APPARENT DIGESTIBILITY COEFFICIENTS OF FEED INGREDIENTS AND ESSENTIAL AMINO ACID REQUIREMENTS OF DUSKY KOB (*ARGYROSOMUS JAPONICUS*)

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

DOCTOR OF PHILOSOPHY

at

Rhodes University

by

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December 2016

ABSTRACT

Important to the evaluation of potential feed ingredients for inclusion in fish diets should be their digestibility and amino acid requirement so that feeds can be formulated on a digestible basis rather than a gross nutrient basis. This thesis established techniques for faecal collection, the validity of digestibility markers, it determined apparent digestibility coefficients for various novel feed ingredients, and it established the optimal lysine requirement for *Argyrosomus japonicus*, which was used to estimate each of the essential amino acid requirements by using the ideal protein concept.

There were no significant differences in dry matter apparent digestibility coefficients when faeces were collected by stripping (77.0 %), dissection (80.1 %) or settlement (83.5 %). Faecal collection by the settlement method produced the most reliable digestibility data. Apparent digestibility coefficients for dry matter, crude protein and energy calculated using acid-insoluble ash were higher (84.0 %, 93.2 % and 93.0 %, respectively) than those using chromic oxide (55.7 %, 79.1 % and 78.2 %) and titanium dioxide (58.6 %, 79.7 % and 80.5 %). The magnitude of variation in digestibility coefficients obtained using acid-insoluble ash was always lower than that obtained with the other markers. Therefore, acid-insoluble ash was preferred as a dietary marker.

Using the above protocol, protein and amino acid digestibility of some animal and plant protein ingredients were evaluated in a series of experiments. The first trial compared the apparent digestibility coefficients of some animal products included as single protein source in the test diets. Apparent protein digestibility values were 84.5 %, 83.8 %, 85.8 % and 83.1 % for fishmeal-prime, fishmeal-standard, poultry meal and pork meal, respectively. Apparent digestibility coefficients for poultry meal were comparable to those of fishmeal, which indicate its potential as a substitute for fishmeal in the diets of *A. japonicus*. The second trial

determined the apparent coefficients of plant and animal protein sources included at 30 % into a practical reference diet (70 %). Apparent protein digestibility ranged from 92.4 % in sunflower meal to 85.5 % in corn gluten meal. Soybean meal is a promising feed ingredient in *A. japonicus* due to the high apparent digestibility of its protein (92.0 %) and essential amino acid digestibility (mean average 91.4 %). A fundamental assumption in fish feed formulation is that the digestibility of nutrients is additive, i.e., digestibility of a nutrient in one ingredient does not interact with the digestibility of the same nutrient in another ingredient. In the third trial, additivity of feed ingredients was tested using pork meal and poultry meal. The results indicate that the apparent digestibility coefficient of animal protein ingredients could be calculated from compound diets to accurately determine protein and amino acid digestibility in *A. japonicus*, and possibly other carnivorous fish species.

Dietary essential amino acid requirements were determined for juvenile *A. japonicus* in two trials. A dose-response study was conducted using crystalline lysine to determine the optimal requirement of dietary lysine for *A. japonicus*. Optimal dietary lysine was estimated at 31.7 g kg^{-1} dry diet, corresponding to 73.5 g kg^{-1} of dietary protein, based on specific growth rate and broken-line segmented regression analyses. Dietary requirements for other essential amino acids ranged from 22 g kg^{-1} (histidine) to 71 g kg^{-1} (leucine) crude protein.

The results of the present study provided a research tool that could be used to assess and verify the conclusions of earlier dietary work on *A. japonicus* and in further studies to develop least cost diet formulations for this species. The study also adds to the knowledge of the nutritional requirements of *A. japonicus* by providing information on the digestibility of plants and animal protein ingredients. It also contributes to future dietary research for this species because this study determines, for the first time, the most suitable methods for investigating the digestibility of raw materials for *A. japonicus*.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

A/E	Ratio of essential amino acids
AA	Amino acid
ADC	Apparent digestibility coefficient
AIA	Acid-insoluble ash
ANOVA	Analysis of variance
APD	Apparent protein digestibility
BLM	Blood meal
CF	Condition factor
CGM	Corn gluten meal
CNM	Canola meal
CP	Crude protein
DL	Dietary level
DM	Dry matter
EAA	Essential amino acid
FBW	Final body weight
FCR	Feed conversion ratio
FI	Feed intake
FM	Fishmeal
HSI	Hepatosomatic index
IBW	Initial body weight
LR	Lysine retention
NEAA	Non essential amino acid
PKM	Pork meal

PLM	Poultry meal
SBM	Soybean meal
SD	Standard deviation
SFM	Sunflower meal
SGR	Specific growth rate
VSI	Viscerosomatic index

PREFACE

This thesis is submitted as a collection of three different papers (each as a separate chapter), preceded by a general introdution. There are repetitive sentences in the introductions, methods and discussions for each of the papers.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all those who helped me during the course of my studies at Rhodes University. In particular, my research supervisors Dr Clifford L.W. Jones and Dr Thomas A. Shipton deserve special thanks, not only for their knowledge, encouragement and dedicated guidance, but also for their sincerity, good sense of humour and care. I thank you for your open-door approach and profound patience.

