.9	6.7	5.1	5.6	6.0	6.5	6.6	5.9
Ash	4.1	4.3	4.2	4.9	5.4	4.4	4.4	7.3	6.3	4.7	4.8	4.1
Gross energy (MJ/Kg)	18.41	19.16	19.11	19.18	19.78	19.04	18.38	19.21	19.18	18.99	20.17	21.06

Dietary components supplied by : [a.] Danish 999 fishmeals, Esbjerg Fiskeindustri, Denmark. [b.] Caldera Ltd. Chile. [c.] Sigma Chemicals, USA. [d.] Abakor Ltd. S.A. [e.] Illova Sugar, S.A. [f.] Western Cape Tanneries, S.A. [g.] EPOL, S.A. [h.] National Sorghum Breweries, S.A.
[i.] 50/50 mixture of sunflower oil and "Marinol R" fish oil. [j.] Composition from Uki *et al*. (1985b). [k.] α cellulose, Sigma Chemicals, USA [m.] Prepared using citric and phosphoric acids and heat treatment according to Viana *et al*. (1993b).

using the figures 14.65 KJ/g and 35.58 KJ/g respectively (Jauncey, 1982). The DE supplied by the protein component varied between diets as DE values were obtained for each of the protein sources under investigation (NRC, 1983; NRC, 1993).

Abalone have a limited ability to utilise high levels of dietary lipid (Uki and Watanabe, 1992; Britz, 1995) and appear to primarily use carbohydrates to satisfy their energetic requirements (Mai *et al*., 1995b; Knauer *et al*., 1996). The energy levels in the diets were balanced by maintaining a constant lipid level across the diets and partial replacement of the starch component with α-cellulose. Cellulose was used as it was assumed to be a non-nutritive filler and therefore did not add to the DE levels in the diets. However, recent evidence suggests that this assumption may be erroneous as there is some evidence that cellulose may be partially available to the abalone (Monje and Viana, 1998).

Statistical analysis

One-way analysis of variance and Student Newman Keuls multiple comparison procedure were used to determine differences in growth rate, condition factor, feed consumption, FCR and PER between dietary treatments. Two-way analysis of variance was used to determine the effects of animal size and dietary treatment on growth, FCR, PER, CF and consumption. Data from experimental replicates were pooled as no significant differences were found between them.

RESULTS

The growth rates of the large and small abalone ranged between 0.33 – 2.12 and 0.24 – 1.68 mm / month respectively (Table 4.3). Two way analysis of variance (Table 4.4) revealed that animal size significantly affected growth rate ($P<0.0001$), with the larger abalone growing at a faster rate than the small abalone.

A significant interaction between dietary protein source and animal size was found ($P=0.02$) indicating that the growth response to a given protein source was influenced by animal size. Within size classes, the highest growth rates (Table 4.3, Figure 4.1) were achieved using the fishmeals, spirulina and sunflower based diets, and growth rates did not differ significantly between these proteins ($P>0.05$). In addition, with respect to the large abalone, there were no significant differences between the growth rates using these proteins sources and the casein based diet. This was not the case in the small abalone where the growth response to casein was significantly reduced with respect to the fishmeals. In the large abalone, no significant differences were found between the growth rates produced by the viscera silage, torula yeast and cotton meal based diets. In contrast, a higher degree of variation in the growth rates was observed in the smaller abalone. Brewery waste and carcass meal performed poorly in both size classes of abalone. In both size classes, the carcass meal produced significantly greater growth than the brewery waste.

The feed conversion ratios (FCR) and protein efficiency ratios (PER) for the large abalone ranged from 1.0 - 6.2 and 0.6 - 5.7 respectively, for the small abalone these

Figure 4.1. Growth rate of abalone reared on 12 protein sources (expressed as shell length).

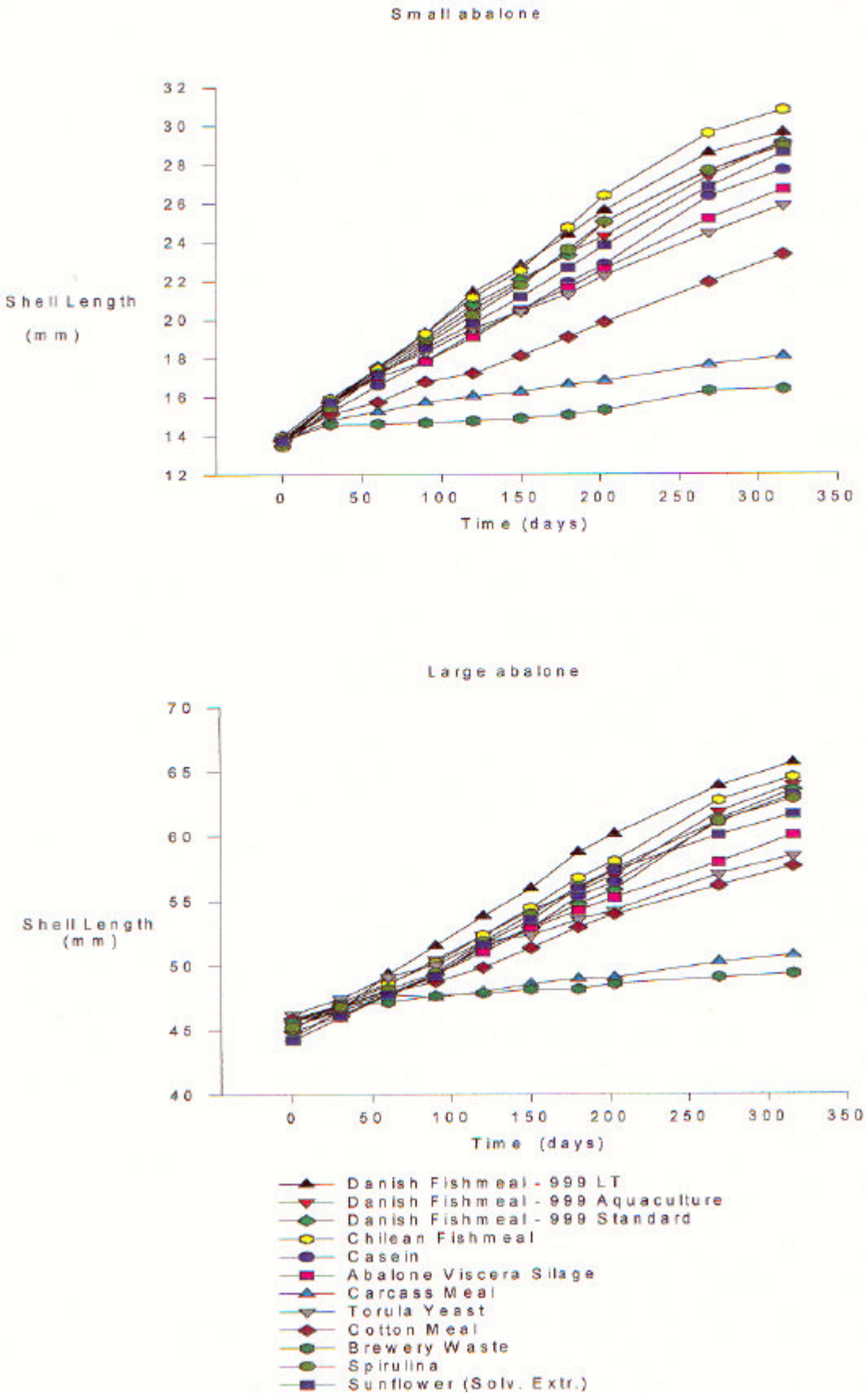


Table 4.3. Growth and nutritional indices for two size classes of abalone fed twelve single protein diets. Values are presented as means with standard errors. Different alphabetic superscripts indicate significant differences between treatments for the parameter concerned ($P<0.05$).

Parameter	Protein Source											
	Danish - LT Fishmeal	**Danish - Std Fishmeal**	**Danish -Aqua Fishmeal**	**Chilean Fishmeal**	**Casein**	**Carcass Meal**	**Viscera Silage**	**Spirulina**	**Sunflower Meal**	**Torula Yeast**	**Cotton Meal**	**Brewery Waste**
Large Abalone												
Weight / length data												
Initial length (mm)	44.4 ± 0.8	45.0 ± 0.8	45.7 ± 0.8	45.1 ± 0.6	45.7 ± 0.7	45.8 ± 0.7	45.3 ± 0.6	45.2 ± 0.6	44.2 ± 0.6	46.2 ± 0.7	45.9 ± 0.7	45.6 ± 0.7
Initial weight (g)	19.6 ± 1.4	19.6 ± 1.6	20.0 ± 1.1	20.0 ± 0.9	20.1 ± 0.9	20.0 ± 1.1	18.8 ± 0.9	19.8 ± 1.0	19.7 ± 0.9	20.2 ± 1.4	19.4 ± 0.8	19.7 ± 1.2
Final length (mm)	65.6 ± 1.4	61.1 ± 1.7	62.9 ± 1.5	64.5 ± 1.6	64.5 ± 1.4	50.7 ± 1.3	60.0 ± 1.1	62.8 ± 2.0	63.3 ± 1.6	58.4 ± 0.8	57.6 ± 1.2	49.3 ± 1.3
Final weight (g)	48.3 ± 3.4	41.1 ± 3.3	44.5 ± 2.8	47.7 ± 3.4	45.5 ± 3.0	22.9 ± 1.9	36.0 ± 1.7	44.9 ± 3.9	43.5 ± 3.0	32.6 ± 1.4	33.3 ± 1.8	19.0 ± 1.6
Small Abalone												
Weight / length data												
Initial length (mm)	13.6 ± 0.1	13.7 ± 0.1	13.8 ± 0.1	13.9 ± 0.1	13.8 ± 0.2	13.7 ± 0.1	13.4 ± 0.1	13.5 ± 0.1	13.7 ± 0.1	13.6 ± 0.2	13.8 ± 0.2	13.8 ± 0.1
Initial weight (g)	0.48 ± 0.01	0.50 ± 0.02	0.50 ± 0.01	0.52 ± 0.02	0.52 ± 0.02	0.49 ± 0.01	0.46 ± 0.02	0.48 ± 0.01	0.49 ± 0.01	0.48 ± 0.02	0.48 ± 0.02	0.48 ± 0.02
Final length (mm)	29.4 ± 0.4	29.1 ± 0.4	29.1 ± 0.4	30.8 ± 0.4	27.7 ± 0.4	18.1 ± 0.2	26.7 ± 0.4	28.9 ± 0.6	28.6 ± 0.3	25.9 ± 0.4	23.3 ± 0.3	16.4 ± 0.2
Final weight (g)	4.27 ± 0.10	4.10 ± 0.16	4.06 ± 0.17	4.72 ± 0.10	3.48 ± 0.16	0.92 ± 0.03	2.93 ± 0.11	3.93 ± 0.15	3.60 ± 0.11	2.52 ± 0.09	1.87 ± 0.06	0.64 ± 0.02
Growth												
Length increment (mm/month)												
Large abalone	2.12^{a}	1.62^{abc}	1.73^{ab}	1.94^{ab}	1.66^{abc}	0.43^{e}	1.47^{bcd}	1.82^{ab}	1.72^{ab}	1.24^{cd}	1.18^{d}	0.33^{f}
Small abalone	1.59^{ab}	1.53^{ab}	1.52^{ab}	1.68^{a}	1.28^{c}	0.43^{e}	1.24^{c}	1.54^{ab}	1.49^{ab}	1.23^{c}	0.97^{d}	0.24^{f}
Food conversion efficiency												
Large abalone	1.1^{a}	1.2^{a}	1.0^{a}	1.0^{a}	1.4^{ab}	4.0^{d}	1.0^{a}	1.3^{a}	1.2^{a}	2.6^{c}	1.8^{b}	6.2^{e}
Small abalone	1.1^{a}	1.2^{a}	1.2^{a}	1.0^{a}	2.0^{b}	6.2^{d}	1.0^{a}	1.2^{a}	1.4^{ab}	1.2^{a}	3.1^{c}	7.1^{e}
Protein efficiency ratio												
Large abalone	5.5^{a}	5.4^{a}	5.7^{a}	5.3^{a}	4.9^{a}	1.4^{c}	5.1^{a}	5.8^{a}	4.8^{a}	2.3^{c}	3.3^{b}	0.6^{d}
small abalone	5.4^{a}	4.9^{ab}	5.1^{ab}	5.1^{ab}	4.0^{cd}	0.8^{f}	4.6^{abc}	5.6^{a}	4.1^{bc}	3.3^{d}	2.0^{e}	0.4^{f}
Consumption (% body wt./d.)												
Large abalone	0.40^{a}	0.35^{a}	0.33^{a}	0.34^{a}	0.40^{a}	0.37^{a}	0.28^{a}	0.34^{a}	0.36^{a}	0.27^{a}	0.37^{a}	0.16^{b}
small abalone	1.20^{a}	1.02^{ab}	1.00^{ab}	0.95^{b}	0.99^{ab}	1.36^{a}	0.87^{bc}	1.17^{a}	1.23^{a}	0.86^{bc}	1.28^{a}	0.67^{c}
Condition factor (day 316)												
Large abalone	0.97^{a}	0.99^{a}	1.02^{a}	1.01^{a}	0.96^{a}	0.99^{a}	0.95^{a}	0.99^{a}	0.97^{a}	0.95^{a}	1.00^{a}	0.89^{b}
Small abalone	0.93^{ab}	0.93^{ab}	0.94^{ab}	0.93^{ab}	0.93^{ab}	0.90^{c}	0.91^{bc}	0.96^{a}	0.89^{c}	0.82^{d}	0.83^{d}	0.81^{d}
Mortality (%)												
Large abalone	12.5	0.0	4.2	8.3	8.3	12.5	4.2	4.2	12.5	8.3	8.3	16.7
Small abalone	1.1	1.1	2.2	1.1	1.1	4.4	0.0	4.4	0.0	3.3	4.4	4.4

Table 4.4. Effect of protein source and animal size on growth (length increment), feed conversion ratio (FCR), protein efficiency ratio (PER), Condition factor (CF) and consumption (% bd.wt.day^{-1}). Means for each effect were calculated using Two-way ANOVA. For the respective effects, significant differences between the means ($P<0.05$) are indicated by different alphabetic superscripts.

Parameter	Effect	Df	*F*	*P*	Protein	Mean	Abalone Size	mean
Growth	Protein	11	64.77	<0.0001	LT Fish.	1.85^{a}	Large	1.45^{x}
Length increment	Size	1	32.38	<0.0001	Standard Fish.	1.58^{bc}	Small	1.24^{y}
(mm / month)	Protein x Size	11	5.43	0.017	Aqua Fish.	1.62^{abc}		
					Chilean Fish.	1.81^{ab}		
					Casein	1.47^{cd}		
					Carcass Meal	0.43^{g}		
					Viscera Silage	1.36^{de}		
					Spirulina	1.68^{abc}		
					Sunflower Meal	1.61^{abc}		
					Torula Yeast	1.24^{ef}		
					Cotton Meal	1.07^{f}		
					Brewery Waste	0.29^{g}		
FCR	Protein	11	16.61	<0.0001	LT Fish.	1.08^{a}	Large	1.61^{x}
	Size	1	5.55	0.019	Standard Fish.	1.21^{a}	Small	1.82^{y}
	Protein x Size	11	2.44	0.0066	Aqua Fish.	1.11^{a}		
					Chilean Fish.	1.03^{a}		
					Casein	1.44^{ab}		
					Carcass Meal	5.06^{d}		
					Viscera Silage	1.03^{a}		
					Spirulina	1.24^{a}		
					Sunflower Meal	1.30^{a}		
					Torula Yeast	1.90^{b}		
					Cotton Meal	2.46^{c}		
					Brewery Waste	6.60^{e}		
PER	Protein	11	26.82	<0.0001	LT Fish.	5.45^{ab}	Large	4.19^{x}
	Size	1	5.41	0.021	Standard Fish.	5.18^{ab}	Small	3.78^{y}
	Protein x Size	11	0.95	0.491	Aqua Fish.	5.47^{ab}		
					Chilean Fish.	5.22^{ab}		
					Casein	4.38^{b}		
					Carcass Meal	1.09^{d}		
					Viscera Silage	4.88^{ab}		
					Spirulina	5.71^{a}		
					Sunflower Meal	4.47^{b}		
					Torula Yeast	2.82^{c}		
					Cotton Meal	2.66^{c}		
					Brewery Waste	0.50^{d}		
Condition	Protein	11	16.65	<0.0001	LT Fish.	0.95^{abc}	Large	0.97^{x}
Factor	Size	1	193.70	<0.0001	Standard Fish.	0.96^{ab}	Small	0.90^{y}
(CF)	Protein x Size	11	4.95	<0.0001	Aqua Fish.	0.98^{a}		
					Chilean Fish.	0.97^{a}		
					Casein	0.95^{abc}		
					Carcass Meal	0.94^{abc}		
					Viscera Silage	0.93^{bc}		
					Spirulina	0.98^{a}		
					Sunflower Meal	0.93^{bc}		
					Torula Yeast	0.88^{d}		
					Cotton Meal	0.92^{c}		
					Brewery Waste	0.85^{e}		
Consumption	Protein	11	15.43	<0.0001	LT Fish.	0.80^{abc}	Large	0.33^{x}
(% bd.wt. day^{-1})	Size	1	1410.71	<0.0001	Standard Fish.	0.69^{bcde}	Small	1.05^{y}
	Protein x Size	11	5.34	<0.0001	Aqua Fish.	0.66^{cde}		
					Chilean Fish.	0.65^{de}		
					Casein	0.69^{bcde}		
					Carcass Meal	0.87^{a}		
					Viscera Silage	0.57^{e}		
					Spirulina	0.76^{abcd}		
					Sunflower Meal	0.80^{abc}		
					Torula Yeast	0.56^{e}		
					Cotton Meal	0.82^{ab}		
					Brewery Waste	0.41^{f}		

figures were 1.0 - 7.2 and 0.4 - 5.6. As anticipated, two way analysis of variance (Table 4.4) revealed that protein source significantly affected both feed conversion and protein efficiency ($P<0.0001$). Likewise, these parameters were significantly affected by animal size ($P<0.05$). The larger animals yielded significantly lower FCRs and higher PERs than the smaller animals indicting greater feed conversion. A significant interaction ($P<0.05$) was found between protein source, animal size and feed conversion indicating that the FCR response to a given protein is dependent upon animal size. Interestingly, no such interaction was found with regards to protein efficiency, indicating that the PER response to a given protein source is independent of animal size.

With respect to FCR and PER values for individual protein sources, significant differences between the ratios were generally the same when the ratios were calculated either on an individual size class basis (Table 4.3) or as a combination of both size classes (Table 4.4). The fishmeals, viscera silage and spirulina yielded significantly lower FCR and correspondingly higher PERs than the other protein sources ($P<0.05$, Tables 4.3, 4.4). In the large abalone, no significant differences were found between the FCR and PER ratios of these protein sources and casein. However, this was not the case with the smaller abalone where the respective FCR and PER ratios were found to be significantly higher and lower indicating not only poorer utilisation of the casein, but also illustrating the effect of animal size on protein utilisation.

Sunflower meal performed well in terms of feed conversion, and no significant differences were found between the FCRs of either size class and the top performing protein sources (Tables 4.3, 4.4). In terms of protein efficiency, a similar situation was

found with respect to the FCRs, the large abalone and the top performing proteins. In contrast, the protein efficiency of the smaller abalone fed the sunflower was lower than that of the larger animals, and although it was similar to a number of the fishmeals, it was significantly lower than the Danish-LT fishmeal and the spirulina based diets.

The remaining four protein sources (torula yeast, brewery waste, carcass and cotton meals) did not perform well in terms of either feed conversion or protein efficiency. Performance was poorest with the brewery waste where the respective mean FCR and PER values from both size classes of animal were 6.6 and 0.5 (Table 4.4). Such figures indicate a protein source of very low biological value.

