Towards determining the dietary lysine requirement in the South African abalone, *Haliotis midae*

A thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

at

RHODES UNIVERSITY

by

KYLE EVERETT LLOYD

February 2016

ABSTRACT

Animals generally do not have a requirement for protein, but instead have a requirement for specific essential amino acids (EAAs) and non-essential amino acids (NEAAs). The NEAAs are those that can be synthesised by the animal, however, EAAs cannot be synthesised and must therefore be supplied as part of the diet. When these amino acids (AAs) are supplied in the correct ratios and with the correct level of digestible energy, nutritionists can maximise somatic growth from proteins. This has resulted in increased research into the use of crystalline AAs as a tool in feed formulation research in order to quantify the AA requirements in aquaculture species, and allow for alternative protein sources (other than fishmeal) to be utilised. In common with other water soluble nutrients, leaching of crystalline AAs from diets prior to ingestion is of concern in an aquatic environment. Microencapsulation techniques have been successfully employed to restrict micronutrient leaching, and improve ingestion rates. In this research, LysiPEARL^{TM} (Kemin®) was used as a means to determine the lysine requirement in *Haliotis. midae*. This encapsulated lysine product is used in the dairy cattle industry as an effective source of rumen bypass for intestinal release of lysine. It has previously been proposed that crystalline AAs are not suitable for AA studies in *H. midae* due to the slow feeding rates of the species as well as the solubility of these AAs. However, 90.00 % of supplemented lysine was maintained in this study after a six hour period of leaching, showing that if effective microencapsulation techniques are used, it is possible to use crystalline amino acids to supplement protein bound lysine in abalone feeds. Six isoenergetic (15.90 MJ/kg), isolipidic (6.00 %) and isonitrogenous (29.00 %) diets enriched with 5.52, 6.40, 7.28, 8.14, 9.00 and 9.86 % lysine (as a % of protein) were fed to triplicate groups of 20 *H. midae* (20.41 ± 1.95 mm SL 1.51 ± 0.44 g w.wt) for 90 days. Wet weight and shell length measurements were taken every 30

days and specific growth rate (SGR) (% body weight.day^{-1}), feed conversion ratio (FCR), protein efficiency ratio (PER), feed consumption (% body weight.day^{-1}) and condition factor were calculated for each dietary treatment. Linear regression showed that FCR increased as dietary lysine increased (Regression analysis, p=0.031), and that PER reduced as dietary lysine increased (Regression analysis, p=0.026). Feed consumption also increased as dietary lysine increased (Regression analysis, p<0.001). The inclusion of lysine at 7.28 % of the total protein in the diet resulted in significantly superior SGR (0.57±0.01 % body weight.day^{-1}) to that of 5.52 % (0.42±0.05 % body weight.day^{-1}), FCR (1.51±0.05) to that of 8.14 % (1.99±0.21) and PER (2.45±0.07) to that of 8.14 % (1.99±0.18; ANOVA, p<0.05). There was a significant difference found in feed consumption (% body wt.d^{-1}), with consumption increasing significantly between the first three dietary treatments and the last three dietary treatments (ANOVA, p<0.001). There was no significant improvement in SGR when dietary lysine increased above 7.28 % of the dietary protein in the diet, indicating that dietary lysine requirement was being met at 7.28 %, after which excess lysine promoted no growth response. The diet producing the best SGR, PER and FCR in this study was diet 3 which had a measured lysine content of 6.90 %. The results of the present study suggest that the lysine requirement in *H. midae* is in the range of 6.00 - 7.00 % of dietary protein. From these data amino acid ratios were used to estimate optimum inclusion levels of other essential amino acids. However, lysine availability in LysiPEARL^{TM} may have resulted in over estimations due to the lipid encapsulation technique used, and haliotids limited ability to efficiently digest lipids. For this reason EAA requirements were suggested based on three different hypothetical scenarios of lysine availability from LysiPEARL^{TM}.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr Tom Shipton and Dr Cliff Jones for all of their effort and guidance they provided me throughout my masters. These past two years have been one of the most enjoyable yet challenging times of my life at the Port Alfred Marine Research Laboratory. I feel I have learnt more about animal husbandry, system and personnel management than your average MSc. candidate, So thank you for the opportunity. Cliff, the past three years of supervision from you since honours have been an absolute pleasure. Thanks for always taking the time to see me, even during your sabbatical, and a big thank you for always making sure my "travel claims" were eventually processed. Thank you both for your patience.

I would also like to thank Marifeed (Pty) Ltd for the support and funding. I also gratefully acknowledge the National Research Foundation for the innovation and scarce skills scholarship received during my second year of masters study. I would also like to thank the Technology and Human Resource for Industry Program (THRIP) for the additional funding provided during my masters.

Thank you to my fellow co-workers. Firstly Justin Kemp, you were like having another supervisor on hand whenever I needed. Thank you for always taking the time to speak to me about system design, or explaining to me the best way not to be electrocuted by the Labs very dicey distribution boards. Thanks must also go to my partner at the lab Adejoke Adesola, thank you for your help with some of my statistical analyses and taking care of the system when I went on a much needed holiday. And lastly, Matthew Farthing, you were always a shoulder to lean on, someone to fish with (maybe a bit too often) or someone to bounce ideas off of, thank you.

A special mention must go to my Port Alfred mom, Angela Farthing. You treated me like another son, and I always felt welcome in your wonderful home and coffee shop when the loneliness bug hit me, thank you.

Saving the best till last, I would like to thank my mom and granny. Not only for the extra funding when my pockets were running a bit dry, but also for giving me the motivation to pursue my passion throughout my academic career. I would like to dedicate this thesis to you. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Investigations into artificial feeds for the abalone culture industry have shown that it is possible to apply animal feed science models to haliotid nutrition (Britz, 1995). Furthermore, while issues pertaining to the gross requirements for nutrients such as lipid (Uki and Watanabe, 1992; Mai *et al*. 1995b; Durazo-Beltran *et al*. 2003; Durazo-Beltran *et al*. 2004; Green *et al*. 2011), protein (Uki *et al*. 1986; Taylor, 1992; Mai *et al*. 1995a; Britz, 1996a; Britz and Hecht, 1997; Gomez-Montez *et al*. 2003; Green *et al*. 2011), vitamins and minerals (Uki *et al*. 1985b; Coote *et al*. 1996; Tan and Mai, 2001) and essential fatty acids (Mai *et al*. 1996; Durazo-Beltran *et al*. 2003) have been identified and resolved, there is still a gap in the literature concerning the more specific requirements of abalone for particular essential amino acids. Therefore the essential amino acid requirements of abalone were chosen as the focus of this study.

Review: Research on abalone culture and feed development

The South African abalone *Haliotis midae* (Linnaeus, 1758), is an important aquaculture species. It is the largest of the six haliotid species found in South African waters and has the second largest distributional range (from St Helena Bay on the west coast to north of Port St Johns on the east coast) exceeded only by *Haliotis spadicea* (Wood, 1993). The distribution of *H. midae* is affected by temperature, ranging from 12ºC in the Atlantic Ocean off the Western Cape province to 21ºC in the Indian Ocean off the Eastern Cape province (Sales and Britz 2001). The remaining five South African species either occur in too few numbers, or are too small to be viable for commercial harvesting (Cook, 1998).

H. midae characteristically inhabit the shallow intertidal and littoral zones of rocky coastlines, and can typically be found to a depth of about 20 m. The sedentary nature of haliotids, combined with their sometimes easily accessible habitat, makes them vulnerable to exploitation from both legal as well as illegal harvesting practices. It is this trend of decline in abalone fisheries, coupled with their high value, that has stimulated the investigation of abalone ecology, biology and over the past 30 years the development of intensive culture techniques for the species (Sales, 2001).

H. midae is placed among the most highly valued seafood products in the world, with the majority of the demand coming from the South-East Asian countries (Sales, 2001). The availability of abalone on the world market has rapidly increased from 20 000 metric tons in the 1970's to an estimated 97 000 metric tons in 2012 (Gordon and Cook, 2013). Due to this high demand and ease of harvest, the demise of the South African wild abalone fishery follows the general pattern with abalone fisheries in other parts of the world (Japan, China, Australia, North America, Canada), where a decline in the fishery, mainly due to poaching, has stimulated the development of abalone farming to meet the demand for the product (Sales, 2011). In South Africa, wild catches have reduced from a peak of 2800 tons in 1965 (Tarr, 1992) to little more than 600 tons in 1971 when quotas were introduced to the fishery. Subsequently, the fishery declined to such a point where the allocation of long-term rights in the abalone commercial fishery were withdrawn in July 2014.

Abalone farming is a relatively young sector of the South African aquaculture industry, as farming only began in the 1990's (Sales and Britz, 2001). Despite the sectors age, the South African abalone farming industry is the third largest in the world, alongside Asia (Cook, 2014), with 14 farms currently registered with the Abalone Farmers Association of

South Africa (Cook, 1998; DAFF, 2012). Twelve of these farms are land based facilities with independent hatchery and grow out facilities, employing a flow-through design, pumping seawater into tanks on land (Troell *et al.* 2006). The remaining two farms comprise a ranching and sea cage operation (DAFF, 2012).

The abalone farming industry is the most economically important form of aquaculture in South Africa, fetching prices of about $34 to $40 per kilogram for live abalone (Ten-Doeschate and Coyne 2008). In 2011, the abalone subsector contributed to 55 % of South Africa's total aquaculture production, with an output of 1036 tons (DAFF, 2012). Sales were valued at roughly ZAR355 million, representing 93.9 % of the total marine aquaculture sector sales in South Africa (DAFF, 2012).

In order to successfully farm any organism, terrestrial or aquatic, farmers need to provide a diet that is both nutritionally complete and able to satisfy the physiological requirements to allow for somatic growth. The Japanese paved the way for abalone feed development over 60 years ago (Murayama, 1935; Ino, 1952). These initial findings have now been implemented in Taiwan (Chen, 1989), New Zealand and Australia (Hanh, 1989c), Ireland (Hahn, 1989c), China (Nie, 1992), the USA and Mexico (McBride, 1998), Chile (Godoy and Jerez, 1998), South Africa (Cook, 1998). The diet of post-larval juvenile haliotids consists primarily of diatoms and flagellate microalgae which are grown on corrugated plastic surfaces, as well as plastic bags exposed to natural or artificial light sources (Garland *et al*. 1985; Sakata, 1989). After this initial phase, abalone (±10mm SL) are weaned onto macroalgae or artificial feeds. There has been a widespread use of natural macroalgae in commercial abalone farms, however, a number of authors have suggested that the development of nutritionally complete artificial feeds have been fundamental to the

growth and expansion of the haliotid culture industry worldwide (Hahn, 1989b; Fallu, 1991; Britz *et al*. 1994). Subsequently, Britz (1995) highlighted the advantages of artificial feeds over their natural macroalgal counterparts in South Africa, culminating in the development of a commercial abalone feed, Abfeed® (produced at Marifeed (Pty) Ltd, Hermanus, South Africa) that comprises of fishmeal, soya bean meal, starch, vitamins and minerals. The Abfeed® grower formulation consists of approximately 35 % protein, 43 % carbohydrate, 5 % fat, 1 % crude fibre, 6 % ash and 10 % moisture (Ayres, 2013). Commercially grown abalone grow well on Abfeed® until they reach 50 mm shell length and most of the farmers use this diet in the early stages of abalone growth (Francis *et al.* 2008).

A major drawback in abalone farming is the slow growth of the animal. Abalone under aquaculture conditions can grow much faster when compared to natural populations; however they still require four to five years to reach a market size of 80 mm (Ten-Doeschate and Coyne 2008). If the production period were to be shortened through optimisation of feeding and therefore growth, production costs would be reduced and increases in the annual profits achieved (Ten-Doeschate and Coyne 2008).

The cost of formulated feed is one of the largest operational costs on South African farms, and research into optimising these diets is essential if profits are to be maximised (Britz *et al.* 1997). The current abalone feed formulation is a predominantly fishmeal-based diet, and dependency on the commodity will only increase as the overseas market demand grows (Troell *et al.* 2006). South Africa was ranked the thirteenth largest fishmeal consumer in the world in 2004 using approximately 101 000 tons (FIN 2005). Fishmeal destined for aquaculture in South Africa was largely dominated by the abalone industry (Hecht and Jones 2009). Natural seaweed can only delay the demand for fishmeal based diets, as harvesting

will eventually become a limiting factor as the industry grows (Troell *et al.* 2006). Total feed used in the abalone industry during 2011 was 7 830 tons which comprised of 1 009 tons of artificial feed and 6 820 tons of seaweed/kelp (DAFF, 2012). Thus, research into alternative protein sources to reduce the sectors dependence on fishmeal and wild seaweed/kelp is important for the industry to continue expanding (Troell *et al.*2006).

There are currently a number of protein sources being utilised at low inclusion levels in artificial feeds for haliotids. However, fishmeal and soya meal are the most commonly used protein sources incorporated into formulations (Fleming *et al*. 1996). There are implications for the industry having a dependence on only two protein sources, as they become inherently vulnerable to fluctuations in the availability and price of these commodities.

Whilst formulated feeds containing fishmeal have reduced the sector's reliance on wild seaweed stocks, and allow for better feed management practices as well as growth rates on farms (Ten-Doeschate and Coyne 2008), these diets have a high dependence on wild forage fish stocks. Fishmeal originates from wild fish such as sardine, anchovy and herring and is an expensive ingredient in formulated feeds (Miles *et al*. 2006). These forage fish are an important part of oceanic ecosystems and are showing an ever declining trend (Harvey, 1991, Naylor *et al*. 2000). This has resulted in an increasing price of the commodity as there is increasing competition for the resource for inclusion in aqua-feeds, agri-feeds as well as a food source for humans (Rumsey, 1993). It is safe to assume that supplies of fishmeal will not increase in the foreseeable future (Rumsey, 1993). Coupled with these supply issues, consumers are placing aquaculture industries around the world under increasing pressure to develop alternative, more environmentally sustainable diets (WWF, 2011).

The World Wide Fund for Nature (WWF) and the Southern African Sustainable Seafood Initiative (SASSI) promote awareness to reduce the amount of wild fish caught for fishmeal in the aquaculture industry by making retailers, restaurants and consumers aware of a forage fish efficiency ratio (WWF, 2011). The FFER is the wet weight of an individual aquaculture product divided by the wet weight of forage fish meal eaten by that fish in its lifetime (Miles *et al*. 2006). The WWF has set a target that all aquaculture species must have a FFER of one or less (WWF, 2011). The future of aquaculture feeds could largely depend upon lower-grade raw materials that may be further improved by processing and biotechnological transformation to provide a consistent nutrient source for farmed fish species (Costa-Pierce *et al.* 2012). Changing the farm's feed to a diet with less fishmeal, which does not reduce production and meat quality but still has sufficient quality protein to sustain optimal growth, could greatly increase farm profits.