This research was supported by funding from the Marine Finfish Farmers Association of South Africa/DST sector innovation fund (MASSIF), Marifeed (Pty) Ltd, the National Research Foundation, Technology and Human Resources for Industry Programme (NRF-THRIP), and the Department of Agriculture, Forestry and Fisheries (DAFF), South Africa. Special appreciation goes to Pure Ocean East London (Pty) Ltd for the constant supply of experimental fish, Marco Van Jaarsveld (Montego Pet Nutrition) and Dirk van der Linde (TICSA) for provision of feed ingredients.

I thank all staff members of the Department of Ichthyology and Fisheries Science, most especially the past and present administrative staff members. I am also deeply appreciative of the warm reception from the Port Alfred community. Thank you so much for providing my family with so many unforgettable memories.

To my dearest gifts from God, Simeon, Harmony and Roseline, thank you for putting up with me over the period of this study. My husband, more than I can say, supported me both emotionally and spiritually throughout this programme, to him I owe the greatest appreciation.

Above all, I wholeheartedly thank Jesus Christ, my Lord and Saviour, for giving me the vision, good health and endurance to complete this interesting study.

CHAPTER 1

INTRODUCTION

Globally, aquaculture is the most rapidly developing agricultural sector, attaining 73.8 million tonnes of food fish in 2014 compared to the 70.3 million tonnes produced in 2013 (FAO, 2016). Aquaculture production in Asia accounts for 89 % of the world aquaculture production, while Africa has contributed 2.3 % (FAO, 2016). South Africa's aquaculture industry is a small sector with local production of 4,802 tonnes in 2013, the marine aquaculture industry recording 2,985 tonnes and the freshwater aquaculture industry recording 1,816 tonnes (DAFF, 2014). The finfish subsector was the smallest contributor to total production, recording a production of 122 tonnes in 2013. The percentage contributed by the finfish sub-sector was 4.1 % (DAFF, 2014). The finfish sub-sector in South Africa is an emerging industry. Over the years, a number of species have been piloted to assess the feasibility and market access. Currently the only commercial finfish species being cultured in the local industry is dusky kob *Argyrosomus japonicus* (DAFF, 2014).

1.1. Research and development of dusky kob aquaculture in South Africa

Dusky kob (*Argyrosomus japonicus* Temminck and Schlegel, 1843) is a widely distributed sciaenid fish found in both hemispheres, along the entire south and eastern coast of Australia, where it is known as mulloway, and from Hong Kong northwards along the Chinese coast to southern Korea and Japan (Griffiths and Heemstra, 1995). In southern Africa, the species occurs on the eastern coast from Cape Point to southern Mozambique, but is especially abundant between Cape Agulhas and KwaZulu-Natal in South Africa (Griffiths, 1997). It grows to a large size with the maximum reported length being 175 cm TL, weight 75 kg and maximum age of 42 y (Silberschneider and Gray, 2008; Ferguson *et al.,* 2014). It is a highly

sought-after sports-fish species in estuarine and coastal waters in South Africa and southern Australia (Griffiths, 1997). The juveniles are commonly targeted by estuarine and surf zone anglers and the adults are targeted by commercial and recreational line-fishers beyond the surf zone (Hutchings and Lamberth, 2003). *Argyrosomus japonicus* is a euryhaline, gregarious, fast growing and highly fecund species that easily reproduces in captivity (Battaglene and Talbot, 1994; Fielder and Bardsley, 1999; Silberschneider and Gray, 2008). It has a relatively large mouth with caniniform teeth, sharp gill rakers and a short intestine with a large distensible stomach. It is regarded as a benthic carnivore but has been found to feed throughout the water column (Fielder and Bardsley, 1999; Silberschneider and Gray, 2008).

Due to its large size, palatability and food value, *A. japonicus* has been targeted for decades by commercial, recreational and subsistence fisheries (Childs and Fennessy, 2013), which has resulted in exploitation far beyond optimal levels and the collapse of natural populations, with spawner biomass-per-recruit estimates of 1.0-4.5 % pristine values in South Africa (Griffiths, 1997). Due to the declining catches, and market demand, *A. japonicus* has been identified as a suitable candidate species for aquaculture (PIRSA, 2001). This has lead to substantial research effort regarding the potential culture of this species.

1.2. Dusky kob *A. japonicus* as a potential aquaculture species

Dusky kob aquaculture in South Africa has developed over recent years, supported by a limited number of hatcheries in the Eastern Cape, the Western Cape and KwaZulu-Natal (Shipton and Britz, 2007; DAFF, 2014). To date, *A. japonicus* have been grown in pilot, small-scale commercial facilities, in both onshore recirculating systems and sea cages. They have been successfully raised in cages in the warmer waters of Pemba, Mosambique and KwaZulu-Natal (Shipton and Britz, 2007).