Two-way analysis of variance (Table 4.4) revealed that at the end of the experimental period, the condition factors (CF) of the abalone had been significantly influenced by both protein source ($P<0.0001$) and animal size ($P<0.0001$). While the CFs in the larger abalone were significantly higher than those in the smaller abalone, the variability in the CFs were greatest in the small abalone (Table 4.3). In addition, a significant interaction ($P<0.0001$) was found between the protein source, animal size and the CF (Table 4.4), indicating that the CF generated by a given protein source was dependent upon animal size. Nevertheless, with the exception of brewery waste, CFs in the large abalone were not significantly affected by protein source (Table 4.3). This however was not the case with the small abalone where the results were more complex. No significant differences were found between the fishmeals, casein and spirulina which produced the highest CFs, and although a significant difference was found between the CF of the viscera silage and spirulina, no significant differences were found between the viscera silage, fishmeals and

casein. In addition, no significant differences were found between the viscera silage, carcass and sunflower meals which recorded lower CFs than the fishmeals. The lowest CFs were found in the torula yeast, cotton meal and brewery waste based diets where no significant differences were observed ($P>0.05$).

The consumption rates observed between the two size classes of abalone were significantly influenced by animal size ($P<0.0001$, Table 4.4), and as expected, significantly greater in the smaller abalone. In addition, protein source was also found to significantly affect consumption ($P<0.0001$, Table 4.4). While this was clearly demonstrated with the smaller abalone, it was less apparent in the larger abalone where protein source did not significantly affect consumption ($P>0.05$, Table 4.3). The exception being the brewery waste where, with respect to the other protein sources, consumption was significantly reduced ($P<0.05$). Moreover, a significant interaction ($P<0.0001$, Table 4.4) was found between protein source and animal size indicating that the consumption of a given protein source was dependant upon animal size. This was a product of the high variability in consumption rates observed with the small abalone and the more constant rates observed in the larger abalone (Table 4.3).

The mortality of the small abalone was low ranging between 0 - 4.4%. This was not the case for the larger abalone where a higher mortality ranging between 0 - 16.6% was recorded. The high mortality in the larger abalone occurred over the first month of the experiment and attributed to a polychaete worm infestation in the shells of the abalone. The worms were found at the first weigh and measure at day 30, and the problem resolved by physically removing them from the animals.

DISCUSSION

In general, significant differences in the response between the juvenile and young adult abalone to the dietary treatments, indicate the existence of size specific nutritional requirements in *H.midae*. As expected, protein source was found to profoundly affect growth, FCR, PER and consumption rates. Furthermore, while the fishmeals, spirulina and sunflower meals promoted the best growth and feed efficiency, all the protein sources with the exception of the brewery waste and carcass meal, demonstrated potential as commercial feed ingredients.

The high growth rates, PERs and low FCR values observed across all the fishmeal diets indicate proteins of high biological value. These results compare favourably with previous studies with abalone using either processed LT-fishmeal (Britz, 1996b) or unprocessed raw fish (Uki and Watanabe, 1986). The FCR and PER values recorded here were however higher than in these previous studies. This was probably due to the lower dietary protein concentrations in the current study, and therefore conform to the well established trends with respect to FCR, PER values and increasing dietary protein content (Steffens, 1989b; Britz, 1996a).

Detailed fishmeal processing temperature data was not available for all the fishmeals used in this experiment. However, with respect to the other fishmeals that were processed at higher temperatures, the LT-fishmeal was found to be the most digestible (Chapter 3, Table 3.3). Extensive studies have demonstrated that nutrient composition, raw material quality and processing conditions affect the biological value of fishmeals. Of particular

significance are processing temperatures that have been shown to negatively correlate with digestibility in salmonids (McCallum and Higgs, 1989; Pike *et al.,*1990), and the presence of biogenic amines produced during the bacterial degradation of the raw fish. Biogenic amines can be used to determine raw material quality, and hence nutrient quality of the meal (Aksnes and Brekken, 1988; Clancy *et al.,* 1995). Unfortunately, the biogenic amine concentration was not known for all the fishmeals used in the experiment, and its effect on feed quality in abalone therefore requires further clarification. Nevertheless, it appears that as with fin fish, fish meal processing temperatures may be an important factor affecting fishmeal digestibility coefficients in abalone.

Consumption rates using the LT-fishmeal were appreciably higher than those observed with the other fishmeals, and in the absence of quantitative differences in either formulation or energy densities between dietary treatments, differences in consumption rates must be due to the fishmeal component. While Pike (1993) demonstrated that raw material freshness (measured as biogenic amine concentration) affected feed intake in salmon, Moksness *et al*. (1995) proposed that heat treatment and the reduction of free amino acids and other low molecular weight feeding stimulants may have a negative affect on palatability. Although both factors may have played a role in this study, it was not possible to determine the relative importance of either factor on consumption rates, and thus further research is required.

In a review of the role of fishmeals in salmonid diets, Pike *et al.* (1990) found that LT-fishmeals outperformed conventionally processed fishmeals by an average of 10 and 15% in terms of feed conversion and growth respectively. This was not the case in the current

study, where despite marked differences in apparent protein digestibility between fishmeals (Chapter 3, Table 3.3), no significant differences were found in the growth parameters between the LT-fishmeal and the alternative three fishmeals. Previous studies with abalone (Britz, 1996b; Uki and Watanabe, 1986) have demonstrated differential growth responses to fishmeals, however these results are difficult to interpret as not only were they undertaken on different species of abalone, but in addition under different dietary and experimental conditions. In contrast, the fishmeals used in the current experiment were tested on the same species of abalone and under the same experimental conditions. Nevertheless, with a current price differential of approximately 30% between the fishmeals used in the current experiment (C.Clayden, Sea Plant Products Ltd., Per. Com., 1998), selection is an important parameter for commercial feed formulators. Furthermore, as it appears that the premium LT-fishmeal failed to produce the improved growth parameters observed in fish, it is appropriate to use less expensive alternatives.

The growth rate and nutritional indices observed in the large abalone fed the abalone viscera silage diet, were comparable to those obtained using the fishmeal based diets. However, while comparisons with similar growth studies (Viana *et al.,* 1996; Guzmán and Viana, 1998) using viscera silages are difficult due to differences in experimental parameters, the results in this experiment generally confirm previous findings that suggest that the silage promotes good growth in abalone. In contrast to the large abalone, the growth performance in the small abalone fed the viscera silage was reduced with respect to the fishmeals. While the reduced growth rate may have been due to differences in either dietary requirements or nutrient availability between two size classes of abalone, differential consumption rates may have also played a role. Viana *et al.* (1994) found that

abalone viscera silage was an attractive ingredient to *H.fulgens*, and while consumption rates in the large abalone were similar to those of the fishmeals, the consumption rates in the small abalone were reduced with respect to the fishmeals. As it is not apparent why relative consumption rates should vary between the size classes of animals, further investigation is required.

In comparison to the fishmeal based diets, pellet stability with the viscera silage based diet was low in terms of both total solids and protein leaching. Guzmán and Viana (1998) reported a similar effect with viscera silage/soya and fishmeal based diets, and although when compared to the current study they reported superior feed stability, this was probably due to a combination of lower experimental temperatures reducing leaching rates and differences in the feed composition, particularly binders. Furthermore, Rivero and Viana (1996) measured pellet stability in viscera silage diets and concluded that the combination of low pH in the feed mixtures, and the liquefaction of the silage due to the presence of partially hydrolysed proteins, make them difficult to bind.

The abalone fed the sunflower meal and spirulina protein sources performed well in terms of FCR and PER. However, while the spirulina produced growth that was comparable to the fishmeals, growth using the sunflower meal was slightly reduced. Consumption rates can not account for this reduction in growth as the rates in both diets were similar to the fishmeals, and indicate that they pose no palatability problems to the animals. Furthermore, the high apparent protein digestibility coefficients, particularly with respect to the sunflower meal (85.1%), indicate high availability of the protein sources to the abalone. Britz (1996b) tested spirulina as a protein source for juvenile *H.midae* and

obtained similar results to those reported in this study. Although spirulina is currently included in the commercial abalone feed formulations at low levels, the raw material costs are approximately 2.5 times those of fishmeal and preclude its use as a major protein source. In contrast, sunflower meal is approximately 25% the cost of fishmeal, and therefore encouraging with respect to diet development, particularly as a partial fishmeal replacement.

Abalone fed the diets containing carcass meal and brewery waste performed poorly in terms of growth, FCR and PER. Low feed consumption observed with the brewery waste based diet may partially account for the restricted growth with this diet, however this was not a factor with the carcass meal where consumption rates were equal or superior to those observed with the fishmeal based diets. The low protein efficiency ratios indicate proteins of low biological value, and while availability measured as apparent protein digestibility was low in the carcass meal (52.1%), it was not measured in the brewery waste due to low feed consumption precluding faecal collections. A comparison between the growth and nutritional responses between these proteins and the torula yeast and cotton meal based diets show that the latter produced superior growth responses, and correspondingly improved feed conversion and protein efficiency. While this may in part be explained by the high apparent protein digestibility coefficients (torula yeast : 83.4%; cotton meal : 76.2%) and hence availability of these protein sources to the abalone, it does not adequately account for the generally poorer growth and nutritional indices observed with these protein sources when compared to the other protein sources in the study. In the absence of marked protein or energy imbalances between dietary treatments, the differences are probably due to limiting essential amino acids or imbalances within

these amino acids. Allen and Kilgore (1975) demonstrated that abalone require the same ten essential amino acids as other groups of animals, and although no studies have been undertaken with abalone, studies with fish (Ogino, 1980; Jackson *et al*., 1982; Tacon, 1990b) have shown some of these protein sources to be deficient or imbalanced in essential amino acids. Thus, although these limitations or imbalances have not been empirically proven in abalone, it is not unreasonable to suggest that they may play a role in restricting growth in the animals. In addition, it should be noted that with respect to the plant protein sources, antinutritional factors may have played a role reducing protein utilisation.

The growth and nutritional responses to casein were found to be size specific, and while similar growth and nutritional parameters were recorded between the large abalone fed either the casein or fishmeal based diets, this was not the case for the small abalone where both growth and feed conversion were reduced with respect to the fishmeals. Interestingly, within size classes, consumption rates between the casein and fishmeal based diets were similar and therefore discount feed intake as a factor affecting the growth parameters. In previous comparative trials between fishmeals and casein, Britz (1995) recorded a reduction in growth in juvenile *H.midae* fed casein based diets, and obtained similar results to the small abalone in this trial. In contrast, Uki and Watanabe (1986) reported a similar growth response to either casein or raw fish in *H.discus hannai*; a result confirmed by Viana *et al.* (1993a) using a fishmeal based diet fed to *H.fulgens*. Although differential growth responses in these studies may have been due to a number of factors including variability in protein quality across the studies, species specific differences in the ability to digest the protein sources (Britz, 1995), or differences in the

nutritional requirements between species. These factors cannot account for the size specific differences in nutritional parameters recorded in this study.

Animal size was found to be a significant factor determining growth, feed conversion and protein efficiency (Table 4.4). In all cases, poorer performance was observed in the smaller animals. These results conflict with previous work with *H.midae* using fishmeal based diets (Britz and Hecht, 1997) that suggests a reduction in feed conversion and protein efficiency with increasing animal size (Table 4.5). Comparisons with the present results indicate that while differences in the feed conversion in the small abalone were negligible, there was a slight increase in the protein efficiency ratios reported in this study. This can be attributed to the effects of protein leaching that were taken into account during the calculation of the PER values. This however cannot account for the general improvement in feed conversion and protein efficiency observed in the larger size class of animal in the present study (Tables 4.3, 4.4). With respect to the large abalone, the close similarity between the experimental parameters, animal size and dietary formulation between the study conducted by Britz and Hecht (1997) and the current study make it difficult to adequately account for the differential feed conversions reported between the studies.

Table 4.5. Comparison of feed conversion efficiency (FCR) and protein efficiency ratios (PER) by abalone.

Animal Size	FCR	PER	Dietary Protein	Source
0.5 – 4.2g	1.1	5.4	LT-Fishmeal –20%	Present study
19.6 – 48.3g	1.1	5.5	LT-Fishmeal –20%	
0.2 – 1.0g	1.0	4.3	LT-Fishmeal -24%	Britz and Hecht (1997)
7 – 14g	1.7	2.4	LT-Fishmeal -24%	

Nevertheless, the differences in performance between the two size classes of abalone across all the diets (Table 4.4) may be due to a number of factors. Firstly, differential ability to digest proteins between size classes of abalone and therefore the availability of protein to the abalone cannot be discounted. Studies with some fish species (Kitamikado and Tachino, 1960; Rajamani and Job, 1976; Ferraris *et al*., 1986) have demonstrated that digestibility increases with animal size, and while there are currently no extensive studies into the effects of animal size on protein digestibility in abalone, Uki and Wantanabe (1992) reported differences in the ability of two size classes of *H.discus hannai* to digest white fishmeal. However, it was found that digestibility reduced with increasing animal size, and therefore protein availability would be expected to be greatest in the smaller abalone. Furthermore, Knauer *et al.* (1996) found that juvenile *H.midae* responded to an increase in dietary protein by elevating digestive protease levels, thus implying a high degree of plasticity in digestive enzyme profiles in response to dietary protein. Nevertheless, as attempts to determine digestibility coefficients in the small animals failed to produce reliable results due to problems associated with obtaining sufficient faecal samples, and as no attempt was made to determine the digestive enzyme profiles of the two size classes of animals. The results suggest that differential availability of the protein sources to the two size classes of abalone may be a factor affecting the nutritional parameters in this study.

The second factor that may account for the differential performance of the two size classes of abalone to the protein sources, is the presence of quantitative differences in their essential amino acid requirements. Unfortunately, the quantitative amino acid requirements of Haliotids have yet to be determined, and as it was beyond the scope of

this experiment to determine these requirements, further experimentation is required to determine whether this was the case.

To conclude, the results from this study indicate that there are qualitative differences in the protein requirements between juvenile and young adult abalone. However, in order to determine causality, further investigations, particularly with respect to protein digestibility, digestive enzyme profiles and the quantitative amino acid requirements between the size classes of abalone are required. With respect to the protein sources used in the investigation, the superior growth and feed conversion obtained with the fishmeal, spirulina and sunflower meal based diets suggest that, notwithstanding ingredient costs, they show the best potential as primary protein sources in formulations. With the exception of the carcass and brewery waste, the relatively good growth, feed and protein conversion obtained with the other protein sources suggest that, although they may be limiting in available EAAs, they are good candidates to partially replace the primary protein sources. Nevertheless, their efficacy as primary protein replacements needs to be assessed. In particular, the availability of EAA from the protein sources requires determination so that once the EAA requirements of the abalone have been determined, mixed protein diets may be designed that supply the optimal balance and inclusion rates of EAA to support optimal growth and feed conversion in the abalone.

CHAPTER 5

REPLACEMENT OF FISHMEAL WITH SELECTED PLANT PROTEIN SOURCES IN FORMULATED FEEDS FOR JUVENILE *H.MIDAE.*

INTRODUCTION

Increasing concerns about the future supply and demand for fishmeals (Barlow, 1989; Rumsey, 1993) have increased efforts to reduce their use as the major protein source in commercial aquaculture feed formulations. The reduction of the fishmeal component in feeds has met with considerable success in a number of species, and suggests that its partial substitution with alternative protein sources need not compromise growth. The most notable success to date is illustrated by the channel catfish industry, where the substitution of fishmeal with soya, meat and blood meals have reduced current fishmeal inclusion rates to as low as 0-4% compared with >30% in pre-1975 formulations (Stickney *et al*., 1996). Although these levels of substitution have not been as dramatic in other sectors of the industry, it has been demonstrated that significant reductions in dietary fishmeal are possible. Amongst others, these sectors include the salmonids (Moyano *et al*., 1992; Pongmaneerat and Watanabe, 1992) tilapias (Jackson *et al*., 1982; Lim, 1989a) and shrimp (Lim,1989b).

With respect to abalone nutrition, a number of studies have compared fishmeal based diets with other single protein formulations (Uki *et al*., 1985a; Uki and Watanabe, 1986,

1992; Viana *et al.*, 1993a; Britz, 1996b), and although these studies give an indication of which proteins may have the potential to become fishmeal substitutes, they provide no indication as to their efficacy as substitutes. Fleming *et al.* (1996) reviewed the nutritional composition of twelve currently available commercial and experimental dietary formulations, and found that of the eight diets that contained fishmeal, all contained supplementary proteins. However, no indication of the effects that these alternative protein sources had on growth and nutritional indices is available. Guzmán and Viana (1998) demonstrated that a combination of abalone viscera silage and soya meal could be used to replace fishmeal in diets for *H.fulgens* without compromising growth. Furthermore, considering the success of previous growth trials using single plant protein sources (Uki *et al*., 1985a; Britz, 1996b; Present study, Chapter 4), and the fact that Haliotids are naturally herbivorous animals with a digestive and enzymatic physiology equipped for processing plant materials (review by Knauer, 1994), the prospects for replacing dietary fishmeal with plant protein sources are good. This is of particular relevance to commercial feed manufacture in South Africa, where fishmeal currently supplies 100% of the protein component in formulations (C.Clayden, Sea Plant Products Ltd., per. com.). The aim of this study was therefore to determine the effects that the partial and total replacement of the fishmeal component with plant protein sources in the current South African commercial formulation, have on growth, nutritional indices and body composition in juvenile *H.midae*.

The fishmeal substitutes chosen for the study were: soya meal, sunflower meal, torula yeast, spirulina and corn gluten meal. The sunflower meal, spirulina and torula yeast were chosen as they had all performed well in terms of both growth and feed conversion when

fed to abalone as single protein diets (Chapter 4). It could therefore be concluded that all possessed well-balanced profiles of available essential amino acids, and as such were good candidates as fishmeal replacements. Soya meal was included in the study, as of all the plant protein sources, it has one of the best amino acid profiles for meeting the essential amino acid requirements of fish (Lovell, 1989b); and as Allen and Kilgore (1975) found that abalone require the same 10 essential amino acids as fish, it is not unreasonable to assume that the same may hold true for abalone. Furthermore, a preliminary study at this laboratory has suggested that fishmeal could be substituted with soya meal by up to 50% without reducing growth rates in juvenile *H.midae* (McDonald, Rhodes University, unpublished data). Corn gluten was included as it has proved a viable fishmeal replacement in studies with fish (Moyano *et al*., 1992; Robaina *et al*., 1997), and is currently available in South Africa at approximately half the cost of fishmeal.

In addition, two diets containing either ground maize or semolina were formulated. As ground maize and semolina have low protein concentrations - 9.5% and 14.0% respectively - they can only be used to replace 10-15% dietary fishmeal. Nevertheless, they are relatively inexpensive sources of carbohydrates. At present, the commercial abalone feed formulation used in South Africa relies on relatively expensive processed starches as both a dietary energy source and pellet binder. Replacement of these starches with cheaper, and in the case of the ground maize, unprocessed carbohydrates, may reduce formulation costs considerably. The aim of including these supplemental formulations into the trial was therefore to determine whether it was possible to replace existing starches in the formulation with cheaper alternatives without compromising growth and feed conversion.

METHODS

Experimental animals and system

The experimental system and environmental parameters under which the experiment was carried out were identical to those described previously (Chapter 4). Juvenile hatchery-reared abalone (10.6±0.1mm shell length, 0.26±0.01g weight) were used in the experiment. Prior to experimentation, the animals were fed exclusively on a fishmeal based artificial diet (34 % protein, 4.5% lipid) and acclimated to the experimental system for a one month period. Three replicate groups of thirty abalone were assigned to each dietary treatment, and placed in the experimental system according to a randomized block design. The duration of the experiment was 180 days, and the abalone were weighed and measured monthly according to the protocol described in Chapter 4.

Animals were fed daily at 1700h, and the feed consumed over the experimental period recorded and corrected for leaching. Total solids leached from the diets over a six hour period were determined according to the protocol outlined in Chapter 4. Feed consumption, protein efficiency ratio, feed conversion ratio and condition factors were calculated for each treatment using the equations described in Chapter 4. The specific growth rates (% body wt./day) for each treatment were calculated from the slopes of the natural log transformed weight data x 100.