In addition to this, the commercial prospects for casein and fishmeal as sources of protein in South African aqua-feeds is restricted, as cheap supplies are not available (Fleming *et al*. 1996). Soya bean 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 abalone diets (Guzman and Viana, 1998; Bautista-Teruel *et al*. 2003). However, abalone farmers in South Africa that rely on locally produced formulated feed, i.e. Abfeed®, reported a periodic drop in growth just after winter, with presumed links to the production and release of gametes (Ayres, 2013). Coupled with this, a large drop in the overall farm tonnage was noticed after spawning peaks, or when environmental cues initiated mass spawning (Ayres, 2013). This phenomenon only affected abalone of a certain age, and interestingly did not appear to affect abalone that were only fed kelp (Ayres, 2013). It was later shown that formulations with high levels of soya bean meal can produce undesired

increases in gonad development in *H. midae* during reproductive phases of the year (Ayres, 2013). In further research investigating effect of soya bean meal on gonad growth, it was postulated that the combination of soya bean meal and fishmeal may supply an AA balance that promotes gonad growth during these reproductive phases of the year (Wu, 2014). It is also not advisable to completely remove soya bean meal from these formulations, as they result in higher quality meat yields when compared to diets without soya bean meal (Ayres, 2013). Therefore it is of great importance to determine the value of alternative plant protein sources for use in combination with current formulations in aqua-feeds. In order for formulators to successfully utilise alternative plant protein sources in *H. midae* diets, the species' specific requirement for AAs needs to be determined.

Animals generally do not have a requirement for protein, but instead have a requirement for specific amino acids (AA), both essential amino acids (EAA) and non-essential amino acids (NEAA) (Shipton *et al*. 2002). NEAAs are those that can be synthesised by the animal, however, EAAs cannot be synthesised and must therefore be supplied as a part of the diet (Steffens, 1989a). When these AAs are supplied in the correct ratios and with the correct level of digestible energy, it is possible to optimise somatic growth. It is therefore required that nutritionists quantify the EAA requirement of a target aquaculture species prior to formulating any artificial feed (Shipton *et al*. 2002). This is undertaken in order to optimize the protein utilisation of the animal, in turn optimising growth at a least dietary cost.

Abalone, like most other farmed animals (mammals, birds and fish), have the same requirement for 10 EEAs, comprising of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Allen and Kilgore, 1975). There

is a growing literature base defining the availability of EAAs from both single, or mixed protein sources (Fleming *et al*. 1998; Van Barneveld *et al*. 1998; Sales and Britz, 2001). Furthermore, it is also generally accepted that the AA profile of an animals' tissue gives an indication of the relative proportions of the EAAs required in the diet (Cowey and Tacon, 1982; Benitez, 1989). The AA profiles of a number of haliotids have been determined (Allen and Kilgore, 1975; Knauer *et al.* 1995a; King *et al*. 1996). Whilst these findings have demonstrated the specific AAs within haliotids, and the levels at which they are found in the body of the animal, it is difficult to transfer directly to artificial feed formulations. This is because EAA profiles from wild abalone may not directly translate to optimal growth rates within commercial facilities as diets are often different according to species as well as habitat type. However, they do provide a good indication of their dietary requirement. Another important factor that needs to be noted is that growth in commercial facilities is faster when compared to wild growth rates. It is also important to note that whilst the EAA pattern of an animals' tissue gives information on the pattern of EAAs required for growth, they do not supply nutritionists with the absolute dietary requirement of the animal. It is thus important to find out the quantitative EAA requirement of haliotids, in order for the EAA availability data supplied by Sales and Britz (2001) to be utilized effectively. It will then be possible for nutritionist to design diets that provide the optimal balance and inclusion rates of EAAs to the animals.

The most common method used for determining the EAA requirement of a candidate aquaculture species is done by predetermining the animals' requirement for a limiting EAA, and then calculating the requirement for the other EAAs relative to the known as well as the EAA pattern of the soft tissue (Shipton *et al.* 2002). This method of estimation has been carried out in many aquaculture finfish species such as chinook salmon, chum salmon, tilapia,

channel catfish, common carp, Japanese eel, catla, milkfish (National Research Council USA, 1993), European seabass, turbot and gilthead seabream (Kaushik, 1998).

In order to determine an animal's' requirement for a limiting EAA, dose response studies using crystalline AAs have been used to good effect in Japanese flounder, red seabream (Forster and Ogata, 1998), yellowtail (Ruchimat *et al.* 1997), Atlantic salmon (Espe *et al.* 2007), hybrid striped bass (Keembiyehetty and Gatlin, 1991), cobia (Zhou *et al.* 2007) and Rainbow trout (Kim *et al.* 1992). This is where isoenergetic and isonitrogenous basal diets are enriched with crystalline AAs in order to determine the optimum inclusion level of the EAA.

Aims and objectives

This study was a continuation of previous research projects initiated by Rhodes University into the development of artificial feeds for the abalone *H. midae*. The primary aim of the study was to gain a better understanding of the species' quantitative requirement for lysine and subsequently their requirement for all other EAAs.

The objectives were to:

1. Identify a commercially available form of lysine that can remain in the pellet during suspension in water;
2. Determine the lysine requirement in *H. midae* by feeding graded levels of the lysine product in isolipidic, isonitrogenous and isoenergetic diets;
3. Determine the species requirement for other EAAs relative to lysine using amino acid ratios.

Most issues pertaining to the gross requirements for nutrients such as lipid (Uki and Watanabe, 1992; Mai *et al*. 1995b; Durazo-Beltran *et al*. 2003; Durazo-Beltran *et al*. 2004; Green *et al*. 2011), protein (Uki *et al*. 1986; Taylor, 1992; Mai *et al*. 1995a; Britz, 1996a; Britz and Hecht, 1997; Gomez-Montez *et al*. 2003; Green *et al*. 2011), vitamins and minerals (Uki *et al*. 1985b; Coote *et al*. 1996; Tan and Mai, 2001) and essential fatty acids (Mai *et al*. 1996; Durazo-Beltran *et al*. 2003) have been identified and resolved, however there is still a gap in the literature concerning the more specific requirements of abalone for particular essential amino acids. Therefore the essential amino acid requirements of abalone were chosen as the focus of this study.

CHAPTER 2

An assessment of the suitability of LysiPEARL™ as a means to determine the lysine requirement in *Haliotis midae*

Introduction

Unsuccessful attempts have been made to determine the dietary arginine and lysine requirements for *Haliotis midae* (Britz *et al*. 1997, Shitpon *et al*. 2002). This coupled with the paucity of information on any of the other essential amino acid (EAA) requirements in abalone provides motivation for further study on the EAA requirements in haliotids. The EAA requirements of organisms can be established by using a dose-response experiment. In these experiments growth is measured as a response to graded levels of an amino acid (AA) in semipurified, isolipidic, isoenergetic and isonitrogenous diets with a basal formulation that was deficient in the AA that was tested (Wilson, 1989).

There have been multiple studies into the AA requirements of *H. midae* attempting to determine the first limiting EAA (Britz *et al*. 1997; Shipton *et al*. 2002). The first limiting EAA is one that is required for protein synthesis, and if it is not present in adequate amounts, growth is restricted by the level of dietary EAA available (Britz *et al*. 1997). For this reason it is usually assumed that the first limiting EAA is always present in the highest quantities in the animal's tissues (Cowey and Tacon, 1982; Benitez, 1989). In an effort to determine the first limiting EAA of *H. midae*, Britz *et al*. (1997) was unable to promote a growth response in juvenile *H. midae* when feeding graded levels of crystalline arginine in casein based diets. This lack of growth response may have been the result of a number of factors, and although it was not demonstrated conclusively, it was suggested that either arginine was not a limiting

EAA in the formulations, or alternatively that crystalline AAs were unsuitable for use in nutritional studies with abalone (Britz *et al*. 1997).

Britz *et al*. (1997) chose arginine for investigation in their study as this EAA is found in the highest concentrations in abalone tissues, and 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 EAA (Mai *et al.* 1994). However, this assumption may have been erroneous. This is because opposed to protein bound arginine, the relatively high levels of arginine found in abalone body tissues could be attributed to high levels of free arginine, stored as phosphoarginine and used as an energy metabolite (Tjeerdema *et al.* 1991). It was proposed that free arginine may account for up to double that of the protein bound arginine (Florkin and Bricteux-Gre´goire, 1972; Mai *et al.* 1994), and it is therefore plausible that relative to the other EAAs, the level of arginine revealed in body composition studies over estimates the dietary requirement for this EAA (Britz *et al*. 1997). Thus, it was hypothesised that arginine may not be the first limiting EAA in *H. midae*.

Shipton and Britz (2002) found a significant correlation between dietary lysine levels and growth ($r = 0.77$, $p = 0.0005$, $n = 16$) in *H. midae* fed diets containing combinations of whole proteins. It was concluded that combinations of fishmeal and plant protein sources created growth restricting lysine deficiencies within the formulations. These findings not only support the hypothesis that arginine is not the first limiting EAA in *H. midae* diets, but in addition indicate that lysine may be the first limiting EAA in formulated feeds for *H. midae* (Shipton and Britz, 2002).

Shipton *et al.* (2002) designed an experiment to determine whether lysine was the first limiting EAA in formulated feeds for *H. midae.* Accordingly, this experiment was designed to determine the lysine requirements for *H. midae* using cottonseed meal and fishmeal based diets supplemented with incremental levels of crystalline lysine. Cottonseed meal was chosen as the primary protein source because it contains a relatively low lysine content when compared to *H. midae* tissues (5.5 % and 7.9 % of the protein in cottonseed and abalone tissue, respectively). In addition, cottonseed meal has proved an effective protein source in semipurified diets for *H. midae* in previous studies (Shipton and Britz, 2001).

In common with other water soluble nutrients, leaching of crystalline AAs from diets prior to ingestion is of concern in an aquatic environment (Wilson, 1989; Lopez- Alvarado *et al*. 1994). As microencapsulation techniques have been successfully employed to restrict micronutrient leaching (Langdon and Siegfried, 1984; Fu-Lin *et al.* 1987; Lopez-Alvarado *et al.* 1994), and thus improve ingestion rates; two encapsulation techniques employing either a gelatine/acacia colloid (Keipert and Melegari, 1993) or cellulose acetate phthalate (Chen *et al*. 1992) were investigated (Shipton *et al.* 2002). However, irrespective of encapsulation technique used in the study, dietary lysine supplementation was unsuccessful in promoting a growth response in *H. midae*. The failure to produce a change in growth response may have been the result of a number of factors, and due to these factors it was not possible to verify that lysine is not a limiting EAA in *H. midae*. It was proposed that crystalline AAs are unsuitable for AA requirement studies in *H. midae* as leaching rates may have impacted on ingestion of the AA (Shipton *et al.* 2002). Crystalline AAs have been deemed unsuitable for use in diets for common carp (*Cyprinus carpio*; Aoe *et al.* 1970) as well as in the prawn

Palaemon serratus (Cowey and Forster, 1971) as they show high leaching rates when suspended in water.

It has been proposed that poly-L-lysine with a molecular weight of 7500 Dalton or higher may produce less leaching when extruded into diets as it is less soluble in water. However, poly-L-lysine is expensive (ZAR650-690 per 25mg, SIGMA-ALDRICH®). For this reason a commercially available, lipid microencapsulated form of L-lysine monohydrochloride, commercially traded as LysiPEARL™ (Kemin AgriFoods North America, Inc, 2100 Maury Street, Des Moines, Iowa, USA) was used for this research. The product was originally designed as a cost effective (ZAR50 per kg) source of rumen bypass and intestinally released lysine for use in dairy cattle, aimed at increasing milk yields over the long term.

Aims and objectives

This study was a continuation of previous research projects initiated by Rhodes University into the development of artificial feeds for the abalone *H. midae*. The primary aim of the study was to determine whether *H. midae* can assimilate commercially available LysiPEARL™, and whether the species' quantitative requirement for lysine could be determined by feeding graded levels of LysiPEARL™ in a semi purified diet.

The objectives were to:

1. Determine the leaching rate of lysine from LysiPEARL™ and establish if the rate of leaching differs at different levels of LysiPEARL™ inclusion;

2. determine if *H. midae* could assimilate LysiPEARL^{TM} by measuring for a growth response;
3. determine the ideal level of lysine in a LysiPEARL^{TM} diet by feeding graded levels of this product in six isoenergetic, isolipidic and isonitrogenous diets.

Methods and Materials

Experimental system

The experiment was conducted at the Port Alfred Marine Research Laboratory (33°45'S; 26°00'E). Water was drawn on the high tide from the mouth of the Kowie River estuary on the Indian Ocean. The indoor 10 000 L semi-recirculating system was employed for the duration of this experiment. Eighteen fibreglass tanks (60 x 60 x 30 cm, 108 L volume) were used in this experiment. Within each tank, randomly assigned groups of 20 animals were housed in perforated five litre plastic containers (Figure 1). Each container received water via a three millimetre aquarium hose (flow rate 500 ml/min, with one water exchange every 10 min) weighted by a stainless steel hex nut. Water circulation through the containers was maintained by the use of air stones positioned in the middle of the water column within each container (Figure 1). Water quality was maintained in the system using a 1000 l biological filter filled with plastic bio filter media, as well as the use of a 15 watt ultra violet light sterilizer (UltraZAP$^{®}$). Ten percent of the system volume was replaced per day by pumping water from the Kowie River on the high tide, maintaining salinity at 35±0.97 g/l. Temperature was maintained at 19±0.98°C through the use of a thermostatically controlled heater in the 1000 l settlement tank. A photoperiod of 12 h light and 12 h dark was used, and maintained using a timer switch.

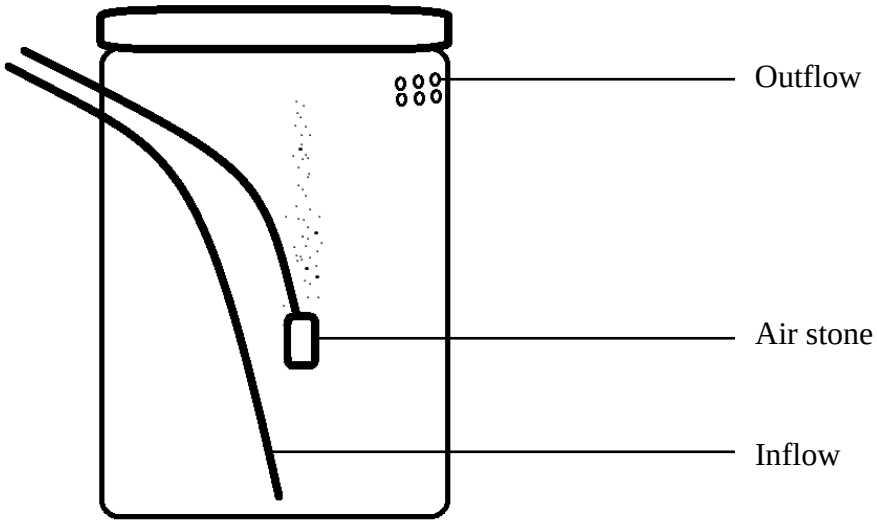

Figure 1: Illustration of the five litre containers in which abalone were housed.