Recent work on optimizing tank-rearing conditions for juvenile *A. japonicus* has shown that densities of 50 kg per m^3 are possible, with best growth and feed conversion at temperatures of 25.3 and 21.7 ^{o}C, respectively (Collett *et al.,* 2008). *Argyrosomus japonicus* readily accept formulated feeds, tolerate a wide range of salinity, temperature and low oxygen levels making them an ideal candidate for farming in sea cages, coastal earthen ponds and recirculating aquaculture systems.

For the dusky kob aquaculture industry to be successful in South Africa, it is important to establish a suitable, least-cost diet for the species. At present, commercial dusky kob farmers use imported seabass feeds as there are no nutritionally complete, cost effective locally manufactured feeds based on locally available feed ingredients in South Africa. As protein is the most expensive dietary component, there is an urgent need to evaluate protein and amino acid digestibility of feed ingredients used in feed formulation.

Generally, protein requirements for growing marine finfish are between 35-55 % of total dietary intake (Rahnema *et al.,* 2005; Kaushik and Seiliez, 2010). Using fishmeal based dietary formulations, Woolley *et al.* (2010) estimated the protein requirement for juvenile dusky kob (100-300 g) at 46 % (dry diet). Fishmeal has a balanced source of essential amino acids, fatty acids, vitamins, minerals and good palatability, and is often in high demand as the protein source for many formulated feeds (Tacon and Metian, 2008; Olsen and Hasan, 2012).

1.3. Fishmeal and other alternative protein sources for fish

Fishmeal has become a major ingredient in fish feeds because it promotes good feed efficiency and better growth and also enhances feed palatability (Tacon *et al.,* 2011; Trosvik *et al.,* 2013). High quality fishmeal has high biological value as a feedstuff, as it has a high

level of digestible essential amino acids, such as lysine, methionine and threonine, which are often deficient in other protein sources (Olsen and Hasan, 2012).

The level of fishmeal in a feed formulation varies depending on a number of factors including the culture species and its trophic feeding level (carnivore or omnivore). In a typical commercial feed for carnivorous fish such as salmon, the required crude protein content varies around 42-50 % (NRC, 1993). What animals require are nutrients, which are provided by ingredients. The majority of the protein requirements in carnivorous species are traditionally made up of fishmeal.

According to FAO (2014), the aquaculture sector is at present the biggest consumer of fishmeal, with a share of 46 % of the total global fishmeal production. The growth of aquaculture and in particular, the focus on high value carnivorous species such as *A. japonicus*, which require high levels of dietary fishmeal is expected to rise because of the public attention that has shifted to the consumption of seafood products.

The use of fishmeal in the aquaculture industry as a major protein source in compound feeds has strongly contributed to increased demand and price for this product. Concern has been expressed that it may not be ethically acceptable to harvest fish for aquaculture feed which could be used directly as food for humans (Tacon and Metian, 2009; Olsen and Hasan, 2012). The ecological impact of overfishing is also a cause for concern. Future dietary inclusion levels of fishmeal within compound aquafeeds need to be decreased as wild supplies diminish and the market price increases (Tacon and Metian, 2008). The inconsistency of supply, and the growing demand and price are limiting the use of fishmeal and putting greater pressure on the feed industry to find economical alternative sources of protein.

In order to reduce the overreliance on fishmeal, researchers are looking towards alternative protein sources for fishmeal in dietary formulations (Gao *et al.*, 2013; Aya *et al.*, 2015; Chor

et al., 2015; Jirsa, *et al.*, 2015; Webster *et al.*, 2015; Hernández and Roman 2016; Krome and Focken, 2016; Minjarez-Osorio *et al.*, 2016; Ribeiro *et al.*, 2016; Rossi *et al.*, 2016). Feed cost can be reduced by developing a better understanding of the nutritional requirements of the species. For example, by measuring the availability of essential nutrients in alternative feed ingredients and knowing the dietary requirements, it is possible to eliminate fishmeal from feed formulations for rainbow trout, *Oncorhynchus mykiss* (Barrows and Frost, 2014; Davidson *et al.*, 2016). Researchers blend plant and animal protein concentrates, and supplement amino acids, to ensure that amino acid content of feeds with reduced levels of fishmeal meets or exceeds the amino acid requirements of the farmed fish (Hardy, 2010).

There are several protein sources that can partially replace fishmeal in aquaculture feeds without affecting the growth performance of the fish (Tacon and Metian, 2009; Kiron *et al.*, 2012). To be a viable alternative for fishmeal, a candidate ingredient must have a relatively high protein content, a favourable amino acid profile, high nutrient digestibility, and be cost effective and readily available in a form that can be in incorporated into commercial feeds (Hardy, 2010; Kaushik and Seiliez, 2010).