Dietary formulations

Fifteen experimental diets were formulated to contain 34% protein, 4.5% lipid; a sixteenth diet containing 34% fishmeal and 4.5% lipid corresponding to a commercial

formulation was manufactured and used as a control (Table 5.1). Formulations between experimental diets varied such that the fishmeal component of the control diet was substituted at either 30, 50, 75 or 100% with the selected plant protein sources previously discussed. The diets were bound using agar according to the methodology described by Knauer (1994). In summary, 20g agar was boiled in 1200ml water for 30 seconds and allowed to cool to 45°C. The agar was then mixed with 80g of dietary mixture, spread into a flat sheet 0.5cm thick and allowed to set at room temperature. Once set, the diet was cut into squares (0.5 x 0.5cm), oven dried at 60°C for 24 hours and stored frozen at –20°C until required.

Carcass and dietary analysis

Proximate analyses of the dietary treatments, and the abalone at the beginning and end of the experimental period were undertaken. While triplicate 5g samples of feed were used to determine the proximate composition of the dietary treatments; 30 animals at the start of the experiment, and 5 animals from each replicate at the end of the experimental period were used to determine the proximate composition of the abalone. The abalone were initially frozen at -20°C for subsequent analysis. On defrosting, the animals were weighed, shucked and the soft body material homogenized using an Ika Turrax T25 homogeniser. With the exception of lipid, the proximate composition of both abalone and the diets were measured according to the standard methods of the AOAC (1984). Thus, crude protein was determined as total Kjeldahl nitrogen (N x 6.25), ash as the residue following the combustion of samples at 550°C for 12 hr, and moisture by oven drying to constant weight at 104°C. Lipid was determined by the chloroform / methanol extraction method (Folch *et al*., 1957), and the nitrogen free extract estimated from the difference.

Table 5.1. Ingredient composition and nutrient analysis of experimental diets. Analysis expressed as a dry weight basis.

Diet	Control Fishmeal 100 (%)	Fishmeal / Soya meal 70 : 30 (%)	Fishmeal / Soya meal 50 : 50 (%)	Fishmeal / Sunflower meal 70:30 (%)	Fishmeal / Sunflower meal 50 : 50 (%)	Fishmeal / Torula yeast 70 : 30 (%)	Fishmeal / Torula yeast 50 : 50 (%)	Fishmeal / Semolina 85:15 (%)
Danish LT fishmeal [a]	47.7	33.0	23.5	33.0	23.5	33.0	23.5	40.0
Soya meal [b]	-	22.5	37.5	-	-	-	-	-
Spirulina [c]	-	-	-	-	-	-	-	-
Sunflower meal [b]	-	-	-	28.6	47.7	-	-	-
Torula yeast [d]	-	-	-	-	-	23.8	39.7	-
Semolina [e]	-	-	-	-	-	-	-	36.6
Starch	31.3	21.5	15.4	15.8	5.9	22.2	15.0	2.4
Lipid [f]	-	2.0	2.6	1.6	1.9	0.9	0.8	0.9
Vit / Min mix [h]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar [g]	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Analysis (calculated)								
Protein	34.2	34.2	34.2	34.2	34.2	34.2	34.2	34.2
Lipid	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Analysis (measured)								
Protein	35.1	35.6	33.0	31.5	33.3	31.4	32.2	32.9
Lipid	4.5	4.4	4.4	4.5	4.3	4.3	4.2	4.5
Ash	7.8	7.5	7.0	7.8	8.1	9.0	10.4	6.4
Moisture	5.2	6.4	6.5	6.3	5.3	6.8	6.2	7.5
Gross Energy (MJ/Kg)	19.62	19.51	19.17	18.52	19.42	18.81	18.94	19.35
Leaching Factor (solids)	0.96	0.81	0.81	0.91	0.93	0.91	0.92	0.96

Table 5.1 continued.

Diet	Fishmeal / Maize 90 : 10 (%)	Fishmeal / Corn gluten 70 : 30 (%)	Fishmeal / Corn gluten 50 : 50 (%)	Fishmeal / Soya meal / Sunflower meal 50 : 25 : 25 (%)	Fishmeal / Soya meal / Sunflower meal 25 : 37.5 : 37.5 (%)	Spirulina / Soya meal / Sunflower meal 50 : 25 : 25 (%)	Spirulina / Soya meal / Sunflower meal 25 : 37.5 : 37.5 (%)	Fishmeal / Spirulina 50 : 50 (%)
Danish LT fishmeal [a]	42.4	33.0	23.5	23.5	11.8	-	-	23.5
Soya [b]	-	-	-	18.8	28.1	18.8	28.1	-
Spirulina [c]	-	-	-	-	-	30.0	15.0	30.0
Sunflower [b]	-	-	-	23.9	35.8	23.9	35.8	-
Corn Gluten [b]	-	15.9	26.6	-	-	-	-	-
Maize meal [b]	36.5	-	-	-	-	-	-	-
Starch	-	28.3	26.7	10.5	0.5	3	0.8	23.9
Lipid [f]	0.1	1.8	2.2	2.3	2.8	3.3	3.3	1.6
Vit / Min mix [h]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar [g]	20.0	20.0	20.0	20.0	20.0	20.0	16.0	20.0
Analysis (calculated)								
Protein	34.2	34.2	34.2	34.2	34.2	34.2	34.2	34.2
Lipid	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Analysis (measured)								
Protein	32.0	33.4	35.6	33.4	33.4	30.6	30.7	30.1
Lipid	4.7	4.7	4.8	4.2	4.2	4.2	4.1	4.2
Ash	7.3	5.9	5.0	7.8	7.6	11.2	9.5	11.4
Moisture	6.0	6.1	4.9	5.8	6.1	6.7	5.3	5.4
Energy (MJ/Kg)	19.38	20.29	19.36	19.65	19.23	19.65	19.23	18.27
Leaching Factor (solids)	0.87	0.93	0.89	0.92	0.88	0.81	0.87	0.83

Dietary components supplied by : [a.] Danish 999 fishmeals, Esbjerg Fiskeindustri, Denmark. [b.] EPOL, S.A. [c.] Western Cape Tanneries, S.A. [d.] Illova Sugar, S.A. [e.] Tiger Milling, S.A. [g.] Continental Lab. Supplies, S.A.
[f.] 50/50 mixture of sunflower oil and "Marinol R" fish oil. [h.] Composition from Uki *et al*. (1985b).

Statistical analysis

Growth rates were determined as the slopes of the natural log-transformed weight data. The slopes of the growth models were compared using analysis of covariance (ANCOVA) followed by a one-tailed F-test (Zar, 1984). One way analysis of variance (ANOVA) and Student Newman Keuls multiple comparison procedure were used to determine significant differences in feed consumption, condition factors, FCR and PER between dietary treatments, and in addition, the effect of dietary treatment on the proximate composition of the abalone at the end of the experimental period. Comparisons between the essential amino acid profiles of the dietary treatments and the whole soft tissue of *H.midae* were determined using linear regression analysis. Pearson product moment correlations were used to determine the strength of association between growth rates, protein efficiency ratios and the levels of individual essential amino acids across all dietary treatments.

RESULTS

The partial and total replacement of dietary fishmeal with the alternative protein sources significantly affected abalone growth over the experimental period (Tables 5.2, 5.3). While six of the formulations produced growth rates that were not significantly different to the control, the remaining nine formulations produced significantly lower growth rates. Growth rates and increases in abalone weight over the experimental period ranged between 1.09 to 1.41%bd.wt.day^{-1} and 600 to 1230% respectively. Feed conversion ratios (FCR) and protein efficiency ratios (PER) ranged from 0.80 to 1.25 and 2.15 to 3.75 respectively.

Table 5.2. Growth and nutritional indices from juvenile abalone fed sixteen multiple protein diets. Values are presented as means with standard errors. Different alphabetic superscripts indicate significant differences between treatments for the parameter concerned. Growth rates are expressed as the slopes of the log-transformed weight data x 100. See Table 5.3 for ANCOVA describing significant differences between growth rates.

Diet	Initial Length (mm)	Final Length (mm)	Initial Weight (g)	Final weight (g)	Growth Rate (%bd.wt.day^{-1})	FCR	PER	Consumption (% bd.wt.day^{-1})	Condition Factor Day 0	Condition Factor Day 180	Mortality (%)
Control – Fishmeal 100 (%)	10.9 ± 0.1	25.8 ± 0.3	0.26 ± 0.01	3.20 ± 0.12	1.38	0.92^{e}	3.39^{b}	0.89^{d}	1.14^{a}	0.99^{a}	0.0
Fishmeal / Soya meal 70 : 30 (%)	10.8 ± 0.1	26.1 ± 0.3	0.27 ± 0.01	3.10 ± 0.09	1.31	0.82^{f}	3.02^{c}	1.11^{bcd}	1.20^{a}	0.99^{a}	1.1
Fishmeal / Soya meal 50 : 50 (%)	10.6 ± 0.1	25.8 ± 0.4	0.26 ± 0.01	2.88 ± 0.10	1.32	0.88^{ef}	2.83^{cd}	1.27^{bc}	1.18^{a}	0.96^{abc}	1.1
Fishmeal / Sunflower meal 70 : 30 (%)	10.5 ± 0.1	26.1 ± 0.3	0.25 ± 0.02	2.98 ± 0.09	1.37	1.00^{cde}	2.83^{cd}	1.23^{bc}	1.19^{a}	0.96^{abc}	2.2
Fishmeal / Sunflower meal 50 : 50 (%)	10.6 ± 0.1	24.5 ± 0.3	0.26 ± 0.01	2.40 ± 0.09	1.23	1.20^{ab}	2.39^{def}	1.38^{abc}	1.20^{a}	0.91^{bcd}	0.0
Fishmeal / Torula yeast 70 : 30 (%)	10.4 ± 0.1	26.7 ± 0.4	0.24 ± 0.01	3.48 ± 0.14	1.41	0.80^{f}	3.75^{a}	1.07^{cd}	1.19^{a}	0.94^{abc}	4.4
Fishmeal / Torula yeast 50 : 50 (%)	10.9 ± 0.1	26.4 ± 0.4	0.27 ± 0.02	3.10 ± 0.12	1.27	0.86^{f}	3.12^{bc}	1.12^{bcd}	1.19^{a}	0.94^{abc}	0.0
Fishmeal / Semolina 85 :15 (%)	10.9 ± 0.1	25.6 ± 0.2	0.27 ± 0.01	2.58 ± 0.08	1.29	1.10^{bcd}	2.41^{def}	1.40^{ab}	1.19^{a}	0.94^{abc}	4.4
Fishmeal / maize 90 :10 (%)	10.6 ± 0.1	26.2 ± 0.3	0.25 ± 0.01	3.02 ± 0.10	1.36	0.90^{e}	2.97^{c}	1.22^{bcd}	1.18^{a}	0.94^{abc}	2.2
Fishmeal / Corn Gluten 70 : 30 (%)	10.5 ± 0.1	23.1 ± 0.3	0.26 ± 0.01	2.39 ± 0.07	1.21	0.98^{cde}	2.84^{cd}	1.16^{bcd}	1.22^{a}	0.94^{abc}	3.3
Fishmeal / Corn Gluten 50 : 50 (%)	10.8 ± 0.1	23.1 ± 0.3	0.26 ± 0.01	1.58 ± 0.07	1.09	1.04^{cd}	2.36^{ef}	1.19^{bcd}	1.19^{a}	0.86^{d}	3.3
Fishmeal / Soya meal / Sunflower meal 50 : 25 : 25 (%)	10.7 ± 0.1	24.5 ± 0.3	0.27 ± 0.01	2.36 ± 0.08	1.19	1.06^{cd}	2.93^{c}	1.20^{bcd}	1.19^{a}	0.90^{bcd}	0.0
Fishmeal / Soya meal / Sunflower meal 25 : 37.5 : 37.5 (%)	10.8 ± 0.1	23.9 ± 0.3	0.26 ± 0.01	2.27 ± 0.09	1.17	1.03^{cd}	2.76^{cde}	1.31^{bc}	1.15^{a}	0.92^{abcd}	0.0
Spirulina / Soya meal / Sunflower meal 50 : 25 : 25 (%)	10.2 ± 0.1	24.4 ± 0.3	0.22 ± 0.02	2.27 ± 0.08	1.25	1.25^{a}	2.15^{f}	1.60^{a}	1.18^{a}	0.92^{abcd}	4.1
Spirulina / Soya meal / Sunflower meal 25 : 37.5 : 37.5 (%)	10.6 ± 0.1	24.4 ± 0.3	0.25 ± 0.01	2.32 ± 0.08	1.22	1.11^{bc}	2.43^{def}	1.42^{ab}	1.20^{a}	0.90^{cd}	4.4
Fishmeal / Spirulina 50 : 50 (%)	10.5 ± 0.1	25.2 ± 0.3	0.26 ± 0.01	2.80 ± 0.09	1.28	0.97^{de}	2.89^{c}	1.23^{bc}	1.22^{a}	0.98^{ab}	3.6

Table 5.3. Analysis of covariance describing differences in growth rates between dietary treatments. Growth models expressed as the natural log of the weight (y in grams) as a function of time (x in days) for the abalone fed the 16 experimental diets. Superscripts next to the *F*-values indicate which models were compared (*n* = number of values in model, r^2 = correlation coefficient of model, d.f. = degrees of freedom, *P* = significance). In order to avoid accumulated error, significance was denoted at 0.0166 for 3 model comparisons, and 0.0125 for 4 model comparisons.

Code	Diet	Growth model	*n*	r^2	*F*-statistic	d.f.	*P*	* Denotes significance
A	Control – Fishmeal 100(%)	ln y = -1.26 + 0.0138x	628	0.89				
B	Fishmeal / Soya meal 70 : 30 (%)	ln y = -1.32 + 0.0131x	621	0.86	4.72^{AB}	1,1245	P=0.03005	
C	Fishmeal / Soya meal 50 : 50 (%)	ln y = -1.24 + 0.0132x	622	0.90	5.59^{AC}	1,1246	P=0.01823	
					0.00^{BC}	1,1239	P=0.96	
D	Fishmeal / Sunflower meal 70 : 30 (%)	ln y = -1.38 + 0.0137x	602	0.90	0.16^{AD}	1,1226	P=0.68	
E	Fishmeal / Sunflower meal 50 : 50 (%)	ln y = -1.32 + 0.0122x	616	0.83	29.48^{AE}	1,1240	P<0.00001	*
					27.63^{DE}	1,1209	P<0.00001	*
F	Fishmeal / Torula yeast 70 : 30 (%)	ln y = -1.28 + 0.0141x	613	0.82	0.55^{AF}	1,1237	P=0.46	
G	Fishmeal / Torula yeast 50 : 50 (%)	ln y = -1.19 + 0.0127x	614	0.85	13.31^{AG}	1,1238	P=0.00027	*
					14.65^{FG}	1,1223	P=0.00013	*
H	Fishmeal / Semolina 85 : 15 (%)	ln y = -1.28 + 0.0129x	622	0.90	11.79^{AH}	1,1246	P =0.00061	*
I	Fishmeal / Maize 90 : 10 (%)	ln y = -1.28 + 0.0136x	594	0.90	0.52^{AI}	1,1218	P=0.47	
					7.45^{HI}	1,1212	P=0.00643	*
J	Fishmeal / Corn Gluten 70 : 30 (%)	ln y = -1.28 + 0.0121x	621	0.85	34.49^{AJ}	1,1245	P<0.00001	*
K	Fishmeal / Corn Gluten 50 :50 (%)	ln y = -1.34 + 0.0109x	616	0.82	103.36^{AK}	1,1240	P<0.00001	*
					17.22^{JK}	1,1233	P=0.00003	*
L	Fishmeal / Soya meal / Sunflower meal 50 : 25 : 25 (%)	ln y = -1.23 + 0.0119x	621	0.82	39.04^{AL}	1,1245	P<0.00001	*
M	Fishmeal / Soya meal / Sunflower meal 25 : 37.5 : 37.5 (%)	ln y = -1.34 + 0.0117x	637	0.81	47.06^{AM}	1,1261	P<0.00001	*
					0.43^{LM}	1,1254	P=0.51	
N	Spirulina / Soya meal / sunflower meal 50 : 25 : 25 (%)	ln y = -1.44 + 0.0125x	604	0.86	20.19^{AN}	1,1228	P<0.00001	*
O	Spirulina / Soya meal / sunflower meal 25 : 37.5 : 37.5 (%)	ln y = -1.33 + 0.0122x	600	0.87	31.38^{AO}	1,1224	P<0.00001	*
P	Fishmeal / Spirulina 50 : 50 (%)	ln y = -1.27 + 0.0128x	607	0.84	6.19^{AP}	1,1211	P=0.01298	
					0.92^{NO}	1,1200	P=0.33	
					2.76^{NP}	1,1187	P=0.096	
					6.74^{OP}	1,1283	P=0.00956	*

With respect to the control diet (100% fishmeal), only torula yeast significantly improved feed conversion at both the 30% and 50% fishmeal substitution levels ($P<0.05$, Table 5.2). However, while a 30% substitution of fishmeal with torula yeast did not significantly affect growth, at 50% substitution, growth was significantly lower than the control ($P=0.00027$, Table 5.3). With respect to protein efficiency, a 30% substitution of fishmeal for torula yeast significantly improved efficiency ($P<0.05$), but at a 50% substitution level, protein efficiency did not differ significantly from the control. In addition, consumption rates did not differ significantly between the control and the diets containing either 30 or 50% torula yeast.

With respect to the control, the inclusion of sunflower meal at 30% or soya meal at either 30 or 50% did not significantly affect growth rates ($P>0.016$, Table 5.3). Nevertheless, the inclusion of corn gluten meal at either 30 or 50% or sunflower meal at 50% produced significant reductions in growth rates ($P<0.016$, Table 5.3). In comparison with the control diet, feed conversion was significantly improved when soya meal was used at the 30% substitution level ($P<0.05$, Table 5.2), but did not differ significantly at a 50% level of substitution. In contrast, the opposite was found to be the case when either sunflower or corn gluten meals were used as substitutes. In both cases, a 30% fishmeal substitution produced an insignificant reduction in feed conversion. At the 50% substitution level, feed conversion was further reduced, and in both cases found to be significantly lower than the control ($P<0.05$). With respect to protein efficiency, inclusion of either soya, sunflower or corn gluten meals produced a significant reduction in efficiency at both levels of inclusion. It is however interesting to note that at the 30% fishmeal substitution level, there were no significance differences in the protein efficiency ratios between the

three protein sources. Nevertheless, as observed with the feed conversion efficiencies, an increase in the substitution level to 50% reduced protein efficiency in all cases. This was particularly apparent in the sunflower and corn gluten formulations in which protein efficiency was significantly lower than the soya meal formulation. At the 30% fishmeal substitution level, the consumption rates of these diets were higher than those observed in the control. However, it was only when inclusion rates were increased to 50% that they were significantly higher than the control ($P<0.05$). The exception was the diet containing corn gluten meal, where a comparison with the control diet produced no significant difference in consumption rates ($P>0.05$). As respective consumption rates and final body weights were generally found to increase and decrease across all the diets in which fishmeal had been substituted at either 30 or 50% with soya, sunflower or corn gluten meals, a Pearson product moment correlation was performed between the consumption rates and final body weights to determine whether consumption rates were related to animal size. No significant correlation was found ($r = -0.42$, $P>0.05$, $n =7$), and thus it appears that differences in animal size cannot adequately account for the observed consumption rates. Differences in consumption rates may therefore be attributed to dietary factors.

The results using spirulina as a partial fishmeal replacement, demonstrated that a 50% substitution of fishmeal with spirulina produced neither a significant reduction in growth rate ($P>0.0125$, Table 5.3), nor a significant reduction in feed conversion ($P>0.05$, Table 5.2). Furthermore, while the animals fed this diet consumed significantly more feed than those animals fed the control diet ($P<0.05$, Table 5.2); protein efficiency was significantly reduced ($P<0.05$), indicating less efficient protein utilization. It is interesting

to note that in the four diets containing the soya meal / sunflower meal combinations, and fishmeal or spirulina at either 25% or 50% inclusion; feed conversion and protein efficiency were greater in those diets containing the fishmeal component as opposed to the spirulina. Nevertheless, a comparison between the diets containing spirulina or fishmeal at the 25% inclusion level, revealed that there were no significant differences between the feed conversion or protein efficiency ratios ($P>0.05$, Table 5.2). However, at the 50% fishmeal or spirulina inclusion level, feed conversion and protein efficiency were significantly reduced in those animals fed the spirulina based diets ($P<0.05$, Table 5.2). A comparison between the consumption rates revealed that at the 50% inclusion rate, those animals fed the spirulina based diet consumed significantly more than those fed the fishmeal based diet; however, at the 25% inclusion rate, consumption rates did not differ significantly.