Diet preparation

Six isonitrogenous (29.00 % crude protein) isolipidic (6.00 %) and isoenergetic (15.90 MJ/kg) basal diets were formulated. Cottonseed meal formed the bulk of the basal diet protein, and was supplemented with fishmeal in order to ensure that there was no deficiency in other EAAs (Table 1). The basal diet was supplemented with graded levels of LysiPEARL^{TM} (Kemin AgriFoods North America, Inc, 2100 Maury Street, Des Moines, Iowa, USA). LysiPEARL^{TM} is L-lysine monohydrochloride (50 %) suspended in vegetable oil using a unique MicroPEARL^{TM} brand spray freezing methodology. The LysiPEARL^{TM} was first sieved through a 2.00 mm sieve, followed by a 1.00 mm sieve to ensure that only particles ranging from 0.50 mm – 1.00 mm were used during extrusion. Supplemented lipid was reduced with the increase in LysiPEARL^{TM} supplementation in order to maintain total dietary lipid at 6.00 % (Table 2). The dry dietary ingredients were weighed to the nearest gram and were mixed with water to form a dough. LysiPEARL^{TM} was weighed (0.01 g) and added to the vegetable oil, which were subsequently homogenised with the other ingredients. Six formulations containing calculated lysine concentrations of 5.52 %, 6.40 %, 7.28 %, 8.14

%, 9.00 % and 9.86 % of dietary protein were cold extruded (Table 2) and dried at 38 ^{o}C for 10 h. From here on lysine content is presented as a percentage of protein.

Table 1: Amino acid (g/100 g of diet) profile of each test diet with graded levels of dietary lysine ranging from 5.52 to 9.86 % of the protein source.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Essential						
Arginine	3.12	3.11	3.20	3.14	3.18	3.13
Histidine	0.65	0.69	0.65	0.59	0.71	0.68
Isoleucine	0.82	0.73	0.89	0.82	0.93	0.84
Leucine	1.75	1.66	1.84	1.69	1.86	1.78
Lysine	1.50	1.54	2.00	2.20	2.51	2.64
Phenylalanine	1.29	1.19	1.36	1.24	1.34	1.30
Methionine	0.39	0.40	0.39	0.34	0.46	0.35
Threonine	0.88	0.83	0.89	0.90	1.01	0.94
Valine	1.15	1.03	1.21	1.10	1.27	1.17
Non-essential						
Aspartic acid	2.21	2.04	2.34	2.26	2.22	2.32
Serine	1.13	1.17	1.14	1.22	1.85	1.26
Glutamic acid	4.76	4.89	5.03	4.78	4.91	4.91
Proline	1.06	0.99	1.07	1.02	1.09	1.04
HO-Proline	0.12	0.10	0.12	0.11	0.11	0.12
Glycine	1.18	1.07	1.18	1.13	1.19	1.17
Alanine	1.14	1.11	1.18	1.10	1.32	1.12
Tyrosine	0.91	0.93	0.99	0.62	0.76	0.87

Table 2: Ingredient composition and calculated nutrient analysis of experimental diets (expressed as a dry weight basis).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Formulated						
Cotton meal	65.0	65.0	65.0	65.0	65.0	65.0
Fish meal	5.0	5.0	5.0	5.0	5.0	5.0
Starch	26.6	24.3	24.0	23.7	23.4	23.2
LysiPEARL™	0.0	0.5	1.1	1.6	2.2	2.7
Lipid added	6.6	4.2	3.9	3.7	3.4	3.1
Mineral/Vitamin mix	1.0	1.0	1.0	1.0	1.0	1.0
Total	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
Lipid (% of diet)	6	6	6	6	6	6
Protein (% diet)	29	29	29	29	29	29
Lysine (% protein)	5.52	6.40	7.28	8.14	9.00	9.86

Dietary lysine leaching

The methods according to Shipton *et al*. (2002) were followed in order to assess the efficacy of the encapsulation of LysiPEARL™ in reducing leaching of the lysine monohydrochloride within the micropearls from the pellets. The lysine content of each test diet was determined by a commercial laboratory (ARC Analytical Services Laboratory, Old Olifantsfontein Road, Irene, South Africa) prior to leaching (i.e. after zero hours leaching), as well as after a three and six hour period of immersion in seawater. For the two leaching trials (i.e. three or six hours of immersion), triplicate 1.00 g samples of each test diet were placed into perforated containers containing no abalone, and submerged in the experimental system for the leaching period. After the leaching period the samples were removed and oven-dried to constant weight (0.01 g) at 45°C.

In order to determine the rate at which supplemented lysine leached out of the diets over a six hour feeding period, one gram samples of each feed formulation were placed into 50 mL of seawater at 20°C, and the pH of the water monitored hourly for six hours using a pH-Conductivity meter (ExStik® EC500, FLIR Commercial Systems, Nashua, USA; Shipton *et al*. 2002). Lysine is a basic EAA (isoelectric point: 10.7), and as it leaches into the water it causes a rise in pH (Shipton *et al*. 2002). 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.

Experimental animals and experimental protocol

Three hundred and sixty hatchery-reared juvenile (20.41±1.95 mm) *H. midae* from the same spawning batch at Roman Bay Sea Farm (Pty) Ltd were used in the experiment. Prior to experimentation, the animals were acclimated to the system for two months, and fed exclusively on a commercial diet (Abfeed® S34 grade two weaning diet, Marifeed (Pty) Ltd, Hermanus, South Africa). Each dietary treatment was assigned to three randomly selected treatment containers of 20 abalone (i.e. three replicate groups of 20 animals per dietary treatment) and was fed the assigned diet for 90 days. Each replicate was fed one gram (calculated to be in excess) of assigned feed at 17:00 immediately after lights out, and the remaining feed was removed at 08:00 the following day, dried and weighed. The feed consumed over the experimental period was recorded and corrected for uneaten food and for solids lost to leaching. Correction factors for total solids leached were determined for each diet by placing one gram of feed in a control container containing no abalone, and placing it in the aquaria for 15 h. After the immersion period, the remaining feed was siphoned and oven dried at 25°C. Total solids leached were then calculated as dry weight loss over this period.

Weight and shell length measurements were measured at 30-day intervals throughout the experimental period. Prior to these measurements being taken, the animals were purged for 24 h to ensure that the penultimate meal did not affect the animal's weight. A 10 % (w/w) solution of magnesium sulphate was used to anaesthetize the animals prior to measurement. Weight was recorded to the nearest 0.01 g using an electronic balance and shell length to the nearest 0.01 mm using Vernier callipers.

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. The following growth indices were calculated according to Shipton *et al*. (2002). Daily feed consumption (% body weight per day) was calculated over the experimental period using the formula:

$$Consumption\ \%\ b.wt. = \frac{Cg}{Wt} \times 100$$

where Cg is the mean daily feed consumption corrected for leaching and Wt is the mean abalone weight at time t (days). Weights were calculated using the formula:

$$Wt = W0\ \times \left[\left(\frac{SGR}{100}\right) + 1\right]^{t-1}$$

where W0 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:

$$SGR = \left\{\frac{[\ln(Wf) - \ln(Wi)]}{t}\right\} \times 100$$

where SGR is the specific growth rate (% body weight increase per day), ln (Wf) is the natural log of the mean final weight of abalone, ln (Wi) is the natural log of the mean initial weight of abalone, and t is the time in days. The condition factors of the animals will be calculated according to Britz (1996) using the formula:

$$Condition\ factor = \frac{weight\ (g)}{length\ (mm)^{2.99}} \times 5575$$

Results

The rate of leaching of the crystalline lysine from the dietary formulations, as measured by an increase in pH of the leachate over a six hour feeding period, suggests that leaching fitted a linear model (Figure 2). There was no significant differences between the slopes of the regressions (ANCOVA $p>0.05$), and it was therefore concluded that lysine leaching rates between the dietary treatments did not differ significantly (Figure 3). Based on the assumptions that the abalone feed at a constant rate over six hours (Uki, 1981; Knauer *et al*. 1995b), and that leaching occurred at a constant rate, it was calculated that approximately 90 % of the supplemental lysine was ingested (Figure 2). Using Figure 2, the amount of lysine ingested by the abalone fed each of the experimental diets was calculated (Table 3).

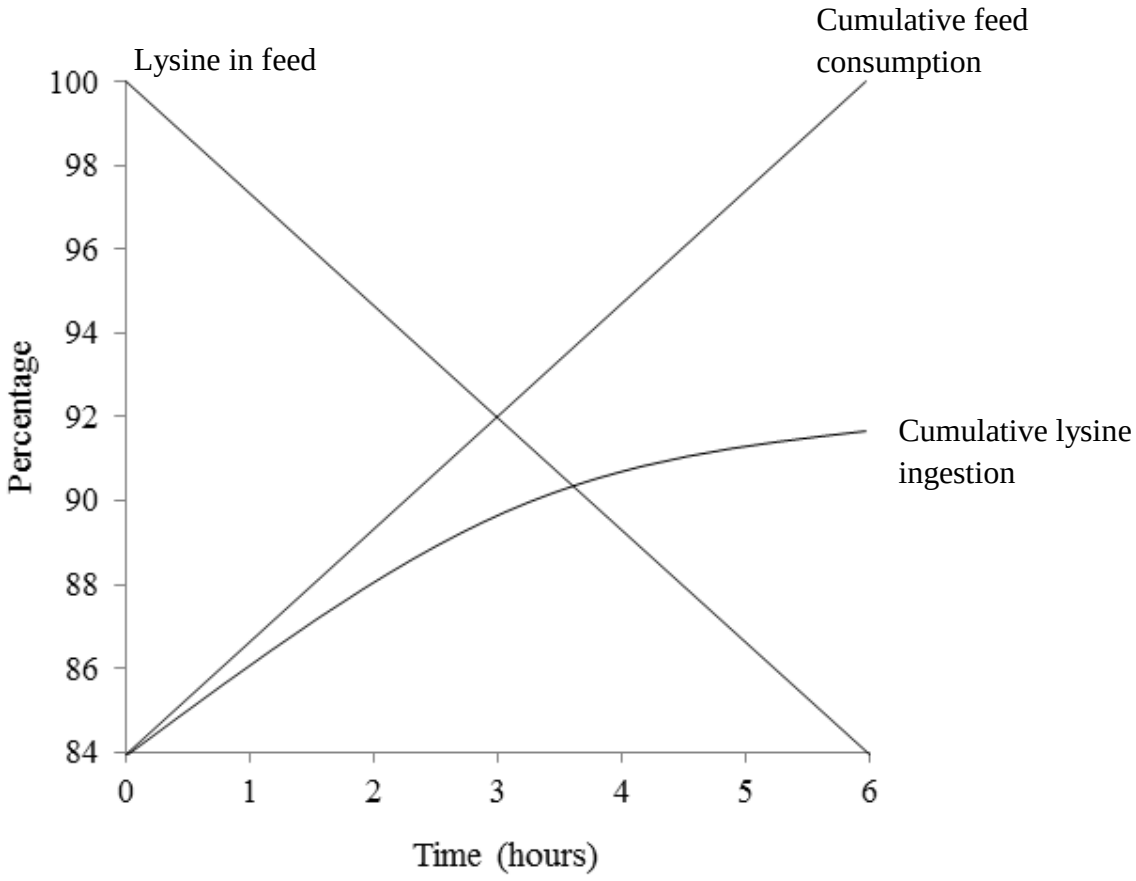

Figure 2: Estimate of the lysine ingestion by abalone over a six hour feeding period. Calculated as a product of feed consumption and the cumulative leaching of lysine from the feed.

Table 3: Dietary lysine concentrations as calculated and observed for the six dietary treatments. The effect of leaching on the observed concentrations and the estimated lysine ingestion based on the rate of leaching over a six hour feeding period (assuming roughly 10 % of supplemented lysine was leached – Figure 3).

	Lysine content (% of dietary protein)				
Diet	Calculated	Measured	After 3 h leaching	After 6 h leaching	Estimated lysine ingestion after 6 h of leaching
1	5.52	5.17	5.17	4.97	4.65
2	6.40	5.31	5.07	5.31	4.78
3	7.28	6.90	6.03	5.62	6.21
4	8.14	7.59	6.76	6.31	6.83
5	9.00	8.66	7.83	6.90	7.79
6	9.86	9.10	7.52	6.90	8.19

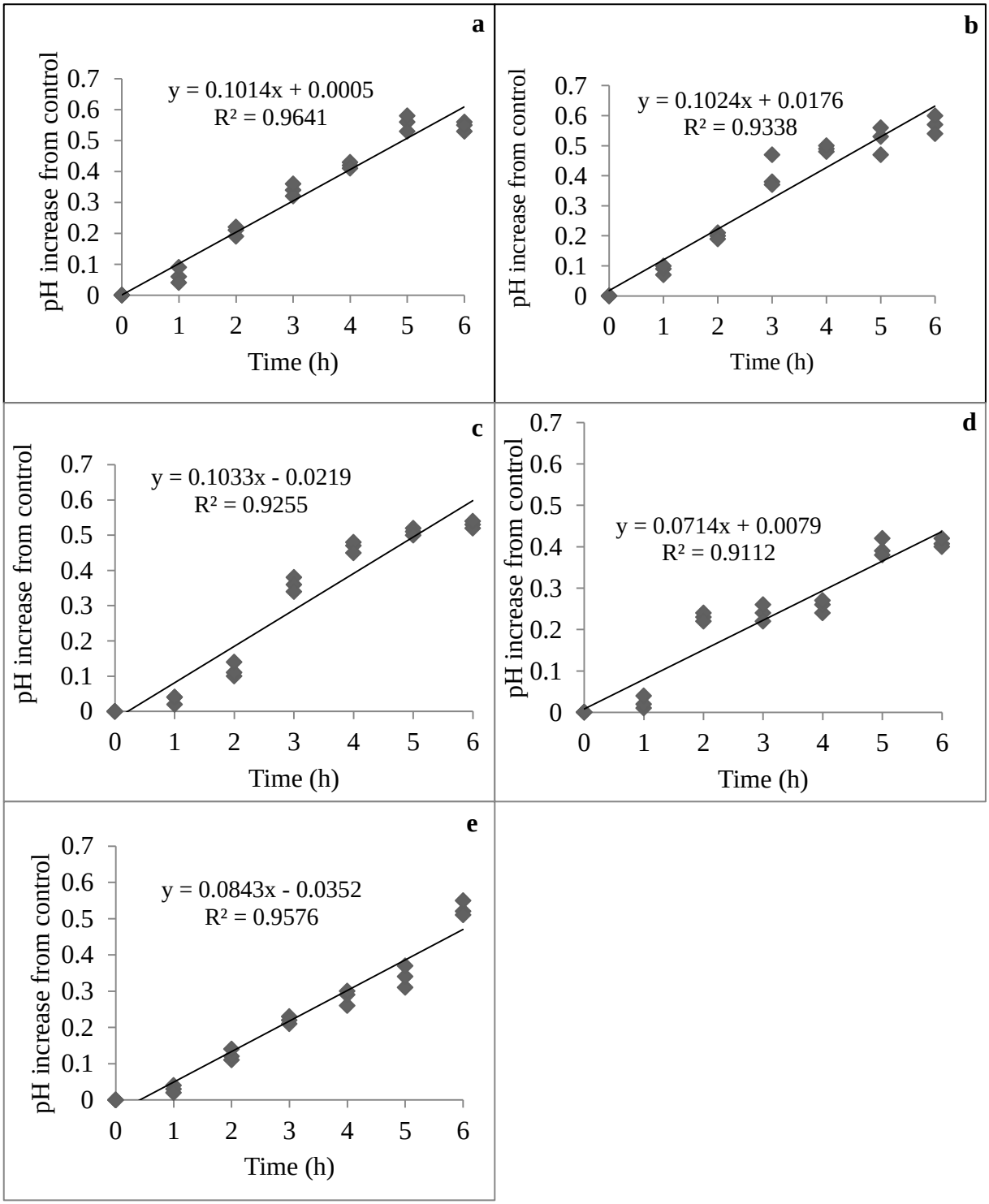

Figure 3: Regression models describing the leaching of lysine from the dietary formulations over a six hour period where a = diet 2, b = diet 3, c = diet 4, d = diet 5 and e = diet 6. Analyses of covariance revealed no significant difference between slopes (ANCOVA, $p>0.05$).