The replacement of fishmeal with alternative protein sources in diets for carnivorous fish species will be more challenging because they require higher levels of dietary protein. Omnivores are typically fed lower protein diets, while carnivores are fed high protein diets (Cowey, 2013).

A number of published reports are available regarding the suitability of plant and animal by-product protein feeds as alternative protein sources for omnivorous and herbivorous fishes such as tilapia *Oreochromis niloticus* and African catfish *Clarias gariepinus* (El-Sayed, 1999; Fagbenro and Davies, 2001; Nyina-wamwiza *et al.*, 2010). It is apparent from these studies that larger amounts of fishmeal could be replaced by plant protein sources in omnivorous

species. However, research with alternative proteins (Gatlin *et al.,* 2007; Murray *et al.,* 2010; Chowdhury *et al.*, 2012; García-Ortega *et al.,* 2016) showed that complete replacement by plant protein has been less successful with carnivorous species. These limitations have been primarily attributed to the presence of anti-nutritional factors in the plant-based materials, specifically soybean (García-Ortega *et al.,* 2016).

The inclusion of plant protein in aquafeeds is challenging due to the presence of anti-nutritional factors. Anti-nutritional factors are defined as substances that interfere in food utilization and digestion, and affect the health and production of the animals (Francis *et al.,* 2001). Major classes of anti-nutritional factors include trypsin inhibitors, phytic acid (makes phosphorus unavailable), phytoestrogens (disrupt fish reproduction), alkaloids (affect feed palatability because of their bitter taste), glucosinolates (affect appetite and disrupt the synthesis of thyroid hormones), lectins (bind to carbohydrate, interfere with nutrient absorption and cause inflammatory phenomena) and phenolic compound (inhibits digestive enzymes) (Francis *et al.,* 2001; Gatlin *et al.,* 2007; Oliva-Teles *et al.,* 2015). The presence of such substances in aquafeeds may significantly affect the nutritional value of ingredient sources and their use may result in a variety of physiological effects on fish (Francis *et al.,* 2001). Another negative characteristic of some plant-based protein sources is that they usually have low palatability, especially when fed to carnivorous fish (Hardy, 1996). Some of these anti-nutritional factors can be inactivated by a variety of methods such as dehulling, soaking and addition or heat treatment such as autoclaving, roasting and extrusion (Francis *et al.,* 2001).

Essential amino acid profiles of plant protein sources do not match the dietary requirements of fish species. For example, corn gluten meal and wheat gluten meal are known to be low in lysine and arginine, whereas soybean meal, the most commonly used plant protein source in

aquaculture feeds, is generally limiting in methionine. To overcome this problem, a common practice is to mix several protein sources in a way that they will give an amino acid profile that better supports normal fish growth (De Francesco *et al.,* 2007).

Replacement of fishmeal with animal protein may be a more suitable alternative for carnivorous fish species. Animal proteins are generally free of anti-nutritional factors, cheaper than fishmeal, and do not have the carbohydrate content that can reduce palatability and digestibility. Animal protein sources such as meat and bone meals, blood meal, pork meal, hydrolysed feather meal and poultry by-product meal are widely used in aquafeeds (Forster *et al.,* 2002). They are high in crude protein; however when used in isolation, they tend not to meet the requirements of the fish species because of the limited amount of essential amino acids. Lysine, methionine and isoleucine are generally the limiting amino acids in animal sources of fishmeal alternatives (NRC, 1993).

1.4. Nutrient requirements of fish

Proteins are an essential component of animal diets; they are needed for growth, development, reproduction and survival (Wu *et al.,* 2013). In fish, proteins are the primary constituents of structural and protective tissue, soft organs, body fluids, enzymes and hormones (Anderson and Lall, 2005; Sabahelkhier *et al.,* 2014). Protein is the major component of carnivorous fish diets and the most expensive component of formulated diets. The protein requirement needed to achieve maximum growth has been measured in fish species (Table 1.1).

Excessive protein in the diet results in excess nitrogenous waste and this can affect fish growth and feed costs, by decreasing feed performance and overall feed efficiency (Cho and Bureau, 2001). The optimum dietary protein level varies with a number of factors including

fish species, life stage, quantity of non-protein energy, dietary protein quality and dietary amino acid composition. The dietary requirement for protein is essentially a requirement for amino acids, depending on the non-protein energy content of the feed.

Table 1.1. Dietary protein requirement (%) of selected juvenile finfish.