Comparisons between the control and the diets containing the soya / sunflower combinations with fishmeal at either 25% or 50%, revealed that both formulations produced significant reductions in growth rates ($P<0.00001$, Table 5.3), feed conversion, protein efficiency and consumption ($P<0.05$, Table 5.2). Nevertheless, it is interesting to note that between these formulations, no significant differences were found between either growth rates or the nutritional indices ($P>0.05$, Tables 5.2, 5.3).

In comparison to the control, the addition of either maize or semolina to the formulations, and the respective replacement of 10 or 15% of the fishmeal combined with the total substitution of the starch component, produced significant reductions in protein efficiency ($P<0.05$). Feed conversion was only significantly reduced in the diet containing the

semolina. In addition, while the presence of maize in the formulation did not significantly affect growth (P=0.47, Table 5.3), growth was significantly reduced in the diet containing the semolina (P<0.016, Table 5.3). Furthermore, as a comparison between the semolina and maize based diets demonstrated that the latter promoted significantly higher feed and protein efficiency (P<0.05), it may be concluded that the maize is a superior candidate as a protein / starch replacement.

In order to determine whether the dietary essential amino acid balance had an effect on feed and protein utilization by the abalone, a preliminary analysis using linear regressions between the essential amino acid profile of the soft body tissues of *H.midae*, and the calculated essential amino acid profiles of the dietary treatments was undertaken (Table 5.4). Correlation coefficients (r^2) from these regressions indicated that with respect to the amino acid balance of the commercial formulation, and with the exception of those diets containing corn gluten or semolina, the amino acid balances of all the dietary treatments produced equal or closer approximations to the balance of amino acids found in the soft body tissues of the abalone. Secondary analyses, using Pearson product moment correlations were performed between the dietary/abalone EAA correlation coefficients and either the respective growth rates or the protein efficiency ratios. As no significant correlations were observed (P>0.05, Table 5.5), it can be assumed that that in this study, dietary essential amino profiles could not be used to predict potential feed utilization by the abalone.

Table 5.4. Essential amino acid profile of the whole soft body tissues of *H.midae*, and the calculated essential amino acid profiles of the experimental diets. A comparison between dietary essential amino acid profiles and the essential amino acid profile of abalone are presented as the coefficients of variation between the dietary and abalone EAAs (r^2). Amino acid values are presented as a percentages of protein (dry weight).

	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine + Cystine	Threonine	Phenylalanine + Tyrosine	Tryptophan	Valine	r^2
H.midae	**7.91**	**1.82**	**4.11**	**6.93**	**6.21**	**3.44**	**4.99**	**7.71**	**0.82**	**4.61**	-
Fishmeal control : 100	5.62	2.92	3.97	7.25	7.52	3.66	4.13	6.89	1.15	4.78	0.81
Fishmeal / Soya meal 70 : 30	5.91	2.74	4.11	7.21	7.00	3.38	3.97	7.07	1.22	4.66	0.87
Fishmeal / Soya meal 50 : 50	6.20	2.65	4.26	7.28	6.75	3.24	3.92	7.29	1.29	4.64	0.90
Fishmeal / Sunflower meal 70 : 30	6.74	2.82	4.20	7.49	6.44	3.52	4.10	7.19	1.19	4.99	0.93
Fishmeal / Sunflower meal 50 : 50	7.58	2.79	4.42	7.76	5.83	3.48	4.14	7.49	1.23	5.20	0.94
Fishmeal / Torula yeast 70 : 30	5.49	2.83	4.48	7.16	7.48	3.37	4.46	7.72	1.11	5.11	0.82
Fishmeal / Torula yeast 50 : 50	5.49	2.81	4.89	7.21	7.56	3.23	4.75	8.38	1.11	5.40	0.81
Fishmeal / Corn gluten 70 : 30	4.87	2.70	4.32	10.07	5.76	3.73	3.85	7.43	0.94	4.84	0.64
Fishmeal / Corn gluten 50 : 50	4.44	2.59	4.61	12.09	4.69	3.83	3.73	7.89	0.82	4.95	0.50
Fishmeal / Semolina 85 :15	5.23	2.73	3.95	7.10	6.68	-	3.93	6.80	-	4.70	0.70
Fishmeal / Maize 90 : 10	5.50	2.90	3.93	7.78	7.02	3.70	4.08	7.09	1.12	4.76	0.81
Fishmeal / Spirulina 50 : 50	6.04	2.41	4.80	7.89	6.10	3.49	4.27	7.23	1.22	5.61	0.87
Fishmeal / Soya meal / Sunflower meal 50 : 25 : 25	6.95	2.74	4.37	7.58	6.33	3.59	4.06	7.45	1.27	4.96	0.94
Fishmeal / soya meal / Sunflower meal 25 : 37.5 : 37.5	7.51	2.62	4.52	7.66	5.69	3.51	3.98	7.64	1.31	4.99	0.94
Spirulina / Soya meal / Sunflower meal 50 : 25 : 25	7.31	2.21	5.16	8.17	4.88	3.39	4.17	7.73	1.33	5.75	0.90
Spirulina / Soya meal / Sunflower meal 25 : 37.5 : 37.5	7.73	2.36	4.94	7.98	4.97	3.43	4.05	7.81	1.35	5.41	0.87

The amino acid profiles of *H.midae*, spirulina and torula yeast were taken from Britz (1995). The fishmeal amino acid profile was supplied by 999 Esbjerg Fiskeindustri, Denmark, and those of soya meal, sunflower meal, corn gluten and maize taken from NRC 1993. Semolina was determined by the Department of Poultry Science, University of Natal, SA.

In contrast, Pearson product moment correlations between the levels of individual dietary essential amino acids and either the growth rates or protein efficiency ratios (Table 5.5), revealed that dietary lysine levels were significantly and positively correlated to both growth and protein efficiency (growth rate : r = 0.77, P=0.0005; PER : r = 0.52, P=0.04). Furthermore, while leucine was found to be negatively correlated to the growth rate (r = -0.68, P=0.003), the other essential amino acids were not significantly correlated with either growth or protein efficiency (P>0.05).

Table 5.5. Pearson product moment correlations between either the dietary essential amino acids or the regression coefficients (r^2) between dietary / abalone EAA profiles, and growth rates and protein efficiency ratios.

	Factor			
Amino Acid	Growth Rate		Protein Efficiency Ratio	
Arginine	r=-0.13	P>0.05	r=-0.40	P>0.05
Histidine	r= 0.42	P>0.05	r= 0.26	P>0.05
Isoleucine	r=-0.39	P>0.05	r=-0.05	P>0.05
Leucine	r=-0.68	P=0.003	r=-0.19	P>0.05
Lysine	r= 0.77	P=0.0005	r= 0.52	P=0.04
Methionine + Cystine	r=-0.39	P>0.05	r=-0.06	P>0.05
Threonine	r= 0.46	P>0.05	r= 0.42	P>0.05
Phenylalanine + Tyrosine	r=-0.41	P>0.05	r= 0.04	P>0.05
Tryptophan	r= 0.04	P>0.05	r=-0.10	P>0.05
Valine	r=-0.15	P>0.05	r=-0.04	P>0.05
Regression coefficients (r^2) between dietary and abalone EAA profiles	r=0.31	P>0.05	r=-0.17	P>0.05

The body composition of the abalone at the end of the experimental period revealed that diet did not significantly affect the protein, lipid, carbohydrate (nitrogen free extract), ash or moisture levels in the soft body tissues of the animals (P>0.05, Table 5.6).

Table 5.6. Proximate composition of the soft tissue of the abalone at the end of the experimental period. With the exception of moisture, all values are expressed on a dry weight basis. Different alphabetic superscripts indicate significant differences between treatments for the parameter concerned. Initial values for the abalone at the start of the experimental period were : Protein – 52.6%; Lipid – 4.1%; Ash – 15.2%; Nitrogen free extract – 28.1; Moisture – 75.6%.

Diet	Protein	Lipid	Nitrogen Free Extract	Ash	Moisture
Control – Fishmeal 100 (%)	55.4[a]	5.2[a]	26.1[a]	13.3[a]	74.6[a]
Fishmeal / Soya meal 70 : 30 (%)	56.6[a]	5.4[a]	23.4[a]	14.6[a]	75.6[a]
Fishmeal / Soya meal 50 : 50 (%)	54.9[a]	5.7[a]	26.5[a]	12.9[a]	76.6[a]
Fishmeal / Sunflower meal 70 : 30 (%)	55.9[a]	5.1[a]	25.0[a]	14.0[a]	76.3[a]
Fishmeal / Sunflower meal 50 : 50 (%)	52.8[a]	5.6[a]	28.1[a]	13.5[a]	75.2[a]
Fishmeal / Torula yeast 70 : 30 (%)	55.3[a]	5.3[a]	25.1[a]	14.3[a]	75.6[a]
Fishmeal / Torula yeast 50 : 50 (%)	55.9[a]	5.9[a]	23.6[a]	14.6[a]	76.3[a]
Fishmeal / Semolina 85 :15 (%)	54.9[a]	5.3[a]	24.8[a]	15.0[a]	76.8[a]
Fishmeal / maize 90 :10 (%)	55.1[a]	5.6[a]	25.7[a]	13.6[a]	75.9[a]
Fishmeal / Corn Gluten 70 : 30 (%)	54.6[a]	5.4[a]	25.8[a]	14.2[a]	77.9[a]
Fishmeal / Corn Gluten 50 : 50 (%)	51.6[a]	5.6[a]	29.1[a]	13.7[a]	75.6[a]
Fishmeal / Soya meal / Sunflower meal 50 : 25 : 25 (%)	54.6[a]	5.7[a]	25.8[a]	13.9[a]	74.9[a]
Fishmeal / Soya meal / Sunflower meal 25 : 37.5 : 37.5 (%)	56.9[a]	5.3[a]	23.5[a]	14.3[a]	75.8[a]
Spirulina / Soya meal / Sunflower meal 50 : 25 : 25 (%)	55.3[a]	5.5[a]	24.6[a]	14.6[a]	76.1[a]
Spirulina / Soya meal / Sunflower meal 25 : 37.5 : 37.5 (%)	51.0[a]	5.3[a]	29.9[a]	13.8[a]	77.2[a]
Fishmeal / Spirulina 50 : 50 (%)	55.2[a]	5.4[a]	26.4[a]	13.0[a]	76.4[a]

Although condition factors were found to reduce in all treatments over the experimental period, at the end of the experiment, there were no significant differences in condition factors between the control diet and the experimental diets ($P>0.05$, Table 5.2). The exceptions being those diets in which 50% of the fishmeal was substituted with either sunflower meal or corn gluten meal, and in addition, those diets containing the mixture of soya and sunflower meals in combination with 50% fishmeal or 25% spirulina. In these

diets, condition factors were found to be significantly reduced with respect to the control diet ($P<0.05$).

Mortality of the abalone was low ranging from 0 - 4.4%, and was attributed to the small animal size and the consequent susceptibility to damage due to handling stress during weigh and measures. There was no evidence of disease in any of the replicates.

DISCUSSION

Without exception, the substitution of dietary fishmeal with plant protein sources increased consumption rates in the abalone. However, as feed consumption may be affected by a number of factors, it is difficult to determine causality. Amongst others, factors that have been shown to affect consumption in poikilotherms include dietary energy density (Lee and Putman, 1973; Weisberg and Lotrich, 1982), and feed attractiveness and palatability (Harada *et al*., 1996).

If it is assumed that animals eat to satisfy their energetic requirements (Smith, 1989), it is reasonable to suggest that the differences in digestible energy across the dietary treatments provide an explanation for the observed consumption rates. Morales *et al*. (1994) demonstrated an increase in consumption rates in rainbow trout when fishmeal was partially replaced with plant protein sources, and concluded that the differences in consumption rates were due to available dietary energy. High levels of indigestible carbohydrates present in the plant protein sources reduce the dietary digestible energy density. Thus as the animals adjust their feed intake to satisfy their energetic

requirements, they consume more of the fishmeal / plant protein diets which contain less available energy than the fishmeal based diets. It is therefore possible that the consumption rates observed in this study are a reflection of the dietary digestible energy densities. However, as no attempt was made to determine the dietary digestible energy levels, it is not possible to clarify the issue, and further research is required.

It has also been demonstrated that dietary protein source may effect feed consumption. While Uki and Watanabe (1986) reported higher feed consumption in *H.discus hannai* fed a fishmeal as opposed to a casein based diet, Viana *et al.* (1994) demonstrated that *H.fulgens* found abalone viscera and fish silages more attractive but less palatable than fishmeal, kelp, soya and corn meals. They speculated that the release of soluble proteins and amino acids enhanced the attractiveness of the silages, and that the palatability problems observed with these protein sources may be overcome by the addition of alternative protein sources that were more palatable to the animals. It is possible that the same argument may hold true in this study. Thus, differences in attractiveness and palatability between individual protein sources may have increased overall feed consumption when they were presented to the animals in composite diets. As opposed to individual presentation, nitrogenous attractants have been demonstrated to have synergistic properties when supplied as mixtures to both abalone (Harada *et al.,* 1987) and other gastropods (Croll, 1983). Nevertheless, although the effects of combining protein mixtures into dietary formulations may have improved their attractiveness and palatability, the general trend of increased consumption across all the fishmeal / plant protein formulations suggests that it provides only a partial explanation, and that the digestible energy density of the diets was also a factor.

The efficacy of soya meal as a partial fishmeal replacement has been well documented in fish (Reinitz, 1980; Jackson *et al.*, 1982; Watanabe *et al.*, 1992), and although it has been incorporated into a number of formulations for Haliotids (Fleming *et al*., 1996), this is the first detailed information suggesting that it is a viable fishmeal replacement for use in diets for *H.midae*. While feed and protein efficiency were reduced at the 50% substitution level, growth rates were not significantly affected. Although an increase in consumption rate at the 50% fishmeal substitution level may account for the stability in the growth rates between the two soya inclusion levels, it fails to account for the general drop in feed and protein efficiency observed in the abalone fed this diet. Increasing dietary EAA deficiencies or lower bioavailability of nutrients may explain this reduction in feed conversion. While a number of studies have demonstrated that amino acid supplementation can improve nutritional indices in soya based diets for salmonids (Dabrowska and Wojno, 1977; Rumsey and Ketola, 1975); methionine has been found to be deficient in soya based formulations for Japanese eel (Lovell, 1989b) and carp (Murai *et al*., 1989). Therefore, it is probable that at the higher inclusion level, the soya meal based diet may have been deficient in EAAs, and in particular methionine. However, it is not possible to verify whether this was the case until both the essential amino acid requirements of the species, and the availability of the amino acids in the soya and fishmeal have been established. An alternative or perhaps additional factor may have been the presence of antinutritional factors in the soya. Trypsin inhibitors can probably be discounted as a major factor affecting amino acid availability as the apparent protein digestibility of the soya meal was found to be high (80.7%, Chapter 3), and in addition, the soya had undergone heat treatment during the lipid extraction process that would have destroyed these inhibitors. Coote *et al*. (1996) proposed that the levels of available

phosphorus in a diet containing 40% soya meal may be limiting in diets for *H.laevigata.* It was concluded that phosphorus in the soya meal was not available to the abalone as it was bound up as phytate, and that supplementation with available phosphorus (NaH_2PO_4) was necessary to meet the dietary requirements of the animals and improve growth. It is possible that the same holds true in this study, and that the supplementation of P at the higher soya inclusion level could improve the nutritional indices. Nevertheless, further research is required. Fishmeal is a good source of dietary P containing between 2.5 - 6.5 times the amount of P present in soya meal (NRC, 1993), and although the dietary P content of the experimental diets were not determined; even at the 50% soya inclusion level, there may have been sufficient P in the remaining fishmeal component to satisfy the dietary requirement.

The growth and nutritional indices using the sunflower meal as a fishmeal replacement demonstrate that it has good potential for future use in formulations. Although feed and protein efficiency were reduced with respect to the control, the higher consumption rates may have improved growth rates, which, at the 30% fishmeal substitution level was not significantly different to the control. However, the reduction in growth and protein efficiency which became apparent when the inclusion level of the sunflower was increased from 30 to 50% may have been due to either a reduction in digestible protein or energy, or imbalances in essential amino acids. Although there is no information concerning the effects of sunflower meal in diets for Haliotids, there have been a number of studies with fish. Using rainbow trout, Sanz *et al.* (1994) cited imbalances between leucine and isoleucine as the primary factor reducing protein efficiency both in their study and an additional study carried out previously by Tacon *et al*. (1984). It is well

documented that a leucine / isoleucine imbalance has a negative impact on growth and nutritional indices in fish (Harper *et al*., 1970; Hughes and Rumsey, 1983; Robinson *et al*., 1984). However, until more detailed information regarding the essential amino acid requirements of abalone is available, it is not possible to determine whether this was the case in this study. An additional issue associated with the use of sunflower meals is their high crude fiber content (18% of meal weight – C.Clayden, per. com., Sea Plant Products Ltd). Although it was not quantified, the fecal material from those abalone fed the sunflower based diets revealed the presence of large quantities of undigested seed hulls, thus effectively reducing the digestible nutrient density of the diets. Future studies should therefore investigate meals in which the hulls have either been predigested or removed from the meal prior to formulation. This approach may improve the dietary digestible nutrient density, and have a positive affect on growth and feed conversion.

The combination of soya and sunflower meals as a partial fishmeal replacement significantly affected both feed and protein efficiency. These indices were respectively higher and lower than the control indicating poorer dietary utilization of the feed which, with the exception of the 50% corn gluten substitution, produced the lowest growth rates in the study. Notwithstanding gross differences in digestible energy between the diets, these reductions in nutritional indices were probably due to a combination of factors previously discussed when either the soya or sunflower meals were tested individually. It is however interesting to note that when the fishmeal component was completely removed and replaced with spirulina, there was an improvement in the growth rates. This suggests that as more information concerning the dietary requirements of the abalone, and

the availability of nutrients from plant protein sources becomes available, the complete removal of the fishmeal component may become a viable proposition.

In comparison to the control diet, a 50% fishmeal substitution for spirulina significantly reduced protein efficiency, but produced no significant effect on either growth or feed conversion. With the exception of the reduction in protein efficiency, a similar result was observed in Chapter 4 when diets containing either fishmeal or spirulina as the sole protein sources were compared. Despite the similarity in the comparative growth and feed conversion ratios between the two studies, it is difficult to determine why protein efficiency was reduced in this study. The spirulina used in this study is a by-product of the tanning industry and as such, produced in the waste waters. Variations in environmental parameters within these waters can produce quantitative differences in both the crude protein level and amino acid composition of the final product (Maart, 1992). Thus, as the two experiments used different batches of spirulina, it is possible that quantitative differences between the batches may account for the relative differences in protein efficiencies recorded in the two studies. However, further research focusing on the nutrient composition and availability from different sources of spirulina is required to determine whether this was the case.

The substitution of fishmeal with torula yeast produced some of the best growth rates and nutritional indices in the study. This was most apparent at the 30% fishmeal substitution level where the diet out performed all the alternative formulations. The high apparent protein digestibility coefficient of the torula yeast (83.4 % - Chapter 3), combined with the high protein and feed conversion suggests not only high availability of

the protein source to the animals, but that it contains a well balanced essential amino acid profile that supports good growth in this context. Nevertheless, at the 50% substitution level, the growth and nutritional indices were reduced with respect to the 30% substitution level. As consumption rates between the two formulations were not significantly different, this reduction in efficiency was probably due to an imbalance of EAAs at the higher torula yeast inclusion level. Evidence to support this position can be found by comparing the apparent protein digestibility coefficients of the protein sources. As the apparent digestibility coefficients of torula yeast was higher than fishmeal (Chapter 3); and if it is assumed that digestibility values are additive in compound feeds for abalone (Fleming *et al*., 1998), then the amount of digestible protein available to the abalone would have been greater in the diet containing 50% torula yeast as opposed to the diet containing 30% torula yeast. Thus, the amount of digestible protein available to the abalone cannot account for the observed results, and notwithstanding energy imbalances in the diets, the reduction in nutritional indices was probably due to increasing imbalances in dietary EAA profile.