Across dietary treatments, mean increases in abalone weight over the experimental period were 147 % for diet 1, 153 % for diet 2, 167 % for diet 3, 157 % for diet 4, 157 % for diet 5 and 158 % for diet 6 (Table 4). Feed conversion ratio showed an increasing trend as dietary lysine was increased (Linear regression, $p=0.032$; Table 5). However, the FCR was significantly lower in abalone fed diet 3 (1.51±0.05), after which FCR increased significantly in abalone fed diet 4 (1.99±0.21; ANOVA, $p=0.028$; Figure 4). As dietary lysine increased across treatments, PER showed a decreasing trend (Regression analysis, $p=0.026$; Table 5). The PER was significantly higher in animals fed diet 3 (2.45±0.07) when compared to that of animals fed diet 4 (1.99±0.18; ANOVA, $p=0.015$; Figure 5). The animals fed diet 3 had a significantly higher SGR (0.57±0.01 % body wt.d^{-1}) when compared to animals being fed diet 1 (0.43±0.05 % body wt.d^{-1}; ANOVA, $p=0.025$; Figure 6). There were significant differences found in feed consumption (% body wt.d^{-1}) between dietary lysine treatments, with animals fed diet 4 and diet 6 consuming significantly more feed than animals fed diets one, two and three (ANOVA, $p<0.01$; Figure 7). Regression analysis showed feed consumption (% body wt.d^{-1}) increasing significantly as lysine concentration in the diet increased (Regression analysis, $p<0.001$, Table 5). There was no significant difference found in condition factor and mortality across dietary treatments (ANOVA, $p>0.05$; Table 4).

Table 4: Growth and nutritional indices of juvenile abalone fed graded levels of LysiPEARL^{TM}. Values are presented as means with standard errors. Different alphabetic superscripts indicate significant differences (ANOVA, $p<0.05$).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Initial length (mm)	21.56±2.16	20.62±1.87	19.87±1.82	20.02±1.85	20.34±1.77	20.03±1.72
Initial weight (g)	1.81±0.49	1.56±0.46	1.39±0.39	1.40±0.40	1.50±0.40	1.45±0.36
Final length (mm)	25.15±2.65	24.18±2.25	23.70±2.03	23.64±2.68	24.03±2.21	23.73±1.92
Final weight (g)	2.66±0.72	2.38±0.66	2.32±0.55	2.20±0.59	2.35±0.61	2.29±0.50
Condition factor (day 90)	0.96^{a}	0.97^{a}	1.00^{a}	0.96^{a}	0.97^{a}	0.98^{a}
Mortality (%)	0.05^{a}	0.03^{a}	0.00^{a}	0.05^{a}	0.06^{a}	0.06^{a}

Table 5: Linear regression analysis for feed consumption (% body wt.d^{-1}), feed conversion ratio (FCR), protein efficiency ratio (PER), condition factor (CF) and specific growth rate (SGR; (% body wt.d^{-1}) of abalone fed diets with a lysine content that ranged from 5.17 to 9.10 as a measured percentage of the total protein, which was 29 % (Regression analysis, $p<0.05$).

Parameter	Model	R^2	$F_{(1,16)}$	p
Feed consumption	y = 0.0451x + 0.384	0.705	38.29	<0.001
FCR	y = 0.0715x + 1.254	0.257	5.54	0.031
PER	y = - 0.0851x + 2.736	0.271	5.93	0.027
CF	N/A	0.021	0.56	0.565
SGR	N/A	0.149	2.82	0.113

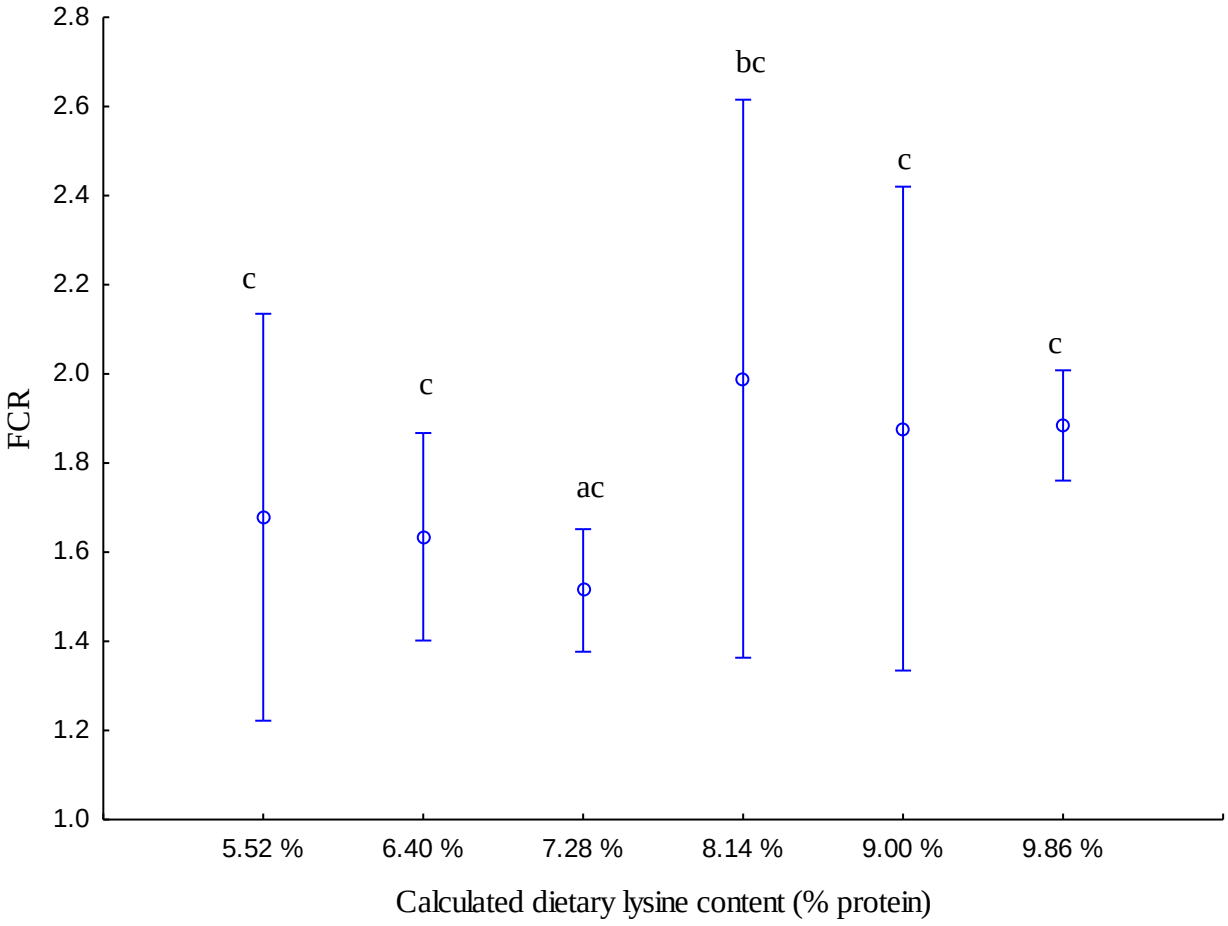

Figure 4: The mean (±95 % confidence intervals) feed conversion ratio (FCR) across dietary treatments. Different alphabetical superscripts denote significant differences between treatment means (ANOVA, $F_{(5, 12)}$=3.72, p=0.028).

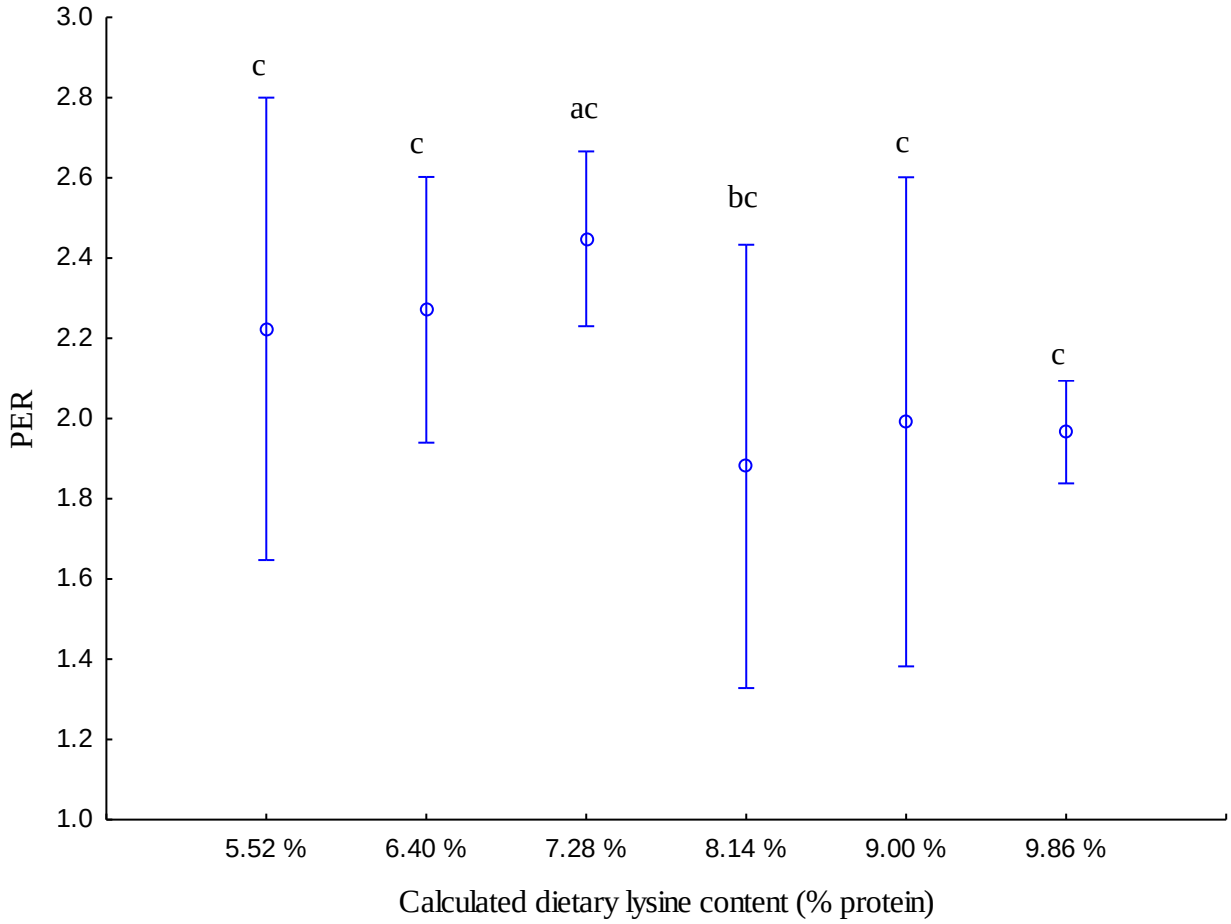

Figure 5: The mean protein efficiency ratio (PER) (±95 % confidence intervals) across dietary treatments. Different alphabetical superscripts denote significant differences between treatment means (ANOVA, $F_{(5, 12)}$=4.47, p=0.015).

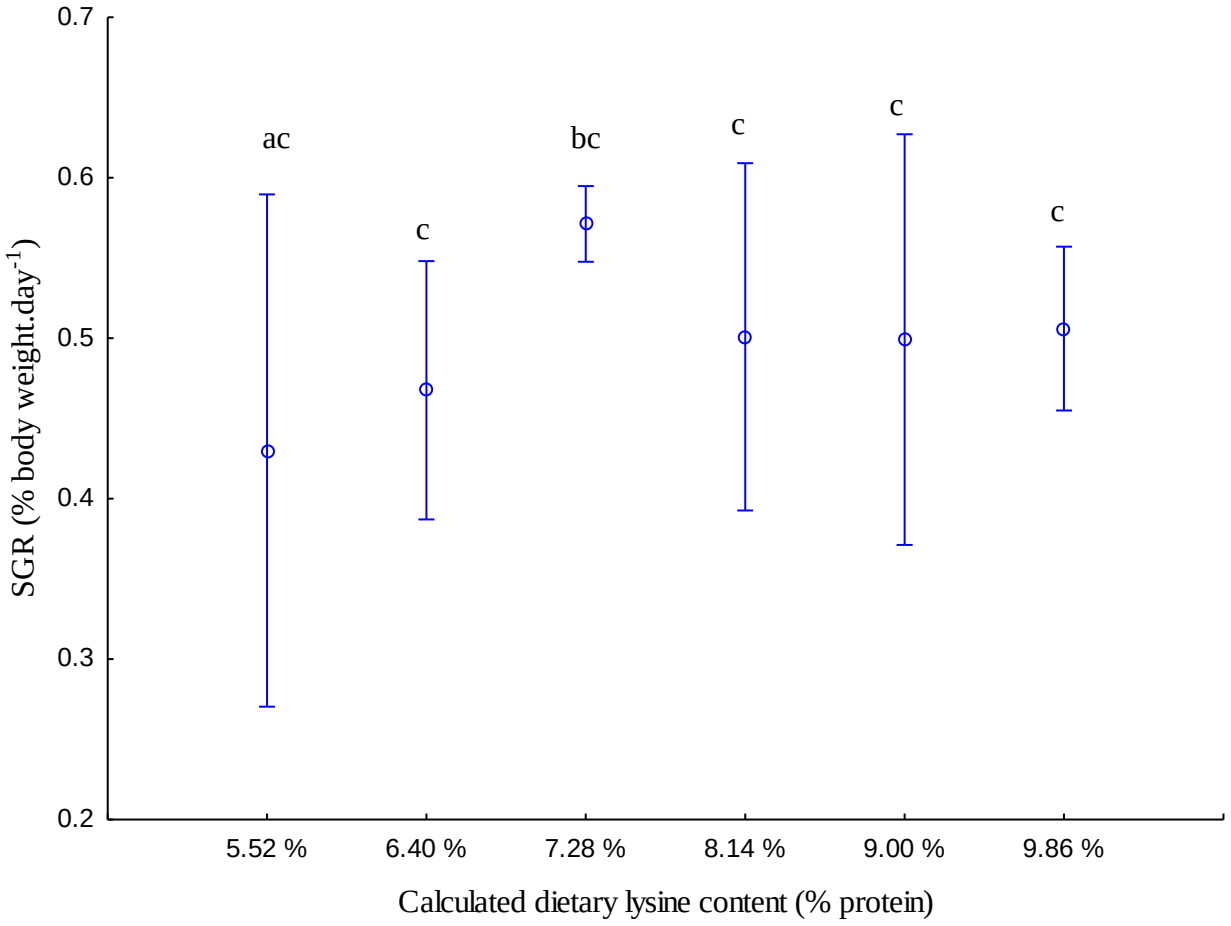

Figure 6: The mean specific growth rate (SGR) (±95 % confidence intervals) across dietary treatments. Different alphabetical superscripts denote significant differences between treatment means (ANOVA, $F_{(5, 12)}$=3.85, p=0.025).