Species	Scientific names	Dietary protein requirements	Average weight (g)	Reference
Dusky kob	*Argyrosomus japonicus*	46	100	Woolley *et al.*, 2010
Asian seabass	*Lates calcarifer*	40-50	1.34	Williams *et al.*, 2003
Gilthead sea bream	*Sparus aurata*	47	42.5	Gómez-Requeni, 2004
Rock fish	*Sebastes schlegeli*	42	22	Song *et al.*, 2014
Mulloway	*Argyrosomus japonicus*	44-49	68	Pirozzi *et al.*, 2010
Sunshine bass	*Morone chrysops x M. saxatilis*	40	2.68	Rawles *et al.*, 2012
Largemouth bass	*Micropterus salmoides*	43.5	14.46	Portz *et al.*, 2001
Shi drum	*Umbrina cirrosa*	45.6	86.3	Akpinar *et al.*, 2012
Red drum	*Sciaenops ocellatus*	40	2	Serrano *et al.*, 1992

Amino acids (AAs) provide nitrogen for the synthesis of protein and other biological molecules. Amino acids can be divided into two functional groups, essential and non-essential (Wilson, 2002; Gatlin, 2010). The non-essential (dispensable) amino acids are those that can be readily synthesized by the fish and comprise alanine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, hydroxyproline, proline and serine (Wu *et al.*, 2013). The other amino acid group is considered as essential amino acids (indispensable) because the animal cannot synthesize them, and thus they have to be supplied in the diet. These comprise arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (NRC, 1993). Some amino acids such as cysteine and tryrosine are considered semi-essential because they can only be synthesised from essential amino acid precursors. The presence of cysteine in the diet has a sparing effect on dietary methionine

requirements. Another example is the sparing effect of tyrosine on dietary phenylalanine. All studies to date on finfish have shown that fish require the same 10 essential amino acids as other vertebrates, which cannot be synthesized and therefore, must be supplied in the diet (Wilson, 2002; Wu *et al.,* 2014). Dietary deficiency in any of the essential amino acids impairs protein synthesis, affects other vital biochemical productions and often suppresses fish growth in general (Wilson, 2002).

All the AAs needed to synthesize protein must be available at adequate balance for protein synthesis to occur. Research evidence has shown that besides their role as building blocks of protein and polypeptides, AAs are important regulators of key metabolic pathways that are necessary for maintenance, growth, reproduction and immunity in organisms, thereby maximizing efficiency of food utilization, enhancing protein accretion, reducing adiposity and improving health (Suenaga *et al.,* 2008; Anderson *et al.,* 2002; Aksnes *et al.*, 2008). Dietary supplementation of AAs is expected to offset adverse effects of replacement of fishmeal in aquafeeds, thereby restoring growth (Li *et al.,* 2009). Research evidence showed a crucial role for specific amino acid in physiology and metabolism in fish (Table 1.2).

Formulating cost-effective feeds that meet the essential amino acid (EAA) requirements of fish can be a challenge (Kaushik and Seiliez, 2010) and will depend on relevant data on both the EAA requirements of the fish and the EAA supplied in the dietary components. Amino acids can be involved in a wide variety of other metabolic processes besides protein synthesis, and are subjected to significant endogenous losses (Rodehutscord *et al.,* 1997). The accurate determination of the requirement for each of the dietary EAAs and the contribution of non-essential amino acids (NEAAs) is considered the fundamental basis of protein and amino acid nutrition.

Table 1.2. Roles of amino acids in physiological functions and metabolism of fish.

Amino acids	Functions	Species	Reference
Arginine	Kill invaded micro organism	Channel catfish	Buentello and Gatlin, 1999
Arginine and methionine	Induce larva intestinal maturation	Seabass	Péres *et al.*, 1997
Alanine, glutamic acid and Serine	Appetite	Many fishes	Shamushaki *et al.*, 2007
Glutamate	Ammonia removal	Rainbow trout	Anderson *et al.*, 2002
Glycine	Increase hepatic T4 5 monodeiodinase	Rainbow trout	Riley *et al.*, 1996
Histidine	Protection against pH change	Salmon	Mommsen *et al.*, 1980
Lysine and methionine	Lipid transporter on mitochondrial membrane	Various fishes	Harpaz, 2005
Leucine	Immunity modulation; Cell signalling	Various fishes	Li and Gatlin, 2007
Methionine	Structure in membrane; neurotransmitter; betaine synthesis	Various fishes	Mai *et al.*, 2006
Phenylalanine and tyrosine	Influence pigmentation	Japanese flounder	Yoo *et al.*, 2000
Tryptophan	Modulate cortisol release, behaviour and feeding	Rainbow trout	Lepage *et al.*, 2002
Taurine	Gut development	Cobia	Salze *et al.*, 2008
Proline	Enhance growth; Collagen function	Salmon	Aksnes *et al.*, 2008