The results using the corn gluten meal suggest that it has potential as a fishmeal replacement at levels below 30%. Comparable studies with fish have indicated that inclusion levels between 20 – 30% dietary protein are viable, and do not negatively impact on growth and feed conversion (Gropp *et al*., 1979; Alexis *et al*., 1985; Robaina *et al*., 1997; Regost *et al*., 1999). However, in comparison with other protein sources, reduced feed conversion and growth at higher inclusion levels (>30%) have been related to its poor essential amino acid profile, and in particular low lysine levels (Robaina *et al*., 1997). This may account for the results in this study in which both feed and protein

efficiency were reduced at the 50% inclusion level. In addition, as the apparent digestibility coefficients of the fishmeal and corn gluten meal were 82.0 and 57.0% respectively (Chapter 3), it suggests that at the higher inclusion level, the amount of digestible protein available to the animals may have been reduced. Furthermore, as no significant difference was found between the consumption rates between the two diets, the total digestible protein available to the abalone was probably reduced at the higher inclusion rate. Thus, a combination of poorer EAA balance and reduced protein availability may account for the reduced growth, feed and protein efficiencies at the higher inclusion rate.

While linear regression analysis between the dietary and abalone EAA profiles revealed a large variation in correlation coefficients (r^2 = 0.50 – 0.94, Table 5.4), Pearson product moment correlations between the r^2 values and the respective growth rates or protein efficiency ratios revealed no significant correlations (Table 5.5). It therefore appears that the balance of dietary EAAs was not a good predictor of either growth or protein utilization in the abalone. In contrast, when similar correlations were performed between growth, protein efficiency and the levels of individual dietary EAAs (Tables 5.4, 5.5); the levels of dietary lysine were significantly and positively correlated to both growth rates and protein efficiency. Thus, although the bioavailability of the EAAs was not taken into account in this study, it can be concluded that as opposed to the dietary EAA profile, dietary lysine levels provide a better indication of which protein sources will promote growth and protein efficiency. Furthermore, the positive correlations between dietary lysine levels and growth and protein efficiency suggest that lysine may have been the first limiting amino acid in these diets. This being the case, and in the absence of detailed

information concerning the EAA requirements of the animal, future dietary formulations should perhaps be calculated on a limiting amino acid basis eg. either lysine or total sulfur amino acid basis.

Carbohydrate replacement study

The nutritional indices obtained using diets containing either ground maize or semolina, suggest that maize has a greater potential than semolina as a carbohydrate replacement in the current commercial formulation. Unfortunately, meaningful comparisons between these carbohydrate sources, and those used in the commercial formulation cannot be made as information pertaining to the commercial processed starches is proprietary knowledge (Sea Plant Products Ltd.). Nevertheless, with respect to the commercial and maize based formulations, there were no significant differences in either the growth rates or feed conversion. However, in comparison with the commercial formulation, significant reductions were observed in the protein efficiency of both the maize and semolina based diets. It is difficult to determine why this should have been the case as not only were the carbohydrate sources completely replaced, but in addition, respectively either 10 or 15% of the fishmeal protein was replaced with either maize or semolina protein. It is therefore possible that the observed protein efficiency ratios were due to differentials in either dietary EAA profiles or in the available protein or energy. However, as only 10-15% of the fishmeal protein component was substituted, it is unlikely that the drop in protein efficiency can be attributed to changes in either EAA profiles or protein availability. Differentials in the amount of digestible energy available to the abalone from the carbohydrate sources and the presence of a protein sparing effect may be a more likely

explanation. In comparison to the processed starches, the starches in the maize and semolina meals probably yielded less digestible energy, and thus a higher proportion of protein was channeled into maintenance requirements, leaving less for somatic growth, and hence the recorded drop in protein efficiency. Nevertheless, although further research must be carried out to determine the digestible energy budgets of the formulations, it appears that unprocessed maize may be a suitable starch substitute in the commercial formulation.

In conclusion, the results suggest that the potential to replace fishmeal with plant protein sources in commercial diets for *H.midae* is promising. Nevertheless, prior to commercialization, a cost-benefit analysis must be undertaken to determine the economic viability of these formulations. The formulation costs to produce one kilogram of each dietary treatment are presented in Table 5.7. In addition, by multiplying these costs with their respective feed conversion ratios, the estimated costs to produce one kilogram of *H.midae*, and the respective changes in production costs with reference to the commercial formulation are presented. With the exception of those diets containing torula yeast and spirulina, the substitution of the fishmeal with the plant proteins reduced formulation costs. Of particular significance are those diets containing soya and sunflower meals, either in combination or as the sole fishmeal substitute. In these formulations, and with respect to the commercial formulation, the combination of low raw material costs, good growth and feed conversion, enabled reductions in production costs between 4.7 – 25.2% to be made. In contrast, the high raw material costs associated with torula yeast and spirulina suggest that despite the promising growth and feed conversion obtained using these protein sources, it is unlikely that they will be used commercially unless

cheaper sources become available. Nevertheless, the success of the spirulina based diets indicate that the total substitution of the fishmeal component with plant protein sources may become a viable proposition. The preliminary study into the efficacy of maize and semolina as carbohydrate replacements, suggests that the low cost associated with maize significantly reduces production costs by removing the costly processed starches from the

Table 5.7. Feed formulation costs and the respective feed costs to produce 1 kg *H.midae* (Expressed as South African Rand).

Diet	Formulation Cost - kg feed [ab] (SA Rand)	Feed cost to produce 1kg *H.midae* (SA Rand)	Percentage change in production costs with reference to Afeed[c] formulation
Control – Fishmeal 100 (%)	5.76	5.30	-
Fishmeal / Soya meal 70 : 30 (%)	5.14	4.21	-20.5
Fishmeal / Soya meal 50 : 50 (%)	4.74	4.17	-21.3
Fishmeal / Sunflower meal 70 : 30 (%)	4.82	4.82	-9.0
Fishmeal / Sunflower meal 50 : 50 (%)	4.21	5.05	-4.7
Fishmeal / Torula yeast 70 : 30 (%)	10.53	8.42	+59.0
Fishmeal / Torula yeast 50 : 50 (%)	13.69	11.77	+122.2
Fishmeal / Semolina 85 :15 (%)	5.44	5.98	+12.9
Fishmeal / maize 90 :10 (%)	3.01	2.71	-48.9
Fishmeal / Corn Gluten 70 : 30 (%)	5.29	5.18	-2.2
Fishmeal / Corn Gluten 50 : 50 (%)	4.97	5.17	-2.5
Fishmeal / Soya meal / Sunflower meal 50 : 25 : 25 (%)	4.48	4.75	-10.4
Fishmeal / Soya meal / Sunflower meal 25 : 37.5 : 37.5 (%)	3.85	3.97	-25.2
Spirulina / Soya meal / Sunflower meal 50 : 25 : 25 (%)	7.39	9.24	+74.3
Spirulina / Soya meal / Sunflower meal 25 : 37.5 : 37.5 (%)	5.29	5.87	+10.8
Fishmeal / Spirulina 50 : 50 (%)	8.62	8.36	+57.8

[a.] Excluding production costs.
[b.] Feed component costs : Fishmeal : R5.90; Soya meal : R2.30; Sunflower meal : R1.50; Spirulina : R15.00; Torula yeast : R25.00; Semolina : R3.20; Corn gluten meal : R2.70; Maize meal : R0.72; Vit / Min mix : R43.00; "Marinol R" fish oil : R4.50; Starch combination : R3.50 and R10.70.
[c] "Abfeed" is a commercially available feed produced by Sea Plant Products Ltd., Hermanus, South Africa.

formulation; and it therefore has good potential for inclusion in commercial formulations. Nevertheless, this study should be viewed as a preliminary investigation into the partial and total replacement of fishmeal; and that prior to determining the optimal protein mixtures required to optimize growth at the least cost, a considerable amount of further research is required. In particular, the digestibility of essential amino acids and energy from the protein sources need to be determined as does the EAA requirements of the abalone. Only then can formulations be designed, that will optimize growth and feed utilization through the delivery of the required essential amino acids and appropriate digestible energy.

CHAPTER 6

INVESTIGATIONS INTO THE QUANTITATIVE AMINO ACID REQUIREMENTS OF *H.MIDAE.*

INTRODUCTION

As animals do not have a requirement for protein *per se*, but a requirement for amino acids, the quantification of the essential amino acid requirements of a species is a prerequisite to the formulation of diets that optimize protein utilization. Abalone require the same 10 essential amino acids as most other animals (Allen and Kilgore, 1975), and although there is currently no published data characterizing the quantitative EAA requirements for haliotids, there is a growing body of work describing the availability of individual EAAs from a variety of single or mixed protein sources (Fleming *et al*., 1998; Van Barneveld *et al.*, 1998; Vandepeer *et al*., 1999). Although these data are of use to feed formulators, it will remain of limited value until the quantitative EAA requirements of the animals are determined. Once these requirements have been established, it will be possible to use the EAA availability data to design diets that supply the optimal balance and inclusion rates of EAAs to the animals.

The quantitative requirement for an EAA is usually determined as dose-response relationship in which growth is measured as a response to graded levels of crystalline amino acid in semi-purified diets deficient in the amino acid under study (Wilson, 1989). In an attempt to determine the arginine requirements of *H.midae*, Britz *et al*. (1997) failed

to promote a growth response to graded levels of crystalline arginine in casein based diets. Failure to produce a response may have been due to a number of factors, and although it was not demonstrated conclusively, it was concluded that either arginine was not a limiting amino acid in the formulations, or alternatively that crystalline amino acids are unsuitable for use in nutritional studies with abalone. Britz *et al*. (1997) chose arginine for investigation as comparisons between the EAA composition of Haliotids (Allen and Kilgore, 1975; Knauer *et al*., 1995a; King *et al*., 1996) and those of conventional feed ingredients, suggest that arginine is the most limiting amino acid (Mai *et al*., 1994). However, this assumption may have been erroneous. As opposed to protein bound arginine, the relatively high levels of arginine found in abalone body tissues are attributed to high levels of free arginine, stored as phosphoarginine and used as an energy metabolite (Tjeerdema *et al*., 1991). As free arginine may account for up to double the protein bound arginine (Florkin and Bricteux-Grégoire, 1972; Mai *et al*., 1994), it is conceivable that relative to the other EAAs, the level of arginine revealed in body composition studies overestimates the requirement. Thus, incremental increases in the dietary arginine may have failed to promote growth as arginine was not a limiting amino acid in the formulations. Nevertheless, as this has yet to be proven empirically, and that failure to promote growth may have been due to the abalone's inability to utilize crystalline amino acids; the first experiment in this chapter was designed to investigate the effects of arginine supplementation in diets for *H.midae* using whole proteins, and in the absence of crystalline arginine.

In the event that the dietary arginine requirement trial proved unsuccessful, a second experiment was undertaken to determine whether lysine was a limiting amino acid in

semi-purified diets for *H.midae*. Although not empirically proven, the results presented in Chapter 5 suggest that the combination of fishmeal and plant protein sources created dietary lysine deficiencies. Accordingly, an experiment was designed to determine the lysine requirements for *H.midae* using cotton meal / fishmeal based diets supplemented with incremental levels of crystalline lysine. Cotton meal was chosen as the primary protein source as with respect to *H.midae* body tissues, it contains a comparatively low lysine content (5.5% and 7.9% of protein respectively). However, as the results presented in Chapter 4 suggest that cotton meal may contain a poor EAA balance when supplied to *H.midae* as the sole dietary protein source; an attempt was made to improve the overall EAA balance of the diets by supplying 10% of the dietary protein as fishmeal.

In common with other water soluble nutrients, leaching of crystalline amino acids from diets prior to ingestion is of concern in an aquatic environment (Wilson, 1989; López-Alvarado *et al*., 1994). As micro-encapsulation techniques have been successfully employed to restrict micronutrient leaching (Langdon and Siegfried, 1984; Fu-Lin *et al*., 1987; López-Alvarado *et al*., 1994), and thus improve ingestion rates; two encapsulation techniques employing either a gelatin/acacia colloid (Keipert and Melegari, 1993) or cellulose acetate phthalate (Chen *et al*., 1992) were investigated for their ability to retard leaching and in the process stimulate growth in the lysine supplemented formulations.

METHODS

Experimental animals and general experimental protocol

Hatchery-reared juvenile abalone (*H.midae*) were used in both experiments (Experiment 1: 15.0±0.1mm shell length, 0.58±0.01g; Experiment 2 : 12.1±0.1mm shell length, 0.37±0.01g). Prior to experimentation, the animals were fed exclusively on a fishmeal based diet (34% protein, 6% lipid). Three replicate groups of 30 abalone were assigned to each treatment, and weight and shell length measurements taken monthly according to the protocol outlined in Chapter 4. The duration of Experiments 1 and 2 were 90 and 80 days respectively. Animals were fed to satiation daily at 17:00, and the feed consumed over the experimental period recorded and corrected for leaching. Growth and nutritional indices were calculated for each treatment according to the equations described in Chapters 4 and 5.

Experiment 1 : Examination of dietary arginine requirement

Experimental system

The experimental trial was carried out in a constant environment room. Four recirculating glass aquaria, similar to those described in Chapter 2 (Figure 2.1), were stocked with four replicate groups of 30 abalone in separate 2 litre perforated plastic jars. Water flow through the jars was maintained using airstones. Water temperature was maintained at 19±1°C using a thermostatically controlled aquarium heater, and salinity at 35±1 p.p.t. The photoperiod was set at 12L:12D with the dark period starting at 18:00. Two thirds of the water in each aquaria was replaced each week with sand filtered seawater.

Dietary formulations

Six isonitrogenous starch-bound experimental diets were formulated to contain 45% protein and 6% lipid (Table 6.1). Fishmeal and gelatin were selected as protein sources as with respect to one another, the arginine concentration in the gelatin was approximately double that found in the fishmeal. The partial and total substitution of the fishmeal component with gelatin produced feeds that contained dietary arginine concentrations ranging between 3.96 - 7.75% dietary protein. As the respective concentrations of the other nine EAAs were lower in the gelatin than the fishmeal, the increased dietary arginine levels were associated with decreases in the levels of the other 9 EAAs.

Statistical analysis

Across dietary treatments, one way analysis of variance followed where appropriate by Student Newman Keuls multiple comparison procedures were used to determine significant differences between specific growth rates, consumption, condition factors, feed conversion and protein efficiency ratios. Pearson product moment correlations were used to establish correlations between the dietary arginine concentrations and the growth and condition factors at the end of the experimental period.

Table 6.1. Ingredient composition of experimental diets (expressed on a percentage dry weight basis).

Ingredient	Diet N^{o}					
	1	2	3	4	5	6
LT-Fishmeal [a]	64.1	57.0	49.8	35.6	21.4	0.0
Gelatin [e]	0.0	5.1	10.2	20.4	30.6	45.9
Starch	32.5	34.0	35.5	38.5	41.6	46.1
Lipid [b]	1.5	2.0	2.5	3.5	4.5	6.0
Chromic oxide	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin / Mineral mix [c]	1.0	1.0	1.0	1.0	1.0	1.0
Analysis (calculated)						
Arginine (% dietary protein)	3.96	4.38	4.80	5.06	6.48	7.75
Crude Protein	45	45	45	45	45	45
Total Lipid	6	6	6	6	6	6
Fishmeal / Gelatin ratios[d]	100 / 0	89 / 11	78 / 22	56 / 44	33 / 67	0 / 100
Leaching correction factors	0.93	0.90	0.86	0.83	0.76	0.69

Dietary components supplied by : [a] 999 Fishmeals, Esbjerg Fiskeindustri, Denmark. [b] Marine Oil Refiners, SA. [e] Davis Gelatin Industries Ltd, SA.
[c] Vitamin and mineral mix - according to Uki *et al*. (1985b).
[d] Fishmeal / Gelatin ratios expressed as percentages of dietary protein.

Experiment 2 : Examination of the dietary lysine requirement

Experimental system

The experiment was carried out at the Port Alfred laboratory in the same system, and under identical environmental parameters as those described previously (Chapters 4, 5).

Amino acid encapsulation and dietary formulations

The encapsulated cellulose acetate phthalate (CAP) preparations were based on the method described by Chen *et al*. (1992). In summary, 2g cellulose acetate phthalate was dissolved in 10ml acetone. Fourteen grams of L-lysine monohydrochloride was added, and the end product collected after removal of the acetone by rotary evaporation. The

gelatin / acacia encapsulation (GAE) was prepared using a modified method described by Keipert and Melegari (1993). In summary, three parts of colloid phase (10% w/w gelatin solution and 10% w/w acacia solution in a ratio of 2:1) was mixed with one part oil phase ("Marinol R" fish oil to which 14g L-lysine monohydrochloride was added) at 40°C and emulsified using an Ika Turrax T25 homogeniser at 8000 rpm for 60 seconds. Under constant agitation (magnetic stirrer), the emulsion was slowly added to a vessel containing eight parts of liquid paraffin at 40°C. The gellification of the microparticles took place as the mixture was allowed to cool. At 10°C, the microparticles were washed two times with four parts of chilled isopropanol. This process dehydrated and further solidified the particles. Finally, the particles were filtered and air dried.

In order to compare the efficacy of the two encapsulation techniques, and in addition, to evaluate the effect of lysine supplementation on growth and nutritional indices in juvenile abalone; cotton and fishmeal basal diets (25% protein, 6% lipid) were supplemented with graded levels of either the CAP or GAE encapsulated lysine. Two series of six formulations containing theoretical lysine concentrations of 5.5, 6.5, 7.5, 8.5, 10.5 and 12.5% dietary protein were manufactured (Tables 6.2, 6.3). In order to augment the encapsulation techniques, the encapsulated lysine was suspended in the dietary oil fraction of the diets before being mixed with the other ingredients.

In order to assess the efficacy of the encapsulation techniques in reducing leaching of the crystalline lysine, the lysine content of each test diet was determined prior to and after

Tables 6.2 and 6.3. Ingredient composition and nutrient analysis of experimental diets (expressed on a dry weight basis).

	Gelatin / Acacia Encapsulation					
Diet	GAE 5.5	GAE 6.5	GAE 7.5	GAE 8.5	GAE 10.5	GAE 12.5
Cotton cake[a]	63.4	63.4	63.4	63.4	63.4	63.4
Fishmeal[b]	3.5	3.5	3.5	3.5	3.5	3.5
Starch	24.0	24.0	24.0	24.0	24.0	24.0
L-Lysine[c] + gelatin / acacia	0.0	1.1	2.3	3.4	5.7	8.0
Lipid[d]	1.3	1.2	1.0	0.8	0.5	0.2
Mineral / vitamin mix[e]	1.0	1.0	1.0	1.0	1.0	1.0
Cellulose[c]	6.9	5.9	4.9	3.9	2.0	0.0
Analysis (calculated)						
Protein	25.0	25.0	25.0	25.0	25.0	25.0
Lysine	5.5	6.5	7.5	8.5	10.5	12.5

Table 6.3.