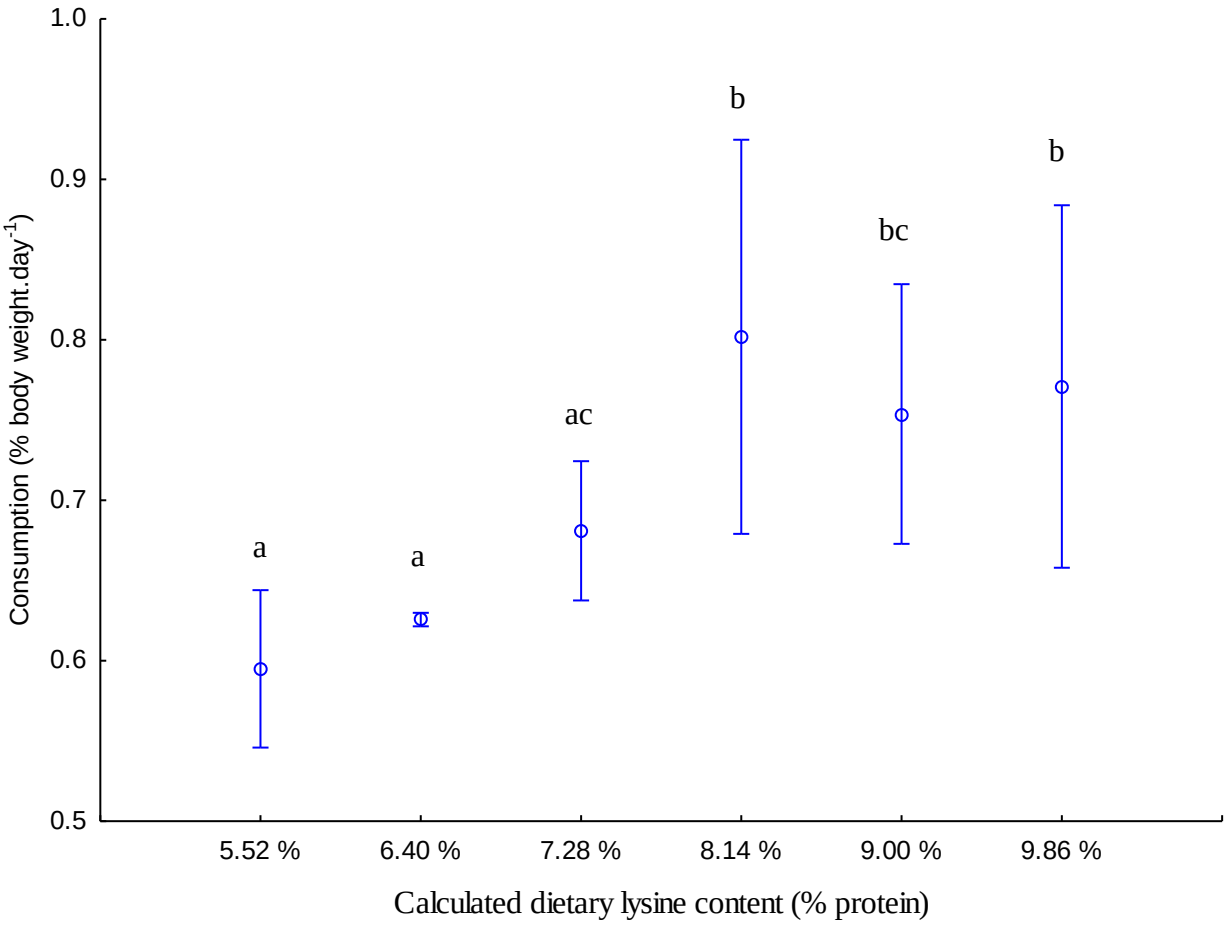

Figure 7: The mean feed consumption (% body wt.d^{-1}) (±95 % confidence intervals) across dietary treatments. Different alphabetical superscripts denote significant differences between treatment means (ANOVA, $F_{(5, 12)}$=20.14, $p<0.05$).

Discussion

The results of this study corroborate the results of Britz *et al*. (1997) where it was suggested that arginine may not be the first limiting EAA in artificial feeds for *H. midae*. The success of diet supplementation with crystalline lysine promoting a significant growth response in this study, suggests that lysine may be the first limiting EAA in the species when fed a formulated diet with cotton seed and fishmeal as the protein sources.

It has previously been thought that crystalline AAs are not suitable for AA studies in *H. midae* (Britz *et* al. 1997; Shipton *et al*. 2002), common carp (Aoe *et al*. 1970) and other invertebrate species (Cowey and Forster, 1971; Deshimaru and Kuroki, 1974, 1975; Deshimaru, 1982). The poor utilization of crystalline AAs in these studies may be due to the differential rates of absorption between supplemented crystalline AAs and the natural protein bound AAs (Cowey and Walton, 1988). A difference in absorption rates would result in different times of 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 AAs in abalone. However, as Mai *et al*. (1994) demonstrated that dietary AA 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 AAs could clarify this issue. Results from this study show that if an effective encapsulation technique is used, it is possible to maintain crystalline AAs within the diets for a sufficient time for slow feeding animals such as abalone.

The availability and digestibility of AAs and other micronutrients can vary greatly between different dietary ingredients and protein sources (Sales and Britz, 2001). Therefore it cannot be assumed that all of the supplemented lysine in LysiPEARL^{TM} as well as protein bound lysine from the cottonseed meal and fishmeal of these diets was digested and converted into somatic growth. For example, the lysine requirement of juvenile yellowtail changed from 4.13 % to 3.85 % of the dietary protein when the lysine availability of the protein sources was considered (Ruchimat *et al.* 1997). The diet producing the best SGR, PER and FCR in this study was diet 3 which had an analysed lysine content of 6.90 %. This

is relatively high when compared to findings of other AA requirement studies in species such as Japanese flounder (*Paralichthys olioaceus*) where best results were found from 4.20 - 4.60 % dietary protein (Forster and Ogata, 1998), 4.30 4.40 % dietary protein for red sea bream (*Pagrus major*; Forster and Ogata, 1998) and 5.30 % dietary protein in juvenile cobia (*Rachycentron canadum*; Zhou *et al*. 2007). This draws into question the possibility that lysine availability in LysiPEARL^{TM} may have resulted in an overestimation of the lysine requirement, as the before mentioned fish species are carnivorous, and have substantially lower lysine requirements than herbivorous *H. midae* if the results from this study are accepted the way they are. However, if it is assumed that only a portion of the lysine in LysiPEARL^{TM} is available to the abalone, the actual lysine requirement will be lower than that represented in the diet, and may in fact show a better representation of the lysine requirement in *H. midae*. A hypothetical example: If 50 % of the lysine in LysiPEARL^{TM} in the diet that produced the best growth (6.90 %) was available to the abalone to digest, coupled with lysine digestibility data of feed ingredients from Sales and Britz (2001) where 86.34 % of lysine in fishmeal, 85.12 % of lysine in cotton meal and 84.84 % of lysine in the starch was available to digest, then the amount of lysine available to the animals is calculated as 6.57 % of dietary protein. The digestibility of LysiPEARL and the subsequent availability of lysine when this product is included in abalone feeds needs to be determined in future research. Only then will it be possible to empirically determine the dietary lysine requirement for the South African abalone.

The assessment of lysine leaching from the test diets suggest that leaching was not a factor in the ability of lysine supplementation to promote growth across all diets, as roughly 90 % of the supplemented lysine was in the pellet after three hours of leaching. It has previously been shown that retention of low molecular weight FAAs bound in microcapsules

of lipid is generally low when these diets are placed in water. It has been shown that micro bound diets containing free crystalline AAs can lose up to 80% of their AAs within the first minute of suspending the feed particles in seawater (Lopez-Alvarado and Kanazawa 1993). In contrast, the diets in this study only lost roughly 10 % of their supplemented lysine within the first three hours of suspension in seawater, implying that the abalone in all of the dietary treatments were effectively supplemented with graded levels of lysine, with 90 % of the supplemented lysine remaining in the pellet at the peak of their six hour feeding period. It has been shown that abalone feeding behaviour is highest roughly three hours after sunset (Lloyd, 2013). Once leaching had been taken into consideration, the amount of lysine ingested by abalone fed the six lysine enriched diets was calculated to range between 4.65 – 8.19 % dietary protein during this six hour feeding period.

An additional source of lysine loss and errors in the subsequent ingestion estimations could have occurred during the feeding process (Shipton *et al*. 2002). During abalone feeding, the animals rasp small particles of feed from the surface of the pellet (approx. 0.50 mm^2; Shipton *et al*. 2002). It has been shown that additional losses (in addition to leaching) of supplemented AAs (Mai *et al.* 1998) and digestibility markers (Sales and Britz, 2001) can occur during feeding due to the nature in which abalone rasp off particles of feed. The particle size of supplemented LysiPEARL™, and composition of the supplemented lysine is of concern in over estimating lysine requirement in this experiment using juvenile abalone. Whilst the LysiPEARL™ was sieved down to particles between 0.50 mm – 1.00 mm in diameter, there might have been additional loss of LysiPEARLS™ when the pellets were ingested that was not accounted for, thus over estimating the level of lysine ingested and therefore dietary requirement. Perhaps using larger abalone which are able to ingest larger

particles during feeding, would allow for more accurate predictions of lysine requirement when using LysiPEARL™ as the lysine source.

LysiPEARL™ is suspended in vegetable oil during the manufacturing process. Unlike most mammals and fish, abalone have a limited ability to digest high levels of lipid and store them as an energy source for later use (Britz *et al*. 1996). It has been shown that lipid levels above 7.00 % significantly decrease the growth of abalone and for *H. midae*, an inclusion level of 10.00 % has shown to be detrimental to the growth of the animal as well as increase mortality rates (Geen *et* al. 2011; Mai *et al*. 1995b; Britz and Hect 1997). Furthermore, lipid levels present in natural alga are very low (generally < 2.00 % of dry weight) (Webber, 1970), and for this reason lipase activity is low in abalone. Therefore their ability to digest lipid and use it as an energy source is limited as they rely on carbohydrate as their main source of energy (Uki and Watanabe 1992, Britz *et al*. 1994, Fleming *et al*. 1996, Knauer *et al*. 1996). Instead, abalone rely on proteins and carbohydrates to fuel metabolism (Vosloo and Vosloo 2010; Green *et al*. 2011). Whilst lipid levels were maintained at 6.00 % of diet by adjusting lipid addition with increasing LysiPEARL™, abalone could only have access to lysine supplementation once the lipid walls of the LysiPEARL™ were broken down, and abalone are not efficient at breaking down lipid (Uki and Watanabe 1992, Mai *et al*. 1995, Britz 1995). This may also have inflated predictions of optimum dietary lysine inclusion. The next phase of this research needs to investigate the digestibility of LysiPEARL™ so as to determine the portion of lysine that becomes available to the abalone after digestion. This will make it possible for one to use these data to more accurately predict the lysine requirement in *H. midae*.

There was a significant difference in the SGR of animals fed diet 1 which had an actual (as determined by the ARC) lysine content of 5.17 % and those fed diet 3 with an actual lysine content of 6.90 %, with animals being fed the 6.90 % dietary ration growing significantly faster. After this initial increase in growth witnessed between the animals fed 5.17 % and 6.90 % diets, there was no significant growth response in diet 4, five and six containing actual lysine contents of 7.59 %, 8.66 % and 9.10 % respectively. This result, coupled with the results from the regression analysis, indicates that an increase of dietary lysine promotes a growth response in juvenile *H. midae* to a point, after which supplementation fails to promote any significant growth. Similar results have been found in turbot (*Scophthalmus maximus*; Peres and Olivia-Teles, 2008), where there was a significant increase in weight gain between a diet with a lysine content of 2.50 % (of protein) and a diet with a lysine content of 4.60 %. After the initial significant increase in growth between these diets, there was no significant increase in growth when lysine was increased further (5.20 %, 5.80 % and 6.40 %) (Peres and Olivia-Teles, 2008). This suggests that similar to turbot (Peres and Olivia-Teles, 2008), abalone in this study may have met their dietary requirement for lysine when being fed diets containing 6.90 % dietary lysine, after which excess supplementation promotes no significant growth response.

There were also significant differences in PER and FCR in the animals fed diets containing lysine levels of 7.59 % when compared to animals fed diets containing lysine levels of 6.90 %, with animals fed 6.90 % performing significantly better in both indices. This might be because arginine and lysine are basic EAAs, sharing a common pathway for transport through the intestinal brush border (Vilella *et al*. 1990), and excess lysine may have had an effect on arginine uptake. Arginine is involved in many metabolic processes such as protein synthesis, urea production, metabolism of glutamic acid and proline, and the synthesis

of creatine and polyamines (Kaushik *et al*. 1988). Arginine has also been shown to have effects on the release of growth hormones as well as play an important role in enhancing immune resistance (Field *et al*. 2002; Wu *et al*. 2004). The AA is also used as an oxidative energy substrate, necessary for nitric oxide synthesis, elevating growth hormone levels, and as a substrate for polyamine synthesis, all of which are vital for healthy growth (Mommsen, 2001). If there was an excess of lysine absorbed in abalone fed the diet containing 7.59 % lysine, arginine may not have been absorbed in sufficient quantities, therefore resulting in a significant decrease in PER and increase in FCR between 6.90 % and 7.59 %. The arginine/lysine interaction was not tested in this work, but it is hypothesised that this might be a possible reason for the results that were observed here.