When the fishmeal component in a fish diet is replaced by alternative protein sources, meeting adequate levels of dietary protein alone does not guarantee adequate levels of essential amino acids. This is because the amino acid profile of alternative proteins tends not to match those of balanced diets to the extent that fishmeal does (Hardy, 2008). Hence, while diets based on alternative protein sources may provide sufficient protein, unless essential amino acids are adequately supplemented, such diets will decrease fish growth. The growing fish fed such a diet would use very few amino acids for energy. The amino acids would be used efficiently for maintenance and the synthesis of new structural proteins which would result in maximum feed efficiency and growth (Halver and Hardy, 2002; Gatlin, 2010). Cowey (1995) reported that non-protein 'energy yielding' nutrients like lipids and

carbohydrates can improve the utilization of dietary protein by means of a protein sparing effect. Insufficent protein can impair fish productivity; however, if too much protein is supplied, only part of it will be used to synthesize new tissue and the remainder will be metabolized and used as an energy source (Wilson, 2002). Therefore, adequate measures should be taken when formulating diets to minimize the amount of protein used for energy. Determining the dietary requirement of EAAs is an essential aspect of developing least-cost complete feeds for a given fish species. Essential amino acid requirements have been established for a number of species of fish, examples of which include bluegill *Lepomis macrochirus* (Masagounder *et al.,* 2011), gilthead seabream *Sparus aurata*, European seabass *Dicentrarchus labrax*, turbot *Psetta maxima* (Kaushik, 1998), pacu *Piaractus mesopotamicus* (Abimorad *et al.,* 2010), seabream (Zhou *et al.,* 2010), chum salmon *Oncorhynchus keta*, Japanese eel *Anguilla japonica* (Akiyama *et al.,* 1997), Atlantic halibut *Hippoglossus hippoglossus*, yellow tail *Seriola quinqueradiata*, Japanese flounder *Paralichtys olivaceus* (Kim and Lall, 2000) and largemouth bass *Micropterus salmoides* (Portz and Cyrino, 2003).

Currently the lack of data on the nutrient requirements of South African *A. japonicus* is one of the major technical constraints for developing low-cost and nutritionally complete diets. Presently, there is no available information on the EAA requirement of the species.

1.5. Ideal protein concept using lysine as a reference amino acid

The ideal protein concept refers to dietary protein that supplies exact requirements of amino acids with no deficiency and no excess, to support optimal growth performance (Wang and Fuller, 1989; Firman and Boling, 1998). The ideal protein concept uses lysine as a reference amino acid, with the requirements for all other essential amino acids expressed as a percentage of lysine (Wilson, 2002). Baker and Han (1994) report that using lysine as reference amino acid has several advantages over other essential amino acids. Lysine is the

first limiting amino acids in many plant protein sources (Hauler and Carter, 2001; Mai *et al.,* 2006). In addition, the sole function of lysine is in protein synthesis, it has no precursor role compared to other essential amino acids (Kerr and Easter, 1995), and therefore, the requirements of other EAAs are generally expressed relative to lysine requirement. On this basis, if the requirement of dietary lysine is known, the requirements of the remaining EAAs can be estimated from the ratio of the whole body amino acid pattern (A/E ratio) of species in a much shorter time and at far less cost (Akiyama *et al.,* 1997). Requirements of EAAs based on this method have been determined in a number of species including colliroja *Astyanax fasciatus* (Furuya *et al.,* 2015); European seabass *Dicentrarchus labrax,* gilthead seabream *Sparus aurata,* turbot *Psetta maxima* (Kaushik, 1998); largemouth bass *Micropterus salmoides* (Dairiki, 2007) and bluegill *Lepomis macrochirus* (Masagounder *et al.,* 2009).

The ideal protein model is aimed at dietary digestible amino acid levels, which supply the correct amount of essential amino acids, whereby nitrogen excretion in waste can be minimized. This will result in significant cost saving in diet formulation for animal production, since if an excess of protein is supplied, only part of it will be used for somatic growth. In addition, the ideal protein concept requires less time and is less costly than determining the amino acid requirements of the fish by conventional methods such as the dose-response method. This is especially valuable and meaningful for those emerging fish species for which nutritional work has not yet been fully conducted.

Applying the ideal protein concept, the evaluation of dietary lysine requirement must be carried out with great accuracy. This is because it is the basis of the requirements for all the remaining essential amino acids. Any error in the lysine requirement will translate into an erroneous estimate for all the other essential amino acids (Barker and Han, 1994; Emmert and Baker, 1997).

Evaluation of lysine requirement is of particular importance in the research of fish nutrition (NRC, 2011). This is because lysine is an EAA that is extensively required for optimum growth and is one of the most limiting EAAs in protein sources commonly used in fish feedstuffs (Wilson, 2003); especially in those feed formulations that are based on plant protein sources. These plant proteins are utilized to replace fishmeal in the formulation of low-cost and economical feed for fish. Deficiency in lysine typically results in loss of appetite and reduction in growth performance (Mai *et al.,* 2006). Feeding a diet that has higher than the requirement level of dietary lysine does not improve performance and feed utilization, but rather reduces growth, and feed efficiencies (Mai *et al.,* 2006; Deng *et al.,* 2010). Therefore, an optimal dietary inclusion level of lysine is required in advance to formulate nutritionally complete and cost effective fish feeds.