	Cellulose Acetate Phthalate Encapsulation					
Diet	CAP 5.5	CAP 6.5	CAP 7.5	CAP 8.5	CAP 10.5	CAP 12.5
Cotton cake[a]	63.4	63.4	63.4	63.4	63.4	63.4
Fishmeal[b]	3.5	3.5	3.5	3.5	3.5	3.5
Starch	29.1	29.1	29.1	29.1	29.1	29.1
L-Lysine[c] + CAP	0.0	0.3	0.5	0.8	1.3	1.8
Lipid[d]	1.3	1.3	1.3	1.3	1.3	1.3
mineral / vitamin mix[e]	1.0	1.0	1.0	1.0	1.0	1.0
Cellulose[c]	1.8	1.5	1.3	1.0	0.5	0.0
Analysis (calculated)						
Protein	25.0	25.0	25.0	25.0	25.0	25.0
Lysine	5.5	6.5	7.5	8.5	10.5	12.5

Dietary components supplied by : [a.] EPOL, S.A. [b.] Danish 999 Fishmeals, Esbjerg Fiskeindustri, Denmark.
[c.] Sigma Chemicals, USA.
[e.] Composition from Uki *et al*. (1985b).

an eight hour period of immersion in seawater. Triplicate 10g samples of each test diet were placed in perforated containers containing no abalone, and submerged in the experimental system for an eight hour period. To ensure continuity in environmental parameters, this process was undertaken during the growth experiment. After the eight hour period, the samples were removed and oven dried to constant weight at 45°C. The lysine content of both the leached and unleached feed samples were determined according to Simpson *et al.* (1976) using HPLC on a Beckman 6300 High Performance Analyzer. In order to determine the rate of lysine leaching over an 8hr feeding period, 2g samples of each feed were placed in 50mls of seawater at 20°C, and the pH of the water monitored hourly for an 8hr period. Lysine is a basic amino acid (isoelectric point : 10.7), and as it leaches into the water causes a rise in pH. Thus, with respect to the control, the change in pH was used as an indication of the rate of lysine leaching. The leaching rates for each diet were undertaken in triplicate.

Statistical analysis

Growth rates were determined as the slopes of the natural log-transformed weight data. Analysis of covariance (ANCOVA) followed by one-tailed F-tests (Zar, 1984) were used to compare the slopes of the log-transformed weight data and the dietary lysine leaching rates. One way analysis of variance (ANOVA) followed by Student Newman Keuls multiple comparison procedures were used to determine significant differences in feed conversion, protein efficiency, consumption and condition factors.

RESULTS

Experiment 1 : Examination of dietary arginine requirement

Increases in dietary arginine and the associated replacement of fishmeal with gelatin produced significant reductions in specific growth rates, consumption, feed conversion, protein efficiency and condition factors ($P<0.05$, Table 6.4). In those diets containing the highest arginine concentrations (6.49-7.75% dietary protein), loss of body weight produced negative growth indices, and precluded the calculation of feed conversion or protein efficiency ratios. Correlation analysis revealed significant negative correlations between the dietary arginine level and both specific growth rates ($r=-0.97$, $n=6$, $P<0.001$) and condition factors ($r=-0.87$, $n=6$, $P<0.02$).

Table 6.4. Growth and nutritional indices. Values expressed as means and standard errors. Different alphabetic superscripts indicate significant differences ($P<0.05$) for the parameter concerned.

Parameter	Diet (Arginine % dietary protein)					
	1 (3.96)	2 (4.38)	3 (4.80)	4 (5.06)	5 (6.48)	6 (7.75)
Initial weight (g)	0.56 ± 0.02	0.54± 0.02	0.62± 0.02	0.59± 0.04	0.58± 0.02	0.56± 0.01
Initial shell length (mm)	15.0± 0.1	14.7± 0.1	15.4± 0.3	15.0± 0.3	14.7± 0.4	14.9± 0.16
Final weight (g)	1.15± 0.05	1.07± 0.03	1.04± 0.01	0.71± 0.05	0.57± 0.03	0.49± 0.02
Final shell length (mm)	19.1± 0.3	18.5± 0.2	18.4± 0.1	16.2± 0.40	15.4± 0.3	14.9± 0.2
Specific Growth Rate (% bd.wt.day^{-1})	0.81^{a}	0.76^{a}	0.58^{b}	0.21^{c}	- 0.02^{d}	- 0.14^{d}
Consumption (% bd.wt.day^{-1})	0.53^{a}	0.57^{a}	0.52^{a}	0.37^{c}	0.34^{c}	0.43^{b}
Feed Conversion Ratio	1.25^{a}	1.52^{b}	1.57^{b}	6.22^{c}	0	0
Protein Efficiency Ratio	1.88^{a}	1.60^{a}	1.56^{a}	0.41^{b}	0	0
Condition factor	0.91^{a}	0.94^{a}	0.93^{a}	0.92^{a}	0.88^{b}	0.84^{b}

Experiment 2 : Examination of dietary lysine requirement

Irrespective of encapsulation technique, the growth rates of the abalone fed the L-lysine enriched diets did not differ significantly from the control diets ($P>0.05$, Tables 6.5, 6.6). Across dietary treatments, mean increases in weight over the experimental period were 102% for the CAP encapsulated diets, and 83% for the GAE encapsulated diets. Feed conversion, protein efficiency, condition factors and consumption rates were not significantly affected by increasing dietary lysine levels in either the CAP or GAE formulations ($P>0.05$, Tables 6.5, 6.6). Mortality across all replicates ranged between 0-5.5%, and was attributed to the small size of the abalone and the subsequent susceptibility to damage during weighing and measuring.

The rate of leaching of the crystalline lysine from the dietary formulations, as measured by an increase in pH of the leachate over an eight hour period, suggests that leaching fitted a linear model (Table 6.7). Within encapsulation techniques, analysis of covariance revealed no significant differences between the slopes of the regressions, and it was therefore concluded that leaching rates did not differ significantly (Table 6.7). An estimation of the of the amount of crystalline lysine ingested was made. Based on the assumptions that the abalone feed at a constant rate over an 8hr feeding period (Uki, 1981; Knauer *et al*., 1995b), and that leaching occurred at a constant rate (Table 6.7), it was calculated that approximately 48% of the supplemental lysine was ingested (Figure 6.1). Using this figure, the amount of lysine ingested by the abalone fed each of the experimental diets was calculated (Table 6.8).

Tables 6.5 and 6.6. Growth and nutritional indices of juvenile abalone fed graded levels of L-lysine encapsulated in either cellulose acetate phthalate (Table 6.5) or gelatin/acacia (Table 6.6). Values are presented as means with standard errors. Different alphabetic superscripts indicate significant differences for the parameter concerned ($P<0.05$).

	Cellulose Acetate Phthalate Encapsulation					
Parameter	CAP 5.5	CAP 6.5	CAP 7.5	CAP 8.5	CAP 10.5	CAP 12.5
Initial length (mm)	11.6±0.1	11.6±0.1	11.6±0.1	11.7±0.1	11.8±0.1	11.6±0.1
Initial weight (g)	0.31±0.01	0.32±0.01	0.32±0.01	0.35±0.01	0.35±0.01	0.33±0.01
Final length (mm)	15.9±0.1	15.5±0.2	15.8±0.1	16.0±0.2	15.9±0.2	15.5±0.2
Final weight (g)	0.67±0.02	0.63±0.02	0.69±0.02	0.71±0.02	0.67±0.02	0.64±0.02
Growth (% body wt.day^{-1})	0.97^{a}	0.88^{a}	0.98^{a}	0.89^{a}	0.81^{a}	0.81^{a}
Feed conversion ratio	2.15^{b}	2.11^{b}	2.02^{b}	2.13^{b}	2.40^{b}	2.69^{b}
Protein efficiency ratio	1.91^{c}	1.91^{c}	1.97^{c}	1.95^{c}	1.66^{c}	1.51^{c}
Consumption (% body wt.day^{-1})	1.18^{d}	0.93^{d}	0.97^{d}	1.01^{d}	0.98^{d}	1.06^{d}
Condition factor (Day 80)	0.95^{e}	0.94^{e}	0.98^{e}	0.97^{e}	0.94^{e}	0.95^{e}
Mortality (%)	2.2	5.5	2.2	1.1	2.2	0

Table 6.6.

	Gelatin / Acacia Encapsulation					
Parameter	GAE 5.5	GAE 6.5	GAE 7.5	GAE 8.5	GAE 10.5	GAE 12.5
Initial length (mm)	12.5±0.1	12.6±0.1	12.5±0.1	12.6±0.1	12.4±0.1	12.4±0.2
Initial weight (g)	0.40±0.01	0.41±0.01	0.41±0.01	0.42±0.01	0.40±0.01	0.41±0.01
Final length (mm)	12.6±0.2	17.0±0.2	16.7±0.3	18.7±0.2	16.3±0.2	16.7±0.2
Final weight (g)	0.8±0.02	0.8±0.02	0.7±0.02	0.8±0.02	0.7±0.03	0.8±0.02
Growth (% body wt.day^{-1})	0.82^{a}	0.84^{a}	0.74^{a}	0.86^{a}	0.76^{a}	0.80^{a}
Feed conversion ratio	2.20^{b}	2.25^{b}	2.55^{b}	2.23^{b}	2.83^{b}	2.83^{b}
Protein efficiency ratio	1.87^{c}	2.08^{c}	2.58^{c}	1.79^{c}	1.48^{c}	1.44^{c}
Consumption (% body wt.day^{-1})	1.07^{d}	0.98^{d}	0.95^{d}	1.01^{d}	1.13^{d}	1.15^{d}
Condition factor (Day 80)	0.95^{e}	0.93^{e}	0.95^{e}	0.97^{e}	0.97^{e}	0.92^{e}
Mortality (%)	0	1.1	5.5	2.2	2.2	1.1

Table 6.7. Regression models describing the leaching of L-Lysine from the dietary formulations over an eight hour period. Within encapsulation techniques, analysis of covariance revealed no significant differences between slopes ($P<0.05$).

Diet	Regression model	r^2
CAP 6.5	y = 0.035 + 0.030x	0.86
CAP 7.5	y = 0.053 + 0.027x	0.78
CAP 8.5	y = 0.051 + 0.019x	0.73
CAP 10.5	y = 0.032 + 0.030x	0.75
CAP 12.5	y = 0.020 + 0.029x	0.80
GAE 6.5	y = 0.059 + 0.028x	0.73
GAE 7.5	y = -0.004 + 0.017x	0.81
GAE 8.5	y = 0.033 + 0.022x	0.68
GAE 10.5	y = 0.007 + 0.018x	0.73
GAE 12.5	y = 0.021 + 0.027x	0.48

Figure 6.1. Estimate of crystalline lysine ingestion by abalone over an eight hour feeding period. Calculated as a product of feed consumption and the cumulative leaching of the crystalline lysine from the feed. consumption.

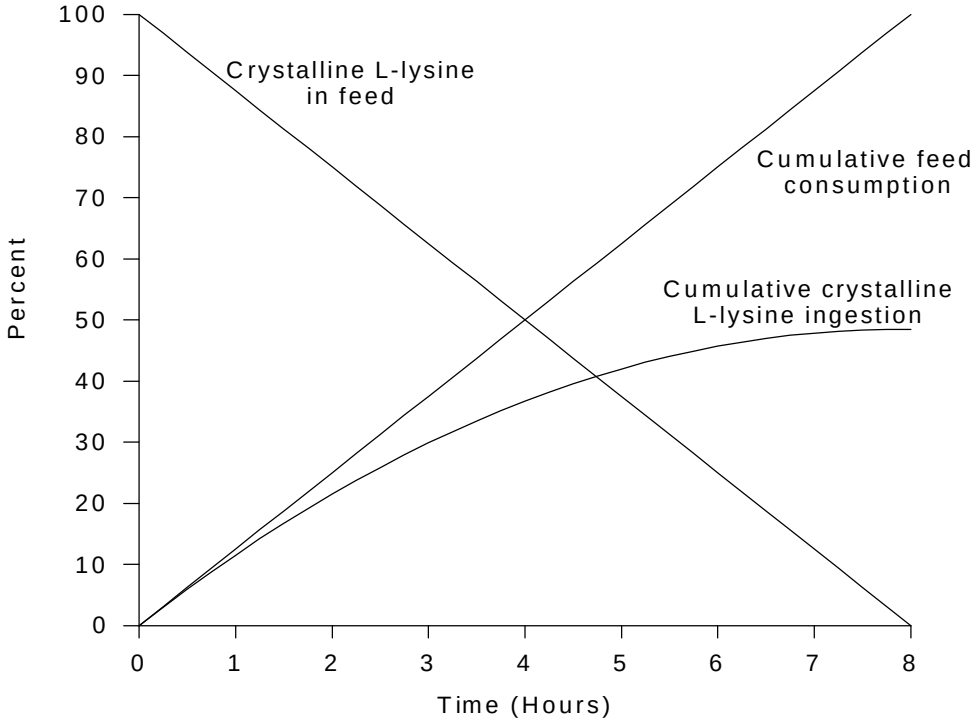


Table 6.8. Dietary lysine concentrations, the effect of leaching on these concentrations and the calculation of ingested lysine over an 8hr feeding period (assuming 48% of supplemental lysine was ingested - Figure 6.1). Figures expressed as percentages of protein.

	Lysine (% of dietary protein)			
Encapsulation	Theoretical	Observed	After 8hr leaching	Ingested over 8hr period
CAP 5.5	5.5	5.7	5.6	5.6
CAP 6.5	6.5	6.4	5.9	5.9
CAP 7.5	7.5	7.1	5.9	6.2
CAP 8.5	8.5	7.7	6.3	6.6
CAP 10.5	10.5	8.8	6.5	4.1
CAP 12.5	12.5	10.1	6.6	7.7
GAE 5.5	5.5	5.7	5.7	5.7
GAE 6.5	6.5	7.2	5.9	6.4
GAE 7.5	7.5	8.5	6.0	7.0
GAE 8.5	8.5	10.0	6.7	7.7
GAE 10.5	10.5	12.2	7.1	8.8
GAE 12.5	12.5	14.4	7.7	9.8

DISCUSSION

While the results of this study corroborate those of Britz *et al*. (1997) that indicated that arginine may not be the first limiting amino acid in formulated feeds for *H.midae*. The failure of the dietary lysine supplementation study to promote growth, suggests that while it is not possible to discount lysine as a limiting amino acid, a more plausible explanation may be that in common with carp (Aoe *et al*., 1970) and invertebrate species (Cowey and Forster, 1971; Deshimaru and Kuroki, 1974, 1975; Deshimaru, 1982), crystalline amino acids are unsuitable for amino acid requirement studies in *H.midae*.

The reduction in the growth responses of the abalone to increasing levels of dietary arginine suggests that either the protein bound arginine supplied in the gelatin was unavailable to the abalone, or alternatively, that arginine was not the limiting amino acid in these diets. Unfortunately due to difficulties associated with amino acid digestibility studies in *H.midae*, it was not possible to obtain arginine digestibility coefficients. While the absence of these coefficients makes it difficult to ascertain whether the abalone were exposed to increasing levels of dietary arginine, it is reasonable to suggest that there was a degree of supplementation, and that furthermore this supplementation did not promote growth. With respect to fishmeal, the EAA profile of gelatin is deficient in nine EAAs. Thus, although the substitution of fishmeal with gelatin increased dietary arginine levels, it was associated with a decrease in the other 9 EAAs. The reduction in growth rates and associated reduction in feed and protein efficiency with increasing dietary gelatin was probably a response to decreasing levels of at least one of these EAAs, and thus provides further evidence to suggest that arginine was not the limiting amino acid in these diets.

Failure of the lysine enriched diets to promote growth may have been due to a number of factors, and while it cannot be discounted that lysine was not the limiting amino acid in these diets, the results presented in Chapter 5 suggest that lysine is a primary candidate as the first limiting amino acid artificial feeds for *H.midae*. More probable explanations may lie with the difficulties associated in using crystalline amino acids in formulated feeds. Principally, these include the high solubility of amino acids and subsequent leaching from the test diets (Yamada and Yone, 1986; Wilson, 1989), differential rates of absorption and catabolism of free and protein bound amino acids (Cowey and Sargent, 1979),

feeding frequency (Tanikitti and March, 1995), and dietary pH and/or electrolyte balance (Murai *et al*., 1983).

The assessment of lysine leaching from the test diets suggest that it was not a factor in the failure of the lysine supplemented diets to promote growth. While there were differences in the efficacy of the encapsulation techniques, the abalone were effectively supplemented with graded levels of crystalline lysine. Once leaching had been taken into consideration, the amount of lysine ingested by abalone fed the CAP and GAE diets ranged between 5.6-7.7% and 5.7-9.8% dietary protein respectively. However, as these figures were derived by measuring leaching rates from intact pellets, an additional source of error in the ingestion estimations could have occurred during the feeding process. Prior to ingestion, abalone rasp small particles from the surface of the feed (approx. 0.5mm^2), and while it is possible that this action may have increased leaching rates, the fact that the particles are immediately ingested probably restricted these losses.

The poor utilization of crystalline amino acids in some fish species has been attributed to differential rates of absorption of crystalline and protein bound amino acids, and their subsequent arrival at the sites of protein synthesis (Cowey and Walton, 1988). To date, it has not been established whether there are differences in the rates of uptake between crystalline and protein bound amino acids in abalone. However, as Mai *et al*. (1994) demonstrated that dietary amino acid intake influenced free amino acid (FAA) patterns in both the visceral and muscle tissues of *H.tuberculata* and *H.discus hannai*, further studies measuring post-prandial FAA levels from abalone fed crystalline and protein bound amino acids could clarify this issue.

Dietary pH has been shown to affect crystalline amino acid utilization in some species of fish (Nose *et al*., 1974; Wilson *et al*., 1977). While Wilson *et al*. (1977) demonstrated that crystalline amino acid utilization improved in channel catfish fed test diets that had been adjusted to pH 7. Murai *et al*. (1983) demonstrated that both dietary pH and electrolyte balance affect amino acid metabolism in carp. However, as there is no information concerning these effects on crystalline amino acid utilization in abalone, it is not possible to evaluate their influence in the current experiment, and clarification must await further investigation.

Although it is evident that a number of outstanding issues must be addressed before it can be demonstrated conclusively that *H.midae* does not have the ability to utilize crystalline amino acids. The results of the present study, combined with those by Britz *et al*. (1997), suggest that they probably cannot. Thus, alternative methodologies such as post-prandial tissue or serum free amino acid studies (Harding *et al.*, 1977; Robinson *et al*., 1981; Ruchimat *et al*., 1997) or serum oxidation studies (Brookes *et al*., 1972; Lall *et al*., 1992) will have to be investigated. Clearly, success will represent a major technical achievement in Haliotid nutrition, and one that is necessary if diets that provide the optimum balance of essential amino acids to the animals are to be developed.

CHAPTER 7

CONCLUDING DISCUSSION

The results of this study have demonstrated that in common with other sectors of the aquaculture feed industry, a reduction in feed formulation costs in diets for *H.midae* can be achieved by the substitution of fishmeal with low-cost protein sources. The acceptance of a diverse array of protein sources reported in both the current and previous studies (Table 1.1), is probably a reflection of the generalist feeding behaviour of Haliotids, and is encouraging in terms of feed development as palatability is unlikely to become a major issue. Nevertheless, differential growth and nutritional responses to the protein sources indicate a high degree of variability in their biological value, and highlight the need for a comprehensive database incorporating digestible protein, amino acids and energy supply from a number of protein sources. Once the requirements for these nutrients have been defined, the least-cost approach to dietary formulation may be applied. While the results from this study fall short of this goal, they represent progress in our understanding of the dietary requirements of *H.midae*, and the availability of dietary nutrients to the animals.

The protein digestibility trials undertaken in this study provide a basis for protein nutrition in *H.midae*. Although chromic oxide was found to produce replicable gross protein apparent digestibility coefficients, the methodology precluded the determination of both the amino acid and digestible energy densities. Although this represents a major technical challenge with this species, comparisons with digestibility coefficients from *H.laevigata* (Vandepeer *et al*., 1999), suggest that there is a degree of similarity in the

digestibility coefficients between Haliotids. Thus, it is not unreasonable to suggest that for dietary formulation purposes, digestible amino acid and energy coefficients obtained from one species of Haliotid may be applicable to others. Furthermore, as the *in vitro* digestibility study demonstrated a high degree of similarity between the digestibility coefficients obtained from *H.midae* and those using an enzyme mixture from evolutionary distant groups of animals (porcine and bovine); it is not unreasonable to suggest that within the Haliotidae, there may be a lack of diversity in general protease digestive enzyme profiles, and as such, digestive efficiencies may be similar. However, although recent work on digestive proteases in Haliotids has been initiated (Serviere-Zaragoza *et al*., 1997; Hernández-Santoyo *et al*., 1998), a comprehensive comparison of proteases between species is required before such assumptions can be validated.