A deficiency in any AA will generally lead to a decrease in weight gain and loss of appetite (Cowey, 1979). Therefore, in order to maximize growth, the diet should contain balanced amounts of high quality protein; this can only be met when the protein in the diet contains a balanced EAA profile. The diets used in this experiment were isonitrogenous, isolipidic and isoenergetic, with the basal protein of cottonseed meal being supplemented with a small amount of fishmeal in order to maintain good levels of other AAs, with only lysine being increased across the dietary formulations (Table 1). Since all of the dietary treatments in this study had similar EAA balances, and the concentrations of lysine never exceeding that of arginine, the significant differences in PER, FCR and SGR witnessed in this study were probably not attributed to a deficiency in any of the other EAAs.

Lysine is a potent inhibitor of the arginine catabolizing enzyme arginase (Abidi and Khan, 2009). The differences in SGR in diets containing 5.17 % dietary lysine and diets

containing 6.90 % dietary lysine, after which SGR levelled off, and the significant decrease in PER, and increase in FCR between diets containing 6.90 % dietary lysine and diets containing 7.59 % dietary lysine may be due to antagonistic effects of lysine on arginine. Antagonism of arginine by lysine has been reported by others in Atlantic salmon (*Salmo salar*) (Berge *et al*. 1999, Berge *et al*. 2002) and poultry (Jones, 1964; Jones *et al*. 1967). A reduction in the utilisation of arginine as a result of high dietary lysine may be due to interactions between the two EAAs at a metabolic or an absorptive level, or both (Berge *et al*. 1999). In *in vivo* studies with poultry fed unbalanced dietary arginine and lysine, absorptive interrupting interactions were suggested when plasma levels did not reflect the AA profile of the diet (Jones, 1964, Jones *et al*. 1967). In fish, the interaction between arginine and lysine is not as clear, but plasma levels of free arginine and lysine do not seem to be affected by dietary levels in experiments with juvenile hybrid striped bass (Griffin *et al*. 1994) and rainbow trout (Barash, 1984, Kaushik and Fauconneau, 1984). Plasma AA concentrations are generally transient in nature and can be affected by factors such as feed intake, time of sampling, and metabolism (Berge *et al*. 1999). Dietary pH (Nose *et al*. 1974, Wilson *et al*. 1977) as well as electrolyte balance in the gut (Murai *et al*. 1983) has been shown to affect crystalline AA utilization in some species of fish. However, as there is no information concerning these effects on crystalline AA utilization in abalone, it is not possible to evaluate their influence in the current experiment, and clarification must await further investigation.

Blood plasma levels of Atlantic salmon are affected by relative amounts of dietary arginine and lysine (Berge et al. 1997, 1998). However, it has been shown in channel catfish fingerlings, that blood plasma arginine and lysine were only recorded in the fish when they were fed unbalanced lysine and arginine levels (Robinson *et al*. 1981). In this study abalone blood plasma was not examined but, all of the diets had a relatively similar arginine and

lysine ratio (Table 1), with arginine being present in slightly higher concentrations across all of the test diets. The lack of significant increase in SGR after 6.90 % and significant decrease in PER between 6.90 % and 7.59 % can therefore not be attributed to excess lysine in the diets reducing arginine absorption.

Irrespective of the actual dietary lysine requirement of *H. midae*, the results from this study allow current feed formulators to produce plant meal and fishmeal basal diets that would otherwise be fairly low in lysine content, and supplement them with a commercial form of encapsulated lysine that is inexpensive, without having a negative impact on growth. The average increase in shell length in animals fed diet 3 was 1.30 mm per month where the trial was run in an indoor, recirculating laboratory system. Growth rates in abalone of a similar size class (around 20 mm SL) on commercial farms that are being fed diets containing high proportions of fishmeal are currently 1.90 mm per month. The diets used in this study had a comparatively low proportion of fishmeal making up the basal protein, with the main component being made up of cottonseed meal (Table 2). For every 13 g of cottonseed meal, one gram of fishmeal was added in the test diets used in this experiment. It has previously been shown that replacing fishmeal with 50% cottonseed meal can result in significant loss in growth (Shipton and Britz, 2001). This decline in growth rate may have been due to a lysine deficiency in the test diets. The promising growth rates that were being witnessed in this diet containing low proportions of fishmeal with lysine supplementation highlights the potential to replace fishmeal with plant proteins.

The annual production of cottonseed is more than six million tons in China (Luo *et al*. 2006). It has been used in diets for both terrestrial animals and fish due to its high protein content and low cost (Yue and Zhou, 2008). Several studies have been conducted to

determine the amount of cottonseed meal that can be incorporated into tilapia diets without negative effects on growth (Jackson *et al*. 1982; Viola and Zohar, 1984; El-Sayed, 1987, 1990; Mbahinzireki *et al*. 2001; El-Saidy and Gaber, 2004). Results have indicated that the amount of cottonseed meal that can be included in an aquafeed depends mainly on the levels of gossypol, cyclopropionic acids, and available lysine (Luo *et al*. 2006,1982; El-Saidy and Gaber, 2004). Planting glandless varieties of cotton can directly decrease the amount of gossypol content of the seed meal (Luo *et al*. 2006). A relatively new method called solvent extracted cottonseed meal has shown advances in detoxifying, as well as improving protein and AA quality of cottonseed meal (Luo *et al*. 2006). The prospects for testing new diets with only plant proteins are promising in haliotid nutrition.

The other potential concern when formulating diets devoid of fishmeal, and with high proportions of cottonseed meal as the main basal protein is the resulting low concentrations of lysine. However, this can be addressed through supplementation with other lysine rich plant proteins such as soya bean meal. Soya bean meal has high lysine concentrations (3.43 % of dry weight) relative to other plant proteins when compared to fishmeal (5.87 %) (Sales, 2001). However, lysine availability suggests that more lysine is available to *H. midae* for digestion from soya bean meal (roughly 98.84 %) when compared to fishmeal (roughly 86.34 %; Sales and Britz, 2001), however fishmeal has nearly double the amount of lysine in soya bean meal. However, a study on juvenile rainbow trout showed that it was possible to completely replace fishmeal with cottonseed meal and soya bean meal and not have significant impacts on growth indices (Lee *et al*. 2002). Thus further research should look at determining dietary lysine requirements in *H. midae* using combinations of whole proteins with known availabilities of lysine, rather than supplementing whole proteins with

encapsulated FAAs where the availability due to lipid encapsulation or losses of entire microcapsules may overestimate the actual dietary requirement.

Irrespective of the actual dietary lysine requirement of *H. midae*, if diets formulated with cottonseed and fishmeal as the basal proteins are fed to abalone, dietary lysine levels should be supplemented with LysiPEARL^{TM} such that an analysed content of roughly 6.90 % lysine makes up the total dietary protein. From here it may also be possible to determine the dietary requirement for the nine other EAAs using soft tissue EAA ratios.

In conclusion, it was shown that there was no significant difference in the leaching rate of the LysiPEARL^{TM} at the different levels of inclusion in the diets. Results show that abalone can assimilate LysiPEARL^{TM} when fed diets containing fishmeal and cottonseed meal as the basal protein. However, it is difficult to give the species quantitative requirement for lysine, as we do not know the digestibility of LysiPEARL^{TM}, but these results have demonstrated the ideal LysiPEARL^{TM} inclusion rate and this has narrowed the quantitative requirement to a range between 6.00 – 7.00 % of the total dietary protein. Future work into the digestibility of LysiPEARL will make it possible to pin the lysine content requirement within the range presented here.

CHAPTER 3

Estimating the essential amino acid requirements of juvenile *Haliotis midae*, using soft tissue essential amino acid ratios.

Introduction

Protein in the form of fishmeal is the most expensive dietary ingredient in aquaculture feed formulations (Miles *et al*. 2006). Animals generally do not have a requirement for a certain protein, but instead have a requirement for specific amino acids (AA) that make up dietary protein (Shipton *et al*. 2002). Abalone, like all other animals, have the same requirement for 10 essential amino acids (EAAs), including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Allen and Kilgore, 1975). There is a growing literature base defining the availability of EAAs from both single or mixed protein sources (Fleming *et al*. 1998; Van Barneveld *et al*. 1998; Sales and Britz, 2001). Furthermore, it is also generally accepted that the AA profile of an animal's' tissue gives an indication of the relative proportions of the EAAs required in the diet (Cowey and Tacon, 1982; Benitez, 1989).

Growth can be defined as the total accretion of AAs, with the AA profile of the animal's tissue bearing a close similarity to the AA profile of the diet of the animal (Fuller *et al*. 1987). The AA profiles of a number of haliotids have been determined (Allen and Kilgore,

1975; Knauer *et al.* 1995a; King *et al*. 1996). Whilst these findings have shown us the specific AAs within the abalone, and the levels at which they are found in the body of the animal, it is difficult to transfer these data directly to artificial feed formulations (Shipton *et al*. 2002). This is because EAA profiles from wild abalone may not directly translate to optimal growth rates within commercial facilities as diets are often different according to species as well as the habitat type in which they feed. The EAA profiles of abalone tissue may give us the relative balance of EAAs but no information on the rate at which they are used. Therefore, a specific EAA could be low in the body tissue, suggesting a low requirement, but if it is used a lot in metabolic processes the requirement rate will be high and therefore there is a higher dietary requirement than what the EAA profile of the tissue explains. Another important factor that needs to be noted is that growth in commercial facilities is faster when compared to wild growth rates. Therefore the EAA acid profile of animals in commercial facilities may be different to that of wild animals.

Whilst it has been suggested that an animal's AA requirement might be deduced from the AA profile of its tissues (King *et al*. 1996), they do not supply nutritionists with the absolute dietary requirement of the animal. It is thus important to find out the quantitative EAA requirement of haliotids, in order for the EAA availability and digestibility data of different dietary ingredients supplied by Fleming *et al*. (1998), Van Barneveld *et al*. (1998) and Sales and Britz (2001) to be utilized effectively. It will then be possible to design diets that provide the optimal balance and inclusion rates of EAAs to the animals.

Many studies have used dose response studies to determine the requirement of a culture species for each individual EAA. However, these are costly and time consuming studies as

each EAA requires a separate experiment (Small and Soares, 1998). An alternative approach has been devised to determine the requirements of an animal for all 10 EAAs simultaneously (Wilson and Poe, 1985). This method is termed the soft tissue essential amino acid ratio (A/E) and can be calculated once the quantitative requirement of a single EAA has been determined using a dose-response study. The A/E ratio methodology has been utilised to effectively determine the EAA requirement of multiple finfish aquaculture species including channel catfish (Wilson and Poe, 1985), common carp (Cowey and Tacon, 1985), coho salmon (Arai, 1981), cherry salmon (Ogata *et al.* 1983), red drum (Moon and Gatlin, 1991), clarias hybrid (Unprasert, 1994), striped bass (Brown, 1995), European seabass, gilthead seabream, turbot (Kaushik, 1998), red sea bream (Forster and Ogata, 1998), bluegill (Masagounder *et al*. 2009) and dusky kob (Adesola, 2015).

Knowing the EAA requirement in *H. midae* will allow for a more appropriate diet formulation allowing for a reduction in the formulation cost, more efficient EAA utilisation as well as reduction in nutrient waste and environmental impact resulting in less ingredients being wasted and money spent on water treamtent. Due to the success of LysiPEARL^{TM} producing a significant growth response in *H. midae*, and the subsequent determination of a lysine requirement, it may be possible to predict the requirement of the species requirement for the remaining nine EAAs using the A/E ratio method.

Aims

Therefore, the aim of this study was to apply the known whole-body AA composition of juvenile *H. midae* (Knauer *et al*. 1994a), and to draw an estimate of their EAA

requirements, based on the relative proportion of EAAs found in the whole soft body tissues and to compare the AA requirements of *H. midae* to other haliotids.

Methods and Materials

This study applied the methodology proposed by Wilson and Poe (1985) and Wilson (1991) where the concept of an ideal protein balance is proposed. This method is based on the idea that there is a correlation between the whole body AA pattern and the dietary AA requirement in animals (Agricultural Research Council, 1981). Based on this theory, Wilson and Poe (1985) and Wilson (1991) introduced a new method for estimating the AA requirement in fish nutrition, where only the lysine requirement is determined from protein accretion during feeding experiments and the other nine EAA requirements are estimated as being proportional to the soft tissue EAA composition pattern including lysine, which is normally the first limiting EAA in most feedstuffs.

Soft tissue essential amino acid content

The AA profiles of juvenile *H. midae* soft tissue determined by Knauer *et al*. (1994a) were used for this experiment. These were compared with the soft tissue AA profiles of *Haliotis rufescens* (Allen and Kilgore 1975), *Haliotis rubra* (King *et al*. 1996), *Haliotis tuberculate* and *Haliotis discus hannai* (Mai *et al*. 1994).

Calculations of lysine availability from feed ingredients

The true availability of lysine for the ingredients used in the test diets were determined using data from (Sales and Britz, 2001) where:

$$True\ lysine\ availability\ (\%)$$

$$= \left\{\left(\frac{concentration\ of\ lysine\ in\ feed}{concentration\ of\ marker\ in\ feed}\right)\right.$$

$$- \left[\left(\frac{concentration\ of\ lysine\ in\ faeces}{concentration\ of\ marker\ in\ faeces}\right)\right.$$

$$\left.\left. - \left(\frac{g\ metabolic\ lysine\ per\ 100g\ feed}{concentration\ of\ marker\ in\ feed}\right)\right]\right\}$$

$$\div \left\{\left(\frac{concentration\ of\ lysine\ infeed}{concentration\ of\ marker\ in\ feed}\right)\right\}$$

Three different hypothetical scenarios of lysine availability of LysiPEARL^{TM} from the diet that produced the best growth rate in chapter two (diet 3 6.90 % of protein) were then coupled with the known lysine availability of the other dietary components in order to calculate the estimated requirements of the other EAAs. These included three hypothetical levels of lysine availability from LysiPEARL^{TM}, including 100 % availability, 75 % availability and 50 % availability.

Calculations of essential amino acid requirement

The whole-body essential AA profile of *H. midae* determined by Knauer *et* al. (1994a) was used to calculate the A/E ratio, which is defined as the content of each EAA divided by the sum of all EAAs, including cysteine and tyrosine, multiplied by 1000 (Wilson and Poe, 1985).

$$A/E\ ratio = \frac{individual\ EAA\ content\ in\ soft\ tissue}{total\ EAA\ content\ (including\ cysteine\ and\ tyrosine)} \times 1000$$

The estimated requirement for each of the other EAAs, besides lysine was calculated using the following equation (Wilson and Poe, 1985; Wilson, 1991):

$$EAA\ requirement = \frac{(requirement\ for\ lysine\ \times specific\ A/E\ ratio)}{A/E\ ratio\ for\ lysine}$$

Three different EAA requirement profiles are given according to the three different theoretical lysine availability indices of LysiPEARL^{TM}. A recommended balance of dietary EAAs according to lysine in *Haliotis midae* and *Haliotis rubra* were compared.