1.6. Digestibility of nutrients

Digestibility is a key factor in evaluating the potential of an ingredient for use in the diet of an aquatic species (Glencross *et al.,* 2007), and should be given priority in nutritional studies that are aimed at evaluating the potential of feed ingredients for use in the diet of an animal. The nutritional value of a feed is not exclusively based on its chemical composition but also depends on the quantity of nutrients and energy that are assimilated and utilised (NRC, 1993). A feed may contain the adequate amount of nutrients formulated for a species but will just be wasted and detrimental to the environment if not efficiently assimilated. An effective feed can be prepared when information on digestibility of nutrients in feedstuffs has been considered in its formulation (Catacutan *et al.,* 2003). Determining the digestibility of nutrients in feedstuffs is important to enable the formulation of diets that maximize the growth of cultured fish, by providing appropriate amounts of available nutrients (Lee, 2002; Glencross *et al.,* 2007). Apparent digestibility coefficients are also necessary to allow

experimental and commercial feeds to be formulated on a digestible rather than a gross nutrient basis.

Recently, there has been much interest in formulating diets on a digestible amino acid basis (Masagounder *et al.,* 2009; Hernández *et al.,* 2010; Lu *et al.,* 2015; Glencross *et al.,* 2016). Formulating diets using this approach will result in a decrease in feed cost as well as a decrease in excess nutrients being excreted into the environment. Nutrient digestibility is the difference between the nutrients ingested and the nutrients excreted in faeces (NRC, 2011).

Protein and amino acid digestibility can be expressed as true or apparent, depending on the corrections made by the contribution of endogenous nitrogen production. True digestibility can be determined by the difference between the amount of amino acids in the diet and the faeces, considering the endogenous losses of amino acids that are subtracted from the total amino acids in the faeces (Sakomura and Rostagno, 2007). When a diet without protein is fed to the animal, the nitrogen observed in faeces is derived from the enzymes, mucins, amides, amines, bacteria and cell desquamation of the intestinal mucosa during the passage of food or chime. Souffrant (1991) confirmed that the main components of these endogenous secretions are digestive enzymes and mucoproteins. There are quite a number of reports on true digestibility values of amino acids in various fish species, as found by Ambardekar *et al.* (2009), Wilson *et al.* (1981) for channel catfish *Ictalurus punctatus* and Anderson *et al.* (1992) for Atlantic salmon *Salmo salar* and red sea bream *Pagrus major*. Their results reflect great variability, which may be related to species, fish size, environmental condition such as water temperature (Bendiksen *et al.,* 2003), dissolved oxygen concentrations, time of faeces collection (Allan *et al.,* (1999) and the method of faeces collection (Austreng, 1978). Apparent digestibility is estimated by subtracting the nutrients contained in the faeces from the nutrients contained in the dietary intake but does not take into account the endogenous

losses, and the higher the dietary protein level, the lower the contribution of endogenous lossess in faecal matter.

The main assumption in the use of protein and amino acid digestibility values is that digestibility coefficients determined for individual feed ingredients are additive in compound diets, suggesting the absence of interactions among ingredients and no influence due to dietary inclusion levels (Sales, 2009). Test diets for evaluating digestibility values for fish usually involve mixing a test ingredient (30 %) with a reference diet (70 %), or 50:50 ratio that includes an indigestible marker for indirect digestibility. A reference diet is included in the test diet to maintain adequate palatability and to satisfy the species requirements for essential nutrients. Although this approach is considered standard, it has been reported that the inclusion of a reference diet may interfere with the nutrient digestibility of the test ingredient with regards to carbohydrate and their level in the diet (Lupatsch *et al.,* 1997) and cause erroneous digestibility values. Some researchers have used single protein sources to measure digestibility of feedstuffs (Masagounder *et al.,* 2009; Lupatsch *et al.,* 1997). Problems associated with this method may include poor water stability, nutrient deficiency and poor acceptability (Masagounder *et al.,* 2009). Researchers have established that protein and amino acid digestibility should be tested for additivity in feed ingredients (Lupatsch *et al.,* 1997; Sales, 2009).

1.7. Digestibility determination techniques

The direct method for determining digestibility coefficients for animals is the total collection method, which involves a quantitative record of the nutrients consumed and those voided in the faeces (Austreng, 1978). The apparent digestibility coefficients are calculated by the quantitative measurement of the nutrients in the feed consumed and in the faeces (Hajen *et al.,* 1993). However, measuring digestibility in an aquatic environment is more difficult than

in terrestrial animals, as nutrients can leach from the faeces into the water before faeces collection and faeces can be contaminated with uneaten feed (Allan *et al.,* 1999). Therefore, an indirect method, which involves the use of an indigestible marker in the diet, has been developed (Maynard and Loosli, 1969). Using the indirect method, the total collection of faeces is unnecessary. The digestibility can be calculated from the ratio of nutrients in a representative portion of the feed and the faeces (Hajen *et al.,* 1993). To be an effective marker, it must not interfere with the digestive process of the animal or gut micro flora; not be absorbed or metabolized; have the same rate of passage through the gut as the experimental diet (Maynard and Loosli, 1969; Alan *et al.,* 2005); and it must be possible to analysed it accurately in feed and faeces (Owens and Hanson, 1992). There are two classes of markers, namely external (those that are added to a diet) and internal (those that are inherently present as natural components of a diet) (Hajen *et al.,* 1993).