The size specific differences that emerged in the protein requirements of the animals is an interesting result that has commercial implications. Body size was found to affect the qualitative protein requirements of the abalone. However, as no attempt was made to determine causality, it was not possible to determine why this should have been the case. Nevertheless, it was proposed that the most probable explanation was that different size classes of animal were undergoing different stages in their physiological development, and that this produced quantitative differences in their essential amino acid requirements. These results, combined with those of Britz and Hecht (1997) that demonstrated quantitative differences in the protein and energy requirements of different size classes of *H.midae*, suggest that future feed development must take into account the effects of animal size. Furthermore, if maximal feed efficiency is to be attained, diets will have to

be designed that satisfy both the quantitative and qualitative protein requirements of the different size classes of abalone.

As abalone are typically slow feeders, nutrient loss through leaching prior to ingestion is of major concern in terms of nutrient delivery to the animals, assessing feed consumption, conversion, and maintaining water quality. The current binding technology used in the commercial production of formulated feeds in South Africa uses starches as the binding agents which, while producing good binding properties when used with fishmeals (87-93% recovery after 12hr submersion at 20°C - current study), were found to be less efficient when plant protein sources were employed (63-89% recovery after 12hr submersion at 20°C – current study). Indeed, in response to the poor performance of the starch binding process in the first growth trial in which plant protein sources were bound into single protein diets, the subsequent fishmeal replacement study employed agar as the binding agent. Although this proved successful in improving water stability in the feeds (81-96% recovery after 12hr submersion at 20°C), the current cost of agar (250Rand/kg) prohibits its use in a commercial situation. Thus, although it may be retained as a binder in experimental formulations, its commercial application is unlikely. Clearly, while this study has demonstrated the efficacy of plant protein sources in formulated feeds for *H.midae*, prior to their commercial application, further investigations must be undertaken to isolate and evaluate novel binding techniques.

In terms of growth and feed efficiency, soya and sunflower meals, torula yeast and spirulina demonstrated promise as fishmeal replacements. Although digestible essential amino acids and energy data was not available, high apparent protein digestibility

coefficients combined with favourable nutritional indices, suggest that in combination with fishmeal, they supply a good balance of available EAAs. It was demonstrated that while growth rates achieved using the commercial 'Abfeed" formulation need not be compromised, 50% of the fishmeal component could be replaced by either soya or spirulina, and 30% by either sunflower or torula yeast. However, current high costs associated with torula yeast and spirulina make the soya and sunflower meals the most promising fishmeal replacements.

Abalone are a high value species in which the organoleptic properties of the final product are of a major consideration to consumers (Fleming *et al*., 1996). While studies have demonstrated that artificial feeds affect these properties (Dunstan *et al*., 1994; Gregorio, 1991), there is some concern that the use of fishmeals and animal proteins in formulations may have an adverse effect on taste and texture. If this is proven, it may make it desirable to reduce or completely remove the fishmeal component in artificial feeds. The results from this study suggest that the complete replacement of the dietary fishmeal component with plant protein sources is a viable proposition. Thus the prospects for "finishing" diets in which the organoleptic properties of the abalone are improved by feeding a fishmeal free diet prior to harvest are promising.

While it was not empirically proven whether either arginine or lysine were limiting in the formulated feeds used in this study, the results indicated that in those cases in which fishmeal had been partially or totally replaced with plant protein sources, lysine was the most likely candidate as the first limiting amino acid. Nevertheless, the failure of the studies undertaken to determine the arginine and lysine requirements of *H.midae*

highlight the problems associated with using either whole proteins or crystalline amino acids in requirement studies. As the results suggest that the prospects for using either technique are poor, future studies should focus on alternative methodologies such as post–prandial tissue or serum free amino acid (Ruchimat *et al*., 1997) or serum oxidation (Lall *et al*., 1992).

To conclude, the prospects for reducing formulation costs in commercial abalone feeds is promising. Four new formulations based on the present research are in production and undergoing trials on commercial farms in South Africa. While the results are encouraging, the current research provides a foundation for further improvements in feed formulation technology.

REFERENCES

Aksnes, A., Brekken, B., 1988. Tissue degradation, amino acid liberation and bacterial decomposition of bulk stored capelin. *J. Sci. Food Agric.,* 45 : 53-60.

Alexis, M.N., Paparaskeva, E., Theochari, V., 1985. Formulation of practical diets for rainbow trout (*Salmo gairdneri*) made by partial or complete substitution of fishmeal by poultry by-products and certain plant by-products. *Aquaculture*, 50 : 61-73.

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

Anderson, J.S., Lall, S.P., Anderson, D.M., McNiven, M.A., 1993. Evaluation of protein quality in fish meals by chemical and biological assays. *Aquaculture*, 115 : 305-325.

AOAC (Association of Official Analytical Chemists), 1984. *Official Methods of Analysis*, 14th ed., AOAC, Washington, DC, pp. 152-160.

AOAC (Association of Official Analytical Chemists), 1990. *Official Methods of Analysis*, 15th ed., AOAC, Washington, DC, pp. 1096-1098.

Aoe, H., Toyoda, T., Kitamura, S., 1970. Nutrition of protein in young carp. I. Nutrative value of free amino acids. *Bull. Jap. Soc. Sci. Fish.*, 39 : 407-413.

ARC (Agricultural Research Council), 1981. The nutrient requirements of pigs. Commonwealth Agricultural Bureaux, Farnham Royal.

Athanasopoulous, N., 1993. Flame methods manual for atomic absorption. GBC Scientific equipment PTY, Victoria, Australia.

Barlow, S. 1989. Fishmeal – World outlook to the year 2000. *Fish Farmer*. October. pp. 40-43.

Bassompierre, M., Kristiansen, H.R., McLean, E., 1998. Influence of weight upon *in vitro* protein digestion in rainbow trout. *J. Fish Biol.*, 52 : 213-216.

Benitez, L.V., 1989. Amino acid and fatty acid profiles in aquaculture nutrition studies. In: S.S. De Silva (Ed.), *Fish Nutrition Research in Asia.* Proceedings of the Third Asian Fish Nutrition Network Meeting. *Asian Fish. Soc. Spec. Publ*., 4 : 23-25.

Bordner, C.E., D'Abramo, L.R., Conklin, D.E., 1983. Assimilation of nutrients by cultured hybrid lobsters (*Homerus* sp.) fed experimental diets. *Proc. World Mar. Soc.,* 14 : 11-24.

Britz, P.J., 1995. *The nutritional requirements of Haliotis midae and development of a practical diet for abalone aquaculture*. PhD Thesis, Rhodes University, South Africa.

Britz, P.J., 1996a. 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.J., 1996b. Suitability of selected protein sources for inclusion in formulated diets for the South African abalone, *Haliotis midae. Aquaculture*, 140 : 63-73.

Britz, P.J., Bacela, N., Hecht, T., 1997. Can crystalline arginine be used to quantify the arginine requirement of abalone?. *Aquaculture*, 157 : 95-105.

Britz, P.J., Hecht, T., Knauer, J., Dixon, M.G., 1994. The development of an artificial feed for abalone farming. *S. Afr. J. Sci*., 90 : 6-7.

Britz, P.J., Hecht, T., 1997. Effect of dietary protein and energy level on growth and body composition of South African abalone, *Haliotis midae.* *Aquaculture,* 156 : 195-210.

Brookes, I.M., Oxens, F.N., Garrigus, U.S., 1972. Influence of amino acid oxidation by the rat. *J. Nutr.*, 102 : 27-36.

Brown, P.B., William, C.D., Robinson, E.H., 1986. Evaluation of methods for determining digestion coefficients for adult red swamp crayfish *Procambarus clarkii. J. World Aqua. Soc.,* 17 : 19-24.

Burgess, E., Main, C., Stevens, P., Christeller, J., Gatehouse, A., Laing, W., 1994. Effect of protease inhibitor concentration and combinations on the survival, growth and gut enzyme activities of the black field cricket, *Teleogryllus commodus.* *J. Insect Physiol*., 40 : 803-811.

Carter, C.G., Bransden, M.P., Van Barneveld, R.J., Clarke, S.M., 1999. Alternative methods for nutrition research on the Southern bluefin tuna, *Thunnus maccoyii* : *in vitro* digestibility. *Aquaculture*, 179 : 57-70.

Chen, H.-C., 1989. Farming the small abalone, *Haliotis diversicolor supertaxa* in Taiwan. In: K.O.Hahn (Ed.), *Handbook of Culture of Abalone and Other Marine Gastropods*, CRC Press, Boca Raton, Florida. pp. 265-283.

Chen, H.Y., Leu, Y,T., Roelants, I., 1992. Effective supplementation of arginine in the diets of juvenile marine shrimp, *Penaeus monodon. Aquaculture*, 108 : 87-95.

Clancy, S., Beams, R., Higgs, D., Dosanjh, B., Haard, N., Toy, B., 1995. Influence of spoilage and processing temperature on the quality of marine fish protein sources for salmonids. *Aquaculture Nutrition*, 1 : 169-177.

Cook, P., 1998. The current status of abalone farming in South Africa. *J. Shellfish Res*., 17(3) : 601-602.

Coote, T.A., Hone, P.W., Kenyon, R., 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., Forster, J.R., 1971. The essential amino acid requirements of the prawn *Palaemon serratus.* The growth of prawns on diets containing proteins of different amino acid compositions. *Mar. Biol.,* 10 : 77-81.

Cowey, C.B., Tacon, A.G.J., 1982. Fish nutrition – relevance to invertebrates. In: G.D. Pruder, C. Langdon and D. Conklin (Eds.), *Proceedings of the Second International Conference on Aquaculture Nutrition.* World Mariculture Society, Special Publication No.2, Louisiana State University, Louisiana. pp. 13-30.

Cowey, C.B., Sargent, J.R., 1979. Nutrition. In : W. Hoar, D. Randall (Eds.), *Fish Physiology*, Vol. VIII. Academic Press, London. pp. 1-69.

Cowey, C.B., Walton, M.J., 1988. Studies on the uptake of ^{14}C amino acids derived from both ^{14}C protein and dietary ^{14}C amino acids by rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.*, 33 : 293-305.

Croll, R.P., 1983. Gastropod chemoreception. *Biol. Rev.*, 58 : 293-319.

Crowder, B., 1990. U.S. demand for fishmeal. In : S. Keller (Ed). *Proceedings of the international conference on fish by-products.* Alaska Sea Grant Program, Fairbanks. pp. 57-60.

Dabrowska, H., Wojno, T., 1977. Studies on the utilization by rainbow trout (*Salmo gairdneri*) of feed mixture containing soybean meal and an addition of amino acids. *Aquaculture*, 10 : 297-310.

Dabrowski, K., Poczyczywski, P., Kock, G., Berger, B., 1989. Effect of partially or totally replacing fishmeal protein by soybean meal protein on growth, food utilisation and proteolytic activity in rainbow trout (*Salmo gairdeneri*). New *in vivo* test for pancreatic secretions. *Aquaculture,* 77(1) : 29-40.

Daniels, R., Floren, R., 1998. Poaching pressures on Northern California's abalone fishery. *J. Shellfish Res.*, 17(3) : 859-862.

Davis, G.E., Haaker, P.L., Richards, D.V., 1998. The perilous condition of white abalone *Haliotis sorenseni,* Bartsch, 1940. *J. Shellfish Res*., 17(3) : 871-875.

Deering, M.J., Hewitt, D.R., Sarac, H.Z., 1996. A comparison of inert markers used to estimate protein digestibility in the leader prawn *Penaeus monodon*.
J. World. Aqua. Soc., 27(1) : 103-106.

Deshimaru, O., 1982. Protein and amino acid nutrition of the prawn *Penaeus japonicus.* In: Pruder, G., Langdon, C.J., Conklin, D.E. (Eds), *Proceedings of the Second International Conference on Physiological Approaches to Shellfish Nutrition*. Lousiana State University, Baton Rouge, pp. 106-122.

Deshimaru, O., Kuroki, K., 1974. Studies on a purified diet for prawn. III. A feeding experiment with amino acid test diets.
Bull. Jpn. Soc. Sci. Fish., 40 : 1127-1131.

Deshimaru, O., Kuroki, K., 1975. Studies on a purified diet for pawn. IV. Evaluation of protein, free amino acids and their mixture.
Bull. Jpn. Soc. Sci. Fish., 41 : 101-103.

DeSilva, S.S., 1988. Digestibility evaluations of natural and artificial diets. In: S.S. DeSilva, *Fish Nutrition Research Asia* - Proc. Third Asian Nutrition Network. Asian Fish. Soc. Special Publ. 4. Asian Fish. Soc. Manila Philippines, pp 36-45.

DeSilva, S.S., Anderson, T.A., 1995. *Fish nutrition in aquaculture*. Chapman and Hall, London. p. 204.

Dimes, L.E. Haard, N.F. 1994. Estimation of protein digestibility : I. Development of an *in vitro* method for estimating protein digestibility in salmonids (*Salmo gairdneri*). *Comp. Biochem. Physiol*., 108A (2/3) : 349-362.

Dimes, L.E., Haard, N.F., Dong, F.M., Rasco, B.A., Forster, I.P., Fairgrieve, W.T., Arndt, R., Hardy, R.W., Barrows, F.T., Higgs, D.A., 1994. Estimation of protein digestibility – II. *In vitro* assay of protein in salmonid feeds. *Comp. Biochem. Physiol*., 108A (2/3) : 363-370.

Dong, F.M., Hardy, R.W., Haard, N.F., Barrows, F.T., Rasco, B.A., Fairgrieve, W.T., Forster, I.P., 1993. Chemical composition and protein digestibility of poultry by-product meals for salmonid diets. *Aquaculture*, 116 : 149-158.

Dunstan, G.A., Baillie, H.J., Barrett, S.M., Volkman, J.K., 1994. Effect of diet on the lipid composition of wild and cultured abalone. *Aquaculture*, 140 : 115-127.

Eid, A.E., Matty, A.J., 1989. A simple *in vitro* method for measuring protein digestibility. *Aquaculture*, 79 : 111-119.

Ezquerra, J.M., García-Carreño, F.L., Civera, R., Haard, N.F., 1997. pH-stat method to predict protein digestibility in white shrimp (*Penaeus vannamei*). *Aquaculture*, 157 : 251-262.

Fallu, R., 1991. *Abalone Farming.* Fishing News Books, Oxford, England. p.195.

Fenucci, J.L., Fenucci, A.C., Lawrence, A.L., Zein-Eldin, Z.P., 1982. The assimilation of protein and carbohydrate from prepared diets by the shrimp *Penaeus stylirostris*. *Proc. World Mariculture Soc.,* 13 : 134-145.

Ferraris, R.P., Catacutan, M.R., Mabelin, R.L., Jazul, A.P., 1986. Digestibility in milkfish, *Chanos chanos* (Forsskal) : effects of protein source, fish size and salinity. *Aquaculture*, 59 : 93-105.

Fleming, A.E., 1994. *Review of the development of artificial feeds for abalone*. Paper presented at the *Second International Symposium on abalone Biology, Fisheries and Culture*, Hobart, Tasmania, February 1994.

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

Fleming, A.E., Van Barneveld, R.J., Hone, P.W., Vandepeer, M.E., Kruck, J.A., 1998. Complimentary additivity of the digestibility coefficients of feed ingredients fed to juvenile greenlip abalone (*Haliotis laevigata*).
J. Shellfish Res., 17(3) : 641-647.

Florkin, M., Bricteux-Grégoire, S., 1972. Nitrogen metabolism in molluscs. In: Florkin, M., Scheer, B.T. (Eds.), *Chemical Zoology, Vol. 7 : Mollusca*. Academic Press, New York, pp. 301-348.

Folch, J., Lees, M., Stanley, S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, 226 : 497-509.

Forester, G.R., 1953. Periotrophic membranes in the *Caridea* (Crustacea, Decapoda).
J. Mar. Biol. Assoc. UK., 32 : 315-318.

Forster, R.M., Gabbott, P.A., 1971. The assimilation of nutrients from compounded diets by the prawn *Palaemon serratus* and *Pandalus platyceros*.
J. Mar. Biol. Assoc. UK, 51 : 943-961.

Fu-Lin, E., Webb, K.L., Hepworth, D.A., Casey, B.B., 1987. Metamorphosis of larvae of *crassostrea virginica* fed microencapsulated diets. *Aquaculture*, 64 : 185-197.

García-Carreño, F.L., Navarrete del Toro, A., Ezquerra, M., 1997. Digestive shrimp proteases for evaluation of protein digestibility *in vitro*. I : Effect of protease inhibitors in protein ingredients. *J. Mar. Biotechnol*., 5 : 36-40.

Garland, C.G., Cooke, S.L., Grant, J.F., McMeekin, T.A., 1985. Ingestion of the bacteria on and the cuticle 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.

Godoy, C., Jerez, G., 1998. The introduction of abalone in Chile : Ten years later. *J. Shellfish Res*., 17(3) : 603-605.

Grabner, M., 1985. An *in vitro* method for measuring protein digestibility of fish feed components. *Aquaculture*, 48 : 97-110.

Grabner, M., Hofer, R., 1985. The digestibility of broad bean (*Vicia faba*) and soya bean (*Glycine max*) under in vitro conditions simulating the alimentary tracts of rainbow trout (*Salmo gairdneri*) and carp (*Cyprinus carpio*). *Aquaculture*, 48 : 111-122.

Gregorio, D., 1991. Development of a commercial abalone aquaculture feed. National Marine Fisheries Service, Final Report, Gregorio Aquatech Inc., Terminal Island, USA.

Gropp, J., Koops, H., Tiews, K., Beck, H., 1979. Replacement of fishmeal in trout feeds by other feedstuffs. In : Pillay, T.V.R., Dill, W.A. (Eds). *Advances in Aquaculture*. Papers presented at the FAO technical conference on aquaculture, Kyoto, Japan. 21 May – 2 June. 1976. Fishing New Books, Farnham, England.

Guzmán, J.M., Viana, M.T., 1998. Growth of abalone *Haliotis fulgens* fed diets with and without fish meal, compared to a commercial diet. *Aquaculture,* 165 : 321-331.

Guzmán del Próo, S., 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 (Eds.), *Abalone of the World. Biology, Fisheries and Culture.* Fishing News Books, Cambridge. pp. 570-582.

Hahn, K.O., 1989a. Survey of commercially important abalone species in the world. In: K.O.Hahn (Ed.), *Handbook of culture of abalone and other marine gastropods.* CRC Press, Florida. pp. 3-11.

Hahn, K.O., 1989b. Abalone aquaculture in Japan. In: K.O. Hahn (Ed), *Handbook of culture of abalone and other marine gastropods.* CRC Press, Florida. pp.185-194.

Hahn, K.O., 1989c. Abalone aquaculture in New Zealand, Australia and Ireland. In: K.O. Hahn (Ed), *Handbook of culture of abalone and other marine gastropods.* CRC Press, Florida. pp. 295-299.

Hahn, K.O., 1989d. Nutrition and growth of abalone. In: K.O. Hahn (Ed), *Handbook of culture of abalone and other marine gastropods.* CRC Press, Florida. pp.135-182.

Harada, K., Eguchi, A., Kurosaki, Y., 1987. Feeding attraction activities in the combinations of amino acids and other compounds for abalone, oriental weatherfish and yellowtail. *Bull. Jpn. Soc. Sci. Fish.,* 53(8) : 1483-1489.

Harada, K., Mikasaki, T., Kawashima, S., Shiota, H., 1996. Studies on the feeding attractants for fishes and shellfishes. XXVI. Probable feeding attractants in allspice *Pimenta offinalis* for the black abalone *Haliotis discus.* *Aquaculture,* 140 : 99-108.

Harding, D.E., Allen, O.W., Wilson, R.P., 1977. Sulfur amino acid requirements of channel catfish : L-methionine and L-cystine. *J. Nutr*., 107 : 2031-2035.

Harper, A.E., Benevenga, N.J., Wohlhueter, R.M., 1970. Effects of ingestion of disproportionate amounts of amino acids. *Physiol. Rev*., 50 : 428-558.

Harvey, D.J., 1991. Outlook for U.S. aquaculture. Annual Agriculture Outlook Conference, U.S. Dept. of Agriculture, Washington, DC.

Hernández-Santoyo, A., Hernández-Arana, A., Arreguín-Espinosa, R., Rodríguez-Romero, A., 1998. Purification and characterization of several digestive proteases from the blue abalone, *Haliotis fulgens*. *Aquaculture*, 159 : 203-216.