Results

-

The most predominant of the EAAs in *H. midae* were arginine, leucine and lysine. These results are similar to those found in other haliotid species such as *Haliotis rubra*, *Haliotis rufescens, Haliotis tuberculuata* and *Haliotis discus hannai* (Table 6). Of these AAs, arginine was the most abundant across all of the abalone species, with leucine the second, and lysine the third most abundant. However, these AAs were found to be consistently higher in *H. discus hannai*. Tryptophan was found to be the least abundant in *H. midae* and *H. rubra* tissues.

The three different scenarios of lysine availability (of diet 3 6.90 %) from LysiPEARL^{TM} and the known availability of the other dietary components from Sales and Britz (2001) are presented (Table 7).

The proposed balances of EAAs relative to lysine in diets for *H. midae* according to three different scenarios of lysine availability from LysiPEARL^{TM} are shown in Table 7. Despite differences in the EAA composition of *H. midae* and *H. rubra* muscle tissues, the proportion of AAs recommended in the diet is relatively similar for both species (Table 9). Recommendations for the EAA requirements of the abalone can be based upon these.

Table 6: Comparison of the essential amino acid (EAA) composition of the soft tissues of *Haliotis midae*, *Haliotis rufescens*, *Haliotis rubra*, *Haliotis tuberculate and Haliotis discus hannai*. Amino acids are represented as % protein.

EAA	*H. midae* [a]	*H. rufescens* [b]	*H. rubra* [c]	*H. tuberculate* [d]	*H. discus hannai* [d]
Arginine	7.91	7.26	9.03±0.47	8.01±0.21	9.39±0.13
Histidine	1.81	1.97	1.78±0.13	1.87±0.13	2.04±0.09
Isoleucine	4.11	3.79	3.62±0.21	4.86±0.26	4.90±0.17
Leucine	6.93	7.86	6.13±0.31	7.55±0.17	7.93±0.25
Lysine	6.21	6.00	5.30±0.26	6.56±0.21	6.97±0.15
Methionine and Cysteine	3.44	3.84	3.38±0.21	3.63±0.14	3.34±0.12
Threonine	4.99	4.64	4.13±0.26	4.43±0.18	6.66±0.07
Phenylalanine and Tyrosine	7.71	7.90	6.00±0.25	7.65±0.17	7.65±0.08
Tryptophan	0.82		0.93±0.06		
Valine	4.61	4.73	4.23±0.21	5.13±0.09	5.27±0.11

[a] Knauer *et al*. (1994a)
[b] Allen and Kilgore (1975)
[c] King *et al*. (1996)
[d] Mai *et al*. (1994)

Table 7: Dietary ingredients and the respective availability of lysine, based on three theoretical scenarios of lysine availability of LysiPEARL™ from diet 3 (measured 6.90%). Numbers in bold and marked with a * are the lysine concentrations used for estimation of other essential amino acids.

Ingredient	g/100g of test diet	g lysine in dietary formulation	True lysine availability (%)	Lysine availabile (g)
Cotton meal	65.00	1.20	85.12	1.02
Fishmeal	5.00	0.33	86.34	0.29
Starch	23.99	0.24	84.84	0.20
LysiPEARL™	1.08	0.54	100.00; 75.00; 50.00	0.54; 0.41; 0.27
Lipid in LysiPEARL™	0.54	0.00	0.00	0.00
Lipid	3.93	0.00	0.00	0.00
Mineral/Vitamin mix	1.00	0.00	0.00	0.00
			Sum lysine (g) (100 % availability)	2.04
			Sum lysine (% protein)	**7.06***
			Sum lysine (g) (75 % availability)	1.91
			Sum lysine (% protein)	**6.40***
			Sum lysine (g) (50 % availability)	1.78
			Sum lysine (% protein)	**6.13***

Table 8: Estimated essential amino acid (EAA) (% of dietary protein) requirement for juvenile *Haliotis midae* assuming different theoretical availabilities of lysine in LysiPEARL™ and the calculated amino acid (A/E) ratios. Numbers in brackets represent lysine requirement as a percentage of protein.

EAA	A/E ratio	EAA requirement if 50% lysine in LysiPEARL™ available (6.13 %)	EAA requirement if 75% lysine in LysiPEARL™ available (6.40 %)	EAA requirement if 100% lysine in LysiPEARL™ available (7.06 %)
Arginine	162.96	7.81	8.41	8.99
Histidine	37.29	1.79	1.92	2.06
Isoleucine	84.67	4.06	4.37	4.67
Leucine	142.77	6.84	7.37	7.88
Lysine	127.94	6.13	6.40	7.06
Methionine and Cysteine	70.87	3.40	3.66	3.91
Threonine	102.80	4.93	5.30	5.67
Phenylalanine and Tyrosine	158.84	7.61	8.19	8.77
Tryptophan	16.89	0.81	0.87	0.93
Valine	94.97	6.65	4.90	5.24

Table 9: Recommended ratio of essential amino acids (EAA; % protein) relative to lysine in *Haliotis midae* and *Haliotis rubra* diets.

EAA	*H. midae*	*H. rubra* [a]
Arginine	1.27	1.70
Histidine	0.29	0.34
Isoleucine	0.66	0.70
Leucine	1.12	1.16
Lysine	1.00	1.00
Methionine and Cysteine	0.55	0.65
Threonine	0.80	0.80
Phenylalanine and Tyrosine	1.24	1.13
Tryptophan	0.13	0.18
Valine	0.74	0.80

[a] King *et al* (1996)

Discussion

Arginine was found to be in the highest levels in all of the studied haliotid species. It is therefore easy to assume that arginine may be the first limiting EAA in haliotids when looking at body composition data. However, it has been found that abalone tissue contains a large pool of free arginine, roughly double that of protein bound arginine (Florkin and Bricteux-Gregoire, 1972; Mai *et al*. 1994). Free arginine is generally cycled within the body as phosphoarginine and is used as a phosphogen (Britz *et al*. 1997). This raises the possibility that the high proportion of arginine, relative to other EAAs, in abalone tissue does not provide an indication of the dietary requirement, as was hypothesised in other studies (Cowey and Tacon, 1982; Mai *et al*. 1994; Britz *et al*. 1997;). Thus the assumption that arginine was the first limiting EAA may be erroneous. This conclusion is supported by the failure of supplemental arginine to boost *H. midae* growth rates (Britz *et al*. 1997).

Leucine was found to be the second most abundant EAA across all of the studied haliotid species. In a study on post-larval Senegalese sole, it was found that leucine diet supplementation in order to balance EAAs, resulted in high retention rates of arginine within the body, and did not increase leucine catabolism (Aragao *et al*. 2004). This effect has also been observed in young pigs (Kim *et al*. 1983). The high leucine level witnessed in haliotid tissues, and the relationship it has in arginine retention, may explain the reason why arginine levels are generally high in haliotid species. This supports the hypothesis that when the AA profile of a test diet are adequately balanced, an increase in AA retention and a decrease in the catabolic losses of AAs can be observed (Aragao *et al*. 2004). The increase in AA retention is probably reflected in an increase in protein retention and therefore an increase in protein accretion and somatic growth (Aragao *et al*. 2004). In future it may be beneficial to

increase the concentration of this AA within diets to test the hypothesis and find evidence of arginine and lysine antagonism.

However, it has been shown that if the concentration of branch chained AAs (valine, isoleucine and leucine) is increased to dietary excess, antagonistic effects may be witnessed in retention of other EAAs (D'Mello, 2003). Antagonistic effects caused by an excess of one of the branch chained AAs in monogastric and preruminant terrestrial animals have been shown. These effects generally result in reduced feed consumption and decreased concentrations of the other two branch chained AAs in the tissues (D'Mello, 2003). In studies on the AA requirements of finfish, it has been shown that an excess of dietary leusine or isoleucine depressed the growth rates of chinook salmon (Chance *et al*. 1964), and common carp (Nose, 1979). A study on lake trout showed that growth depression witnessed in lake trout due to high levels of leusine, could be mitigated if concentrations of valine were increased through supplementation (Hughes *et al*. 1984). Similar relationships have been noted between histidine and phenylalanine. When post larval Senegalese sole were fed a diet with a histidine deficiency, phenylalanine oxidation was elevated (Aragao *et al*. 2004). However, when the dietary concentration of histidine was increased, phenylalanine catabolism decreased to a minimum (Aragao *et al*. 2004). These results highlight the importance of having balanced EAA profiles within diets, especially once limiting AAs have been identified.

Modeling EAA requirement via the A/E method is considered an effective and accurate technique for establishing an ideal dietary protein profile for an aquaculture species (Wilson and Poe, 1985). However, due to the lipid encapsulation of LysiPEARL^{TM}, uncertainties are raised concerning the lysine availability of the product. It is therefore difficult to specify the

exact quantities of each EAA in an ideal diet. Further work where these three formulations are used in a growth trial may reveal true availability of lysine in LysiPEARL™ for *H. midae* and in doing so, provide ideal concentrations of other EAAs required in the diet.

The data from the present study suggests that *H. midae* and *H. rubra* require similar ratios of EAA relative to lysine in their diets. This similarity indicates that two species may have comparable AA requirements for somatic growth. No literature describing the quantitative EAA requirements of any of the other haliotid species was found during the compilation of this thesis. Therefore, success in determining the EAA requirement in *H. midae* may represent a significant step in haliotid nutrition across species.

CHAPTER 4

Concluding remarks

The potential to replace fishmeal with plant protein sources in commercial diets for *Haliotis midae* is promising. Nevertheless, prior to commercialization, a cost-benefit analysis must be undertaken to determine the economic viability of these formulations. It is clear that the quality of a protein for use in aquafeeds for different candidate species is highly dependent on the protein having the correct balance of amino acids (AA) for that candidate species. Understanding both the AA requirement of *H. midae* as well as the availability of AAs in different protein sources is not only important for optimising dietary formulations, but also to farm managers, as different dietary proteins can have different effects on water quality (Sales and Britz, 2001).

In summary the most effective means of reducing feed costs is through improved diet development, by more effective use of available AAs in feed ingredients, and to match them with the requirements of the animal in the correct essential amino acid ratios. An AA may be present in an ingredient, but with no nutritional value if it is unavailable in the correct ratios to the animal (Akiyama, 1991), or if it is made unavailable through the excess provision of another AA. For example, by precisely measuring the availability of essential nutrients in feed ingredients, coupled with determining specific AA requirements, it was possible to completely eliminate fish meal from poultry feed formulations (Scott *et al*. 1982).

Although it is evident that a number of outstanding issues must be addressed before the exact lysine requirement of *H. midae* can be demonstrated empirically, the results of the present study suggest that it ranges between 6.00 - 7.00 % of dietary protein. Nevertheless

since the availability of the lysine in the LysiPEARL^{TM} has not yet been established, these results need to be treated with some caution. However, it has been shown conclusively that LysiPEARL^{TM} can be used to supplement diets that are low in protein bound lysine. In order to gain a full understanding of the lysine requirement in *H. midae*, future research could focus on dose response studies with alternative methodologies such as post-prandial tissue or serum FAA studies (Harding *et al*. 1977; Robinson *et al*. 1981; Ruchimat *et al*. 1997) or serum oxidation studies (Brookes *et al*. 1972) or alternatively determining the digestibility of LysiPEARL^{TM} and conduct another dose response experiment.

Due to the issues of digestibility surrounding crystalline essential amino acid (EAA) encapsulation in lipids, a further alternative would be to vary the EAA levels in abalone diets through appropriate mixtures of whole proteins, whilst ensuring the availability of other EAAs are kept constant. For example, Andrews *et al*. (1977) increased the level of dietary arginine fed to channel catfish by substituting gelatin for casein in a semi-purified diet, and observed increased growth rates as a result. Another option would be to use crystalline lysine with a heavier molecular weight. For example, poly-L-lysine can have a molecular weight of 70 000-150 000 dalton, and therefore have a reduced solubility in water.

Success in determining the EAA requirements of the species will represent a major technical achievement in haliotid nutrition, and one that is necessary if diets that provide the optimum balance of EAAs to the animals are to be developed whilst decreasing the sectors dependence on fishmeal.

References

Abidi S, Khan M. 2009. Dietary arginine requirement of fingerling Indian major carp, *Labeo rohita* (Hamilton) based on growth, nutrient retention efficiencies, RNA / DNA ratio and body composition. *Journal of Applied Ichthyology* 25: 707-714.

Agricultural Research Council. 1981. Protein and amino acid requirements in "The nutrient requirements of pigs", Commonwealth Agricultural Breaux, Slough, England, 1981, pp. 67-124.

Allen WV, Kilgore J. 1975. The essential amino acid requirements of the red abalone, *Haliotis rufescens. Comparative Biochemistry and Physiology* 50A: 771-775.

Aoe H, Toyoda T, Kitamura S. 1970. Nutrition of protein in young carp. I. Nutrative value of free amino acids. *Bulletin of the Japanese Society of Scientific Fisheries* 39: 407-413.

Aragao C, Conceicao L, Martins D, Ronnestad I, Gomes E, Dinis M. 2004. A balanced dietary amino acid profile improves amino acid retention in post-larval Senegalese sole (*Solea senegalensis*). *Aquaculture* 233: 293-304.

Arai S. 1981. A purified test diet for coho salmon, *Oncorhynchus kisutch*, fry. *Bulletin of the Japanese Society of Scientific Fisheries* 47(4): 547-550.

Ayres D. 2013. Effect of diet and sex-sorting on growth and gonad development in farmed South African abalone, *Haliotis midae*. MSc thesis, Rhodes University, South Africa.

Barash H. 1984. The influence of the lysine level in the diet on nitrogen excretion and the concentration of ammonia and free amino acids in the plasma of rainbow trout *Salmo gairdneri*. *Nutrition Reports International* 29: 283-289

Benitez LV. 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 Fisheries Society Special Publication* 4: 23-25.

Berge GE, Lied E, Sveier H. 1997. Nutrition of Atlantic salmon *Salmo salar*. The requirement and metabolism of arginine. *Comparative Biochemistry and Physiology* 117A: 501-509.

Berge GE, Lied E, Sveier H. 1998. Nutrition of Atlantic salmon *Salmo salar*. The requirement and metabolic effect of lysine. *Comparative Biochemistry and Physiology* 120A: 477-485.

Berge G, Bakke-McKellep A, Lied E. 1999. In vitro uptake and interaction between arginine and lysine in the intestine of Atlantic salmon (*Salmo salar*). *Aquaculture* 179: 181-193.

Berge G, Sveier H, Lied E. 2002. Effects of feeding Atlantic salmon (*Salmo salar* L.) imbalanced levels of lysine and arginine. *Aquaculture Nutrition* 8: 239-248.

Britz PJ, Hecht T, Knauer J and Dixon M. 1994. The development of an artificial feed for abalone farming. *South African Journal of Science* 90: 7-8.

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

Britz PJ. 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 PJ, Hecht T, Knauer J. 1996. Gastric evacuation time and digestive enzyme activity in abalone *Haliotis midae* fed a formulated diet. *South African Journal of Marine Science* 17: 297-303.

Britz PJ, 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 IM, Oxens FN, Garrigus US. 1972. Influence of amino acid oxidation by the rat. *Journal of Nutrition* 102: 27-36.