The utilization of internal indigestible components or the addition of external indigestible markers eliminates the need for quantitative faeces collection but requires that a representative faecal sample be collected (Maynard and Loosli, 1969). A number of external markers have been used for nutritional studies, with chromic oxide being the most widely used in work with fish (Reyes *et al.,* 2015; Rossi *et al.,* 2016). Alternative markers include acid-insoluble ash, polyethylene, titanium dioxide, barium oxide and yttrium (Sugiura *et al.,* 1998; Riche *et al.,* 1995; Austreng *et al.,* 2000; Krontveit *et al.,* 2014).

An internal marker is a chosen substance, which forms an integral part of the feed consumed by the animal (Marais, 2000). It has the advantage of being cheap and convenient, and may be particularly useful in experimentation with wild or free-ranging animals, which are difficult to dose with an external marker (Marais, 2000). Examples include acid-insoluble ash

(Tacon and Rodrigues, 1984; Atkinson *et al.,* 1984) and polyethylene (Tacon and Rodrigues, 1984).

Since over 30 % of the faecal nitrogen is highly water soluble (Lied *et al.,* 1982), it is technically difficult to collect faeces without any leaching of soluble compounds, therefore, the faecal collection method can affect digestibility estimates of a diet (Glencross *et al.,* 2005). The effect of the faecal collection method on digestibility estimates has been studied at length with different species of fish (Spyridakis *et al.,* 1989; Rawles *et al.,* 2010).

Several techniques have been used to collect faecal material from fish. In order to avoid any leaching in the water, fish could be taken out of the water and the faeces sampled directly by dissection (Austreng, 1978), stripping or anal suction. Alternative methods for the collection of faeces in the water include the collection of decanted faeces from within the tank (Smith and Lovell, 1971), from effluent water via filtration (Ogino *et al.,* 1973) or decantation columns-the Guelph system (Cho and Slinger, 1979) and from continuous effluent filtration/faeces removal (Choubert *et al.,* 1979).

There has been concern among researchers regarding the degree of nutrient leaching with the various methods of faeces collection. Windell *et al.* (1978) observed that the most nutrient leaching from faeces occurs during the first hour after defaecation. Some authors calimed that systems that remove faeces from the water swiftly cause minimum leaching, while others claimed that breakage of faeces pellets during collection is the major cause of nutrient leaching (Hajen *et al.,* 1993). Cho *et al.* (1982) showed that results obtained by intestinal dissection and by anal suction were not significantly different from the results obtained by the Guelph system for nutrient digestibility. This would indicate that nutrient leaching was not an important source of error with the Guelph system.

1.8. Need for research in dusky kob nutrition

For the dusky kob farming industry to reach its potential in South Africa, locally produced and cost effective feeds must be developed. To date there are no commercial feeds manufactured exclusively for dusky kob and farmers rely on imported commercial finfish feed, which are based on trout and seabass formulations. The cost of purchasing and delivering feeds remains the single highest operating cost for carnivorous fish culture, often accounting for as much as 60 % of on-farm operating costs (De Silva and Hasan, 2007). Globally, aquafeed costs are primarily driven by the international availability and price of fishmeal, and the prevailing currency exchange rates. Low-cost locally available alternatives to fishmeal need to be identified in order to develop the cost effective production of dusky kob in South Africa. The major issues associated with feed development include an understanding of the nutritional requirements of the species, and access to a diverse range of ingredients for which the nutritional limitations, with respect to the ingredient and the species are known (Glencross *et al.,* 2007). The first task in evaluating the potential of any ingredient for inclusion in finfish diets should be the determination of its proximate composition and apparent digestibility (Allan *et al.,* 1999; Glencross *et al.,* 2007). Preferably, amino acid digestibility of an ingredient should be investigated, because high protein digestibility by itself is not necessarily commensurate with the availability of amino acids, in particular the essential amino acids, which should be available in sufficient quantities and in the correct proportions for the fish (De Silva *et al.,* 2000). The use of the apparent digestibility coefficient of an amino acid is necessary to establish the "digestible" AA requirement for growth and maintenance of *A. japonicus*. Collection of faecal matter from fish is a difficult process, and collection methods must ensure digestibility coefficients are accurate (Allan *et al.,* 1999). In order to effectively evaluate available nutrients in feed ingredients, it is

necessary to establish a suitable technique for faecal collection and identify a reliable marker for the species.

Presently, there is no published information on EAA requirements for *A. japonicus*. Typically lysine is one of the principal limiting essential amino acids commonly encountered in fish feed formulations. Knowing the requirement of lysine, then the requirement for other EAAs could be estimated based on the ideal protein concept. Improved dietary formulation could be achieved by measuring the availability of EAAs in alternative feed ingredients and matching it with the EAA requirements of *A. japonicus*.

This thesis