Hsu, H.W., Vaak, D.L., Saterlee, L.D., Miller, G.A., 1977. A multi-enzyme automatic recording technique for *in vitro* protein digestibility.
J. Food Sci., 42(5) : 1269-1273.

Hughes, S.G., Rumsey, G.L., 1983. Dietary requirements of essential branch-chain amino acids by lake trout. *Trans. Am. Fish. Soc*., 112 : 812-817.

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

Ishikawa, M., Teshima, S., Kanazawa, A., Koshio, S., 1996. Evacuation of inert markers in digestibility determination, 5α-cholestane and chromic oxide, in the prawn *Penaeus japonicus. Fisheries Science,* 62(2) : 229-234.

Jackson, A.J., Capper, B.S., Matty, A.J., 1982. Evaluation of some plant protein in complete diets for the tilapia *Sarotherodon mossambicus*. *Aquaculture*, 27 : 97-109.

Jauncey, K., 1982. The effects of varying dietary protein level on the growth, food conversion, protein utilisation and body composition of juvenile tilapias (*Sarotherodon mossambicus*). *Aquaculture,* 27 : 43-54.

Jobling, M., 1983. A short review and critique of methodology used in fish growth and nutrition studies. *J. Fish. Biol.*, 23 : 685-703.

Keipert, S., Melegari, P., 1993. Preparation and characterization of oil containing microparticles. *Drug Dev. Ind. Pharm.,* 19(5) : 603-621.

King, R.H., Rayner, C.J., Kerr, M., Gorfine, H.K., McShane, P.E., 1996. The composition and amino acid balance of abalone (*Haliotis rubra*) tissue. *Aquaculture,* 140 : 109-113.

Kitamikado, M., Tachino, S., 1960. Studies on the digestive enzymes of rainbow trout. II. Proteases. *Bull. Japan. Soc. Sci. Fish*., 26 : 685-690.

Knauer, J., 1994. *Development of an artificial weaning diet for the South African abalone, Haliotis midae (Haliotidae : Gastropoda*). MSc Thesis, Rhodes University, Grahamstown, South Africa.

Knauer, J., Britz, P.J., Hecht, T., 1993. The effect of seven binding agents on 24hr water stability of an artificial weaning diet for the South African abalone, *Haliotis midae* (Haliotidae, Gastropoda). *Aquaculture,* 115 : 237-334.

Knauer, J., Brady, D., Duncan, J.R., Hecht, T., 1995a. Amino acid, fatty acid and mineral element profile of juvenile South African abalone, *Haliotis midae* L. *Aquaculture Res*., 26 : 283-288.

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

Knauer, J., Britz, P.J., Hecht, T., 1996. Comparative growth performance and digestive enzyme activity of juvenile South African abalone, *Haliotis midae*, fed on diatoms and a practical diet. *Aquaculture,* 140 : 75-85.

Lall, S.P., Kaushik, S., Le Bail, P.Y., Keith, R., Anderson, J.S., Plisetskaya, E., 1992. Quantitative arginine requirement of Atlantic salmon (*Salmo salar*) reared in seawater. In : *Proceedings 5th international symposium on fish nutrition and feeding.* 7-10 September 1992, Santiago, Chile. Fundacion Chile, Departamento de Recusos Marinos.

Langdon, C.J., Siegfried, C.A., 1984. Progress in the development of artificial diets for bivalve filter feeders. *Aquaculture*, 39 : 135-153.

Lautenschlager, K.P., Kaushik, N.K., Robinson, J.B., 1978. The periotrophic membrane and fecal pellets of *Gammarus lacustris* Smith. *Freshwater Biol.*, 8 : 207-211.

Leavitt, D.F., 1985. An evaluation of gravimetric and inert marker techniques to measure digestibility in the American lobster. *Aquaculture*, 47 : 131-142.

Lee, D.J., Putman, G.B., 1973. The response of rainbow trout to varying protein/energy ratios in a test diet. *J. Nutr.*, 103 : 916-922.

Lim, C., 1989a. Practical Feeding – Tilapia. In : Lovell, T., (Ed). *Nutrition and Feeding of Fish.* Van Nostrand Reinhold, New York. pp. 163-184.

Lim, C., 1989b. Practical Feeding – Penaeid Shrimps. In : Lovell, T., (Ed). *Nutrition and Feeding of Fish.* Van Nostrand Reinhold, New York. pp. 205-222.

López, L.M., Tyler, P.A., Viana, M.T., 1998. The effect of temperature and artificial diets on growth rates of juvenile *Haliotis tuberculata* (Linnaeus, 1758). *J. Shellfish Res*., 17(3) : 657-662.

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

Lovell, T., 1989a. Practical Feeding – Channel Catfish. In : Lovell, T., (Ed). *Nutrition and the feeding of fish*. Van Nostrand Reinhold, New York. pp. 145-162.

Lovell, T., 1989b. Feed formulation and processing. In : Lovell, T., (Ed). *Nutrition and Feeding of Fish.* Van Nostrand Reinhold, New York. pp. 107-127.

Maart, B.A., 1992. *The biotechnology of effluent grown spirulina and application in aquaculture nutrition.* MSc thesis, Rhodes University, Grahamstown.

Maguire, G.B., Wee, K.L., Hindrum, S.M., 1993. Digestibility studies – The "ins" and "outs" of abalone guts. *Austasia Aquaculture,* 7 : 42-45.

Mai, K., Mercer, J.P., Donlon, J., 1994. Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. II. Amino acid composition of abalone and six species of macroalgae with an assessment of their nutritional value. *Aquaculture*, 128 : 115-130.

Mai, K., Mercer, J.P., Donlon, J., 1995a. 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., Donlon, J., 1995b. 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.

Maynard, L.A., Loosli, J.K., 1956. *Animal nutrition*. McGraw Hill, New York, NY. 4th edition.

McBride, S.C., 1998. Current status of abalone aquaculture in the Californias. *J. Shellfish Res*., 17(3) : 593-600.

McCallum, I., Higgs, D., 1989. An assessment of processing effects on the nutritive value of marine protein sources for juvenile chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture*, 77 : 181-200.

Merican, Z.O., Shim. K.F., 1995. Apparent digestibility of lipid and fatty acids in residual lipids of meals by adult *Penaeus monodon*. *Aquaculture*, 133 : 275-286.

Moksness, E., Rosenlund, G., Lie, O., 1995. Effect of fish meal quality on growth of juvenile wolffish, *Anarhichas lupus* L. *Aquaculture Res.*, 26 : 109-115.

Monje, H., Viana, M.T., 1998. Effect of cellulose on the growth and cellulolytic activity of abalone *Haliotis fulgens* when used as an ingredient in formulated artificial diets. *J. Shellfish Res.*, 17 : 667-671.

Morales, A.E., Gardenete, G., De la Higuera, M., Sanz, A., 1994. Effects of dietary protein source on growth, feed conversion and energy utilization in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture,* 124 : 117-126.

Moyano, F.J., Cardenete, G., De la Higuera, M., 1992. Nutritive value of diets containing a high percentage of vegetable proteins for trout, *Oncorhynchus mykiss. Aquat. Living Res*., 5 : 23-29.

Murai, T., Hirasawa, Y., Akiyama, T., Nose, T., 1983. Effects of dietary pH and electrolyte concentration on utilization of crystalline amino acids by fingerling carp. *Bull. Jpn. Soc. Sci. Fish.*, 49 : 1377-1380.

Murai, T., Daozun, W., Ogata, H., 1989. Supplementation of methionine to soy flour for fingerling carp, (*Cyprinus carpio*). *Aquaculture*, 77 : 373-385.

New, M.B., 1991. Turn of the millenium aquaculture. *World Aquaculture*, 22(3) : 28-49.

Nie, Z.Q., 1992. A review of abalone culture in China. In: S.A. Shepherd, M.J. Tegner, S.A. Guzmán del Próo (Eds.), *Abalone of the World. Biology, Fisheries and Culture*, Fishing News Books, Cambridge. pp. 592-602.

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

NRC (National Research Council), 1983. *Nutrient requirements of warm water fishes and shellfishes*. Rev. Edn., National Academy Press, Washington, USA. pp. 41-42.

NRC (National Research Council), 1993. *Nutrient requirements of fish*. National Academy Press, Washington, USA. pp. 65-66.

Ogino, C., 1980. Requirements of carp and rainbow trout for essential amino acids. *Bull. Japan. Soc. Sci. Fish.*, 46 : 385-388.

Ogino, C., Kato, N., 1964. Studies on the nutrition of abalone – II. Protein requirements for growth of abalone, *Haliotis discus* Reeve with artificial diets. *Bull. Japan. Soc. Sci. Fish.,* 29 : 691-694.

Olli, J., Hjelmeland, K., Krogdahl, A., 1994. Soybean trypsin inhibitor in diets for Atlantic salmon (*Salmo salar,* L.) : Effects on nutrient digestibilities and in pyloric ceaca homogenate and intestinal content. *Comp. Biochem. Physiol*., 109A : 923-928.

Pedersen, B., Eggum, B.O., 1983. Prediction of protein digestibility by an *in vitro* enzymatic pH-stat procedure. *Z. Tierphysiol Tierernahrg. u. Futtermittelkde,* 49 : 265-277.

Pike, I.H., 1993. Freshness of fish for fish meal effect on growth of salmon. In : Kaushik, S.J., Luquet. P., (Eds.), *Fish Nutrition in Practice*. INRA, Paris, pp. 843-846.

Pike, I.H., Andorsdottir, G., Mundheim, H., 1990. The role of fishmeal in diets for salmonids. *International Association of Fish Meal Manufacturers*, 24 : 1-35.

Pongmaneerat, J., Wantanabe, T., 1992. Utilisation of soybean meal as a protein source in diets for rainbow trout. *Nippon Suisan Gakkashi*, 58(10) : 1983-1990.

Rajamani, M., Job, S.V., 1976. Food utilization by *Tilapia mossambica* (Peters) : function of size. *Hydrobiologia*, 50 : 71-74.

Regost, C., Arzel, J., Kaushik, S.J., 1999. Partial or total replacement of fishmeal by corn gluten meal in diets for turbot (*Psetta maxima*). *Aquaculture*, 180 : 99-117.

Reinitz, G., 1980. Soybean meal as a substitute for herring meal in practical diets for rainbow trout. *Prog. Fish Cult*., 42(2) : 103-106.

Rivero, L.E., Viana, M.T., 1996. Effect of pH, water stability and toughness of artificial diets on the palatability for juvenile abalone *Haliotis fulgens.* *Aquaculture*, 144 : 353-362.

Robaina, L., Moyano, F.J., Izquierdo, M.S., Socorro, J., Vergara, J.M., Montero, D., 1997. Corn gluten and meat bone meal as protein sources in diets for gilthead seabream (*Sparus aurata*) : Nutritional and histological implications. *Aquaculture*, 157 : 347-359.

Robinson, E.H., Wilson, R.P., Poe, W.E., 1981. Arginine requirement and apparent absence of a lysine-arginine antagonism in fingerling channel catfish. *J. Nutr.*, 111 : 46-51.

Robinson, E.H., Poe, W.E., Wilson, R.P., 1984. Effect of feeding diets containing an imbalance of branch-chain amino acids on fingerling channel catfish. *Aquaculture*, 37 : 51-62.

Ruchimat, T., Masumoto, T., Hosokawa, H., Itoh, Y., Shimeno, S., 1997. Quantitative lysine requirement of yellowtail (*Seriola quinqueradiata*). *Aquaculture*, 158 : 331-339.

Rudd, M., 1994. *A review of international abalone trade patterns and pricing.* Paper presented at the Second International Symposium on Abalone Biology, Fisheries and Culture, Hobart, Tasmania, February 1994.

Rumsey, G.L. 1993. Fish meal and alternate sources of protein in fish feeds, update 1993. *Fisheries*, 18(7) : 14-19.

Rumsey, G.L., Ketola, H.G., 1975. Amino acid supplementation of casein in diets of Atlantic salmon (*Salmo salar*) fry and of soybean meal for rainbow trout (*Salmo gairdneri*) fingerlings. *J. Fish. Res. Board Can.*, 32 : 422-426.

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

Sakata, K., 1989. Feeding attractants and stimulants for marine gastropods. In : P.J. Scheuer (Ed.), *Bioorganic Marine Chemistry. Vol. 3.* Springer Verlag, Berlin. pp. 115-129.

Sanz, A., Morales, A.E., de la Higuera, M., Cardenete, G., 1994. Sunflower meal compared with soybean meal as partial substitutes for fish meal in rainbow trout (*Oncorhynchus mykiss*) diets : protein and energy utilization. *Aquaculture*, 128 : 287-300.

Saterlee, L.D., Marshall, H.F., Tennyson, J.M., 1979. Measuring protein quality. *J. Am. Oil Chem. Soc*., 56 : 103-109.

Serviere-Zaragoza, E., Navarrete del Toro, M.A., García-Carreño, F.L., 1997. Protein-hydrolysing enzymes in the digestive systems of the adult Mexican blue abalone, *Haliotis fulgens* (Gastropoda). *Aquaculture*, 157 : 325-336.

Simpson, R.J., Neuberger, M.R., Liu, C.-Y., 1976. Complete amino acid analysis of protein from a single hydrolysate. *J. Biol. Chem.,* 251 : 1936-1940.

Smith, R.R., 1989. Nutritional energetics. In: J.E. Halver (Ed.), *Fish Nutrition, Second Ed..* pp. 1-29.

Sopropêche. 1995a. Comparison of biogenic amines and molecular weight of CPSP with other products. 62204 Boulogne sur Mer, France.

Sopropêche. 1995b. Results of experimental trials CPSP – CPSP special G – estrilvo. 62204 Boulogne sur Mer, France.

Steffens, W., 1989a. *Principles of fish nutrition*. Halsted Press, New York. p. 67.

Steffens, W., 1989b. *Principles of fish nutrition*. Halsted Press, New York. p. 111.

Stickney, R.R., Hardy, R.W., Koch, K., Harrold, R., 1996. The effects of substituting selected oilseed protein concentrates for fish meal in rainbow trout *Oncorhynchus mykiss* diets. *J. World Aqua. Soc.* 27(1) : 57-63.

Stuttaford, M., 1997. *Fishing Industry Handbook*. Marine Information C.C. Cape Town. p. 415.

Tacon, A.G.J., 1990a. *Standard methods for the nutrition and feeding of farmed fish and shrimp*. Argent Laboratories Press, Redmond, USA. Vol. 2, p. 3.

Tacon, A.G.J., 1990b. *Standard methods for the nutrition and feeding of farmed fish and shrimp*. Argent Laboratory Press, Redmond, USA. Vol. 1, p. 16.

Tacon, A.G.J., Webster, J.L., Martinez, C.A., 1984. Use of solvent extracted sunflower seed meal in complete diets for fingerling rainbow trout (*Salmo gairdneri* Richardson). *Aquaculture*, 43 : 381-389.

Tanikitti, C., March, B.E., 1995. Dynamics of plasma free amino acids in rainbow trout (*Onconhynchus mykiss*) under a variety of dietary conditions. *Fish Physiol. Biochem.*, 14 : 179-194.

Tarr, R.J.Q., 1992. The abalone fishery of South Africa. In: S.A. Shepherd, M.J. Tegner and S.A. Guzm<n del Próo (Eds.), *Abalone of the World. Biology, Fisheries and Culture.* Fishing News Books, Cambridge. pp. 438-447.

Taylor, B., 1992. Abalone nutrition : Optimal protein level in artificial diets for *Haliotis kamtschatkana. J. Shellfish Res.*, 11 : 556.

Tjeerdema, R.S., Kauten, R.J., Crosby, D.G., 1991. Sublethal effects of hypoxia in the abalone (*Haliotis rufescens*) as measured by *in vivo* ^{31}PNMR spectroscopy. *Comp. Biochem. Physiol*., 100B : 653-659.

Uki, N., 1981. Feeding behaviour of experimental populations of abalone *Haliotis discus hannai*. *Bull. Tohoku Reg. Fish. Res. Lab*., 43 : 53-58.

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

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

Uki, N., Watanabe, T., 1986. Effect of heat treatment of dietary protein sources on their protein quality for abalone. *Bull. Japan. Soc. Sci. Fish.,* 52 : 1199-1204.

Uki, N., Kemuyama, A., Watanabe, T., 1986. Optimum protein level in diets for abalone. *Bull. Japan Soc. Sci. Fish.*, 52 : 1005-1012.

Uki, N., Watanabe, T., 1992. Review of nutritional requirements of abalone (*Haliotis* sp.) and development of more efficient artificial diets. In : S.A. Shepherd, M.J. Tenger, S.A. Guzm<n del Próo (Eds), *Abalone of the World. Fisheries, Biology and Culture*, Fishing News Books, Oxford, UK, pp. 504-517.

Van Barneveld, R.J., Fleming, A.E., Vandepeer, M.E., Kruk, J.A., 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*). *J. Shellfish Res*., 17(3) : 649-655.

Vandepeer, M.E., Van Barneveld, R.J., Hone, P.W., Havenhand, J.N., 1999. The digestibility of raw, autoclaved and phytase treated legumes in greenlip abalone, *Haliotis laevigata*. *Proceedings of the 6th annual abalone aquaculture workshop*. World Aquaculture Society Conference, Sydney, April 1999.

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

Viana, M.T., Nava, C., Solana-Sansores, R., 1993b. Acid fish silages. Effect of preheating and addition of phosphoric and citric acids on the biochemical quality. *Ciencias Mar*., 19(4) : 415-433.

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

Viana, M.T., López, L.M., García-Esquivel, Z., Mendez, E., 1996. The use of silage made from fish and abalone viscera as an ingredient in abalone feed. *Aquaculture*, 140 : 87-98.

Viyakran, V., Wantanabe, T., Aoki, H., Tsuda, H., Sakamoto, H., Okamoto, N., Iso, N., Satoh, S., Takeuchi, T., 1992. Use of soya bean meal as a substitute for fishmeal in a newly developed soft dry pellet for yellowtail. *Bull. Jap. Soc. Sci*., 58(10) : 1991-2000.

Watanabe, T., Viyakarn, V., Kimura, H., Ogawa, K., Okamoto, N., Iso, N., 1992. Utilization of soya bean meal as a protein source in a newly developed soft-dry pellet for yellowtail. *Nippon Suisan Gakkaishi*, 58(9) : 1761-1773.

Wee, K.L., Maguire, G.B., Hindrum, S.M., 1992. Methodology for digestibility studies with abalone. 1. Preliminary studies on the feeding behaviour of Blacklip abalone, *Haliotis rubra*, fed natural and artificial diets. In: G.L Allen and W Dall (eds), *Proc. Aquaculture Nutrition workshop*, Salamander Bay, NSW Fisheries, Brackish Water Fish Culture Research Station, Salamander Bay Australia, pp. 192-196.

Wee, K.L., Maguire, G.B., 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). Proceedings of the Third Asian Fisheries Forum*, Singapore. Asian Fisheries Society, Manila.

Weisberg, S.B., Lotrich, V.A., 1982. Ingestion, egestion, excretion, growth and conversion efficiency for the mummichog, *Fundulus heteroclitus*. *J. Exp. Mar. Biol. Ecol*., 62 : 237-250.

Wilson, R.P., 1989. Amino acids and proteins. In : Halver, J.E., (Ed), *Fish Nutrition*. 2nd Ed., Academic Press, San Diego, USA. pp. 112-151.

Wilson, R.P., Harding, D.E., Garling, D.L., 1977. Effect of dietary pH on amino acid utilization and the lysine requirement of fingerling channel catfish. *J. Nutr.*, 107 : 166-170.

Windell, J.T., Folz, J.W., Sarokon, J.A., 1978. Effect of fish size, temperature and amount fed on nutrient digestibility of a pelleted diet by rainbow trout, *Salmo gairdneri*. *Trans. Amer. Fish. Soc.*, 107 : 613-616.

Yamada, S., Yone, T., 1986. Loss of dietary amino acids during mastication by carp. *Bull. Jpn. Soc. Sci. Fish.*, 52 : 673-676.

Zar, J.H., 1984. *Biostatistical Analysis*, 2nd Ed. Prentice-Hall, Inc. Englewood Cliffs, N.J. pp. 718.