Brown PB. 1995. Using whole-body amino acid patterns and quantitative requirements to rapidly develop diets for new species such as striped bass (*Morone saxatilis*). *Journal of Applied Ichthyology* 11: 342-346.

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

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

Cook P. 2014. The Worldwide Abalone Industry. *Modern Economy* 5: 1181-1186.

Coote TA, Hone PW, Kenyon R, Maguire GB. 1996. The effect of different combinations of dietary calcium and phosphorus on the growth of juvenile *Haliotis laevigata*. *Aquaculture* 145: 267-279.

Cowey CB, Forster JR. 1971. The essential amino acid requirements of the prawn *Palaemon serratus*. The growth of prawns on diets containing proteins of different amino acid compositions. *Marine Biology* 10: 77-81.

Cowey CB, Tacon AGJ. 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 CB, Walton MJ. 1988. Studies on the uptake of 14C amino acids derived from both 14C protein and dietary 14C amino acids by rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* 33: 293-305.

Costa-Pierce BA, Bartley DM, Hasan M, Yusoff F, Kaushik SJ, Rana K, Lemos D, Bueno P, Yakupitiyage A. 2012. Responsible use of resources for sustainable aquaculture. In: Subasinghe RP, Arthur JR, Bartley DM, De Silva SS, Halwart M, Hishamunda N, Mohan CV, Sorgeloos P (eds), *Farming the Waters for People and Food. Proceedings of the Global Conference on Aquaculture 2010, Food and Agriculture Organization of the United Nations (FAO),* Rome. pp 113-148.

Dabrowski K, Poczyczywski P, Kock G, Berger B. 1989. Effect of partially or totally replacing fishmeal protein by Soya bean 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.

Department of Agriculture, Forestry and Fisheries. 2012. Marine Aquaculture Annual Farm Operations Report 2010.

El-Saidy DMSD, Gaber MMA. 2004. Use of cottonseed meal supplemented with iron for detoxification of gossypol as a replacement of fish meal in Nile tilapia, *Oreochromis niloticus* (L.) diets. *Aquaculture Research* 35: 859-869.

El-Sayed A-FM. 1987. Protein and energy requirements of *Tilapia zillii*. PhD Thesis, Michigan State University, East Lansing, MI, USA. 147 pp.

El-Sayed A-FM. 1990. Long-term evaluation of cottonseed meal as a protein source for Nile tilapia *Oreochromis niloticus* (Linn.). *Aquaculture* 84: 315-320.

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

FIN. 2005. *Fishmeal and fish oil facts and Figures*. Fishmeal Information Network. 33pp.

Fleming AE, Van Baneveld RJ, Hone PW. 1996. The development of artificial diets for abalone : a review and future directions. *Aquaculture* 140: 5-53.

Fleming AE, Van Barneveld RJ, Hone PW, Vandepeer ME, Kruck JA. 1998. Complimentary additivity of the digestibility coefficients of feed ingredients fed to juvenile greenlip abalone (*Haliotis laevigata*). *Journal of Shellfish Research* 17(3): 641-647.

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

Forster I, Ogata H. 1998. Lysine requirement of juvenile Japanese flounder *Paralichthys olioaceus* and juvenile red sea bream *Pagrus major*. *Aquaculture* 161: 131-142.

Forster I, Ogata H. 2007. Lysine requirement of juvenile Japanese flounder *Paralichthys olioaceus* and juvenile red sea bream *Pagrus major*. *Aquaculture* 161: 131-142.

Fuller MF, Wang TC. 1987. Amino acid requirements of the growing pig. In: APSA Committee (Editors), Manipulating Pig Production. Australasian Pig Science Association, Werribee, Australia. pp. 97-111.

Garland CG, Cooke SL, Grant JF, McMeekin TA. 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. *Journal of Experimental Marine Biological Ecology* 91: 137-149.

Griffin ME, Wilson KA, Brown PB. 1994. Dietary arginine requirement of juvenile hybrid striped bass. *Journal of Nutrition* 124: 888-893.

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

Gordon HR, Cook PA. 2013. World Abalone Supply, Markets, and Pricing: 2011 Update. *Journal of Shellfish Research* 32: 5-7.

Green AJ, Jones CLW, Britz PJ. 2011a. The protein and energy requirements of farmed South African abalone *Haliotis midae* L. cultured at optimal and elevated water temperatures. *Aquaculture Research* 42: 1653-1663.

Green AJ, Jones CLW, Britz PJ. 2011b. Effect of lipid level on growth and feed utilization in South African abalone *Haliotis midae* L.fed diets with a constant protein-to-energy ratio. *Aquaculture Research* 42: 1501-1508.

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

Hahn KO. 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 KO. 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 KO. 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.

Harding DE, Allen OW, Wilson RP. 1977. Sulfur amino acid requirements of channel catfish: L-methionine and L-cystine. *Journal of Nutrition* 107: 2031-2035.

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

Hecht T, Jones CLW. 2009. Use of wild fish and other aquatic organisms as feed in aquaculture – a review of practices and implications in Africa and the Near East. In: Hasan MR and Halwart M (eds). *Fish as feed inputs for aquaculture: practices, sustainability and implication. FAO Fisheries and Aquaculture Technical Paper No. 518. Rome* 518: 129-157.

Ino T. 1952. Biological studies on the propagation of the Japanese abalone (genus *Haliotis*). *Bulletin Tokai Regulation of Fisheries Research Laboratory* 5: 1.

Jackson AJ, Capper BS, Matty AJ. 1982. Evaluation of plant proteins in complete diets for tilapia, *Sarotherodon mossambicus*. *Aquaculture* 27: 97-109.

Jones JD. 1964. Lysine–arginine antagonism in the chick. *Journal of Nutrition* 84: 313-321.

Jones JD, Petersburg SJ, Burnett PC. 1967. The mechanism of the lysine–arginine antagonism in the chick: effect of lysine on digestion, kidney arginase, and liver transamidinase. *Journal of Nutrition* 93: 103-116.

Kaushik SJ. 1998. Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. *Aquactic Living Resources* 11(5): 355-358.

Kaushik SJ, Fauconneau B. 1984. Effects of lysine administration on plasma arginine and on some nitrogenous catabolites in rainbow trout. *Compendium of Biochemical Physiology* 79A: 459-462.

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

Kim K, McMillan I, Bayley HS. 1983. Determination of amino acid requirements of young pigs using an indicator amino acid. *British Journal of Nutrition* 50: 369-382.

King RH, Rayner CJ, Kerr M, Gorfine HK, McShane PE. 1996. The composition and amino acid balance of abalone (*Haliotis rubra*) tissue. *Aquaculture* 140: 109-113.

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

Knauer J, Hecht T, Britz P. 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.

Lloyd K. 2013. A preliminary look at the feeding behaviour of farmed abalone, *Haliotis midae* L, fed a formulated feed and the effect of stocking density on this behaviour. Honours thesis, Rhodes University, South Africa.

Lopez-Alvarado J, Kanazawa A. 1993. Utilization of crystalline amino acids by Japanese flounder larvae, *Paralichthys olivaceus* (Temminck & Schlegel), fed on zein-microbound diets. In: World Aquaculture '93, Torremolinos, Spain, 1993, European Aquaculture Society, Special Publication no. 19. pp. 410.

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

Luo L, Xue M, Wu X, Cai X, Cao H, Liang Y. 2006. Partial or total replacement of fishmeal by solvent extracted cottonseed meal in diets for juvenile *Oncorhynkus mykiss*. *Aquaculture Nutrition* 12: 418-424.

Mai K, Mercer JP, 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 JP, 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 JP, 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.

Masagounder K, Firman JD, Hayward RS, Sun S, Brown PB. 2009. Apparent digestibilities of common feedstuffs for bluegill and largemouth bass using individual test ingredients. *Aquaculture Nutrition* 15: 29-37.

Mbahinzireki GB, Dabrowski KJ, EL-Saidy D, Wisner ER. 2001. Growth, feed utilization, and body composition of tilapia (*Oreochromis* sp.) fed cottonseed meal based diets in a recirculating system. *Aquaculture Nutrition* 7: 189-200.

McBride SC. 1998. Current status of abalone aquaculture in the Californias. *Journal of Shellfish Research* 17(3): 593-600.

McLean N. 1970. Digestion in *Haliotis rufescens* Swainson (Gastropoda: Prosobranchia). *Journal of Experimental Zoology* 173(3): 303-318.

Moon HY, Gatlin DM. 1991. Total sulphur amino acid requirement of juvenile red drum, *Sciaenops oscillates*. *Aquaculture* 95: 91-106.

Murai T, Hirasawa Y, Akiyama T, Nose T. 1983. Effects of dietary pH and electrolyte concentration on utilisation of crystalline amino acids by fingerling carp. *Bulletin of Japanese Society of Scientific Fisheries* 49: 1377-1380.

Nie ZQ. 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. *Bulletin of Japanese Society of Scientific Fisheries* 40: 903-908.

Ogata H, Arai S, Nose T. 1983. Growth responses of cherry salmon *Oncorhynchus masou* and amago salmon *O. rhodurus* fry fed purified casein diets supplemented with amino acids. *Bulletin of the Japanese Society of Scientific Fisheries* 49: 1381-1385.

Peres H, Olivia-Teles A. 2008. Lysine requirement and efficiency of lysine utilisation in turbot (*Scophthalmus maximus*) juveniles. *Aquaculture* 275: 283-290.

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

Robinson EH, Wilson RP, Poe WE. 1981. Arginine requirement and apparent absence of a lysine–arginine antagonism in fingerling channel catfish. *Journal of Nutrition* 111: 46-52.

Ruchimat T, Masumoto T, Hosokawa H, Itoh Y, Shimeno S. 1997. Quantitative lysine requirement of ye1lowtail (*Seriola quinqueradiata*). *Aquaculture* 158: 331-339.

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

Sales J. 2001. Nutrient Digestibility in the South African abalone *Haliotis* midae. PhD Thesis, Rhodes University, South Africa.

Sales J, Britz PJ. 2001. Research on abalone (*Haliotis midae* L.) cultivation in South Africa. *Aquaculture Research* 32: 863-874.

Sales J, Britz PJ. 2003. Apparent and true availability of amino acids from common feed ingredients for South African abalone (*Haliotis midae* L.). *Aquaculture Nutrition* 9: 55-64.

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.

Scott ML, Nesheim MC, Young RJ. 1982. Nutrition of the Chicken, 3rd edn. M.L. Scott and Assoc., Ithaca, NY. 562 pp.

Shipton T, Britz P, Walker R. 2002. An assessment of the efficacy of two lysine microencapsulation techniques to determine the quantitative lysine requirement of the South African abalone , *Haliotis midae* L. *Aquaculture nutrition* 8: 221-227.

Small B, Soares J. 1998. Estimating the quantitative essential amino acid requirements of striped bass *Morone saxtillis*, using fillet A/E ratios. *Aquaculture nutrition* 4: 225-232.

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

Tarr RJQ. 1992. The abalone fishery of South Africa. In: Shepard SA, Tegner MJ, Guzman del Proo SA (eds), *Abalone of the World. Biology, Fisheries and Culture.* Fishing News Books, London. pp 438-447.

Taylor B. 1992. Abalone nutrition : Optimal protein level in artificial diets for *Haliotis kamtschatkana*. *Journal of Shellfish Research* 11: 556.

Ten-Doeschate KI, Coyne VE. 2008. Improved growth rate in farmed *Haliotis midae* through probiotic treatment. *Aquaculture* 284: 174-179.

Troell M, Robertson-Andersson D, Anderson RJ, Bolton JJ, Maneveldt G, Halling C, Probyn T. 2006. Abalone farming in South Africa: An overview with perspectives on kelp resources, abalone feed, potential for on-farm seaweed production and socio-economic importance. *Aquaculture* 257: 266-281.

Tuerjischer E, Wertheimer E. 1941. Glycogen and Adipose Tissue. *Journal of Physiology* 100: 385-409.

Tjeerdema RS, Kauten RJ, Crosby DG. 1991. Sublethal effects of hypoxia in the abalone (*Haliotis rufescens*) as measured by in vivo 31PNMR spectroscopy. *Comparative Biochemistry and Physiology* 100B: 653-659.

Uki N, Kemuyama A, Watanabe T. 1985b. Development of semipurified test diets for abalone. *Bulletin of the Japanese Society of Scientific Fisheries* 51: 1825-1833.

Uki N, Watanabe T. 1986. Effect of heat treatment of dietary protein sources on their protein quality for abalone. *Bulletin of the Japanese Society of Scientific Fisheries* 52: 1199-1204.

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. Guzman del Próo (Eds), *Abalone of the World. Fisheries, Biology and Culture*, Fishing News Books, Oxford, UK, pp. 504-517.

Unprasert NG. 1994. An evaluation of the ideal protein concept to estimate essential amino acid requirements in the Clarias hybrid (*Clarias macrocephalus* × *Clarias gariepinus*). PhD dissertation, Mississippi State University.

Van Barneveld RJ, Fleming AE, Vandepeer ME, Kruk JA, 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*). *Journal of Shellfish Research* 17(3): 649-655.

Vilella S, Ahearn GA, Cassano G, Maffia M, Storelli C. 1990. Lysine transport by brush border membrane vesicles of eel intestine:interaction with neutral amino acids. *American Journal of Physiology* 259: R1181-R1188.

Viola S, Zohar G. 1984. Nutrition studies with market size hybrids of tilapia *Oreochromis* in intensive culture. *Bamidgeh* 36: 3-15.

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. *Bulletin of the Japanese Society of Scientific Fisheries* 58(10): 1991-2000.

Vosloo D, Vosloo A. 2010. Response of cold-acclimated, farmed South African abalone (*Haliotis midae*) to short-term and long-term changes in temperature. *Journal of Thermal Biology* 35: 371-323.

Webber HH. 1970. Changes in metabolite composition during the reproductive cycle of abalone *Haliotis cracheroidii* (Gastropoda: Prosobranchiata). *Physiological Zoology* 43: 213-231.

Wilson RP. 1991. Amino acid nutrition of fish: A new method of estimating requirement values. 12th U.S.-Japan Symposium on Aquaculture Nutrition, Newport, Oregon, Oct. 1991. pp. 49-54.

Wilson RP, Harding DE, Garling DL. 1977. Effect of dietary pH on amino acid utilization and the lysine requirement of fingerling channel catfish. *Journal of Nutrition* 107: 166-170.

Wilson RP, Poe WE. 1985. Relationship of whole body and egg amino acid patterns to amino acid requirement patterns in channel catfish (*Ictalurus punctatus*). *Comparative Biochemistry and Physiology* 80B: 385-388.

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

Yue Y, Zhou Q. 2008. Effect of replacing Soya bean meal with cottonseed meal on growth, feed utilization, and hematological indexes for juvenile hybrid tilapia, *Oreochromis niloticus*×*O. aureus*. *Aquaculture* 284: 185-189.

Zhou Q, Wu Z, Chi S, Yang Q. 2007. Dietary lysine requirement of juvenile cobia (*Rachycentron canadum*). *Aquaculture* 273: 634-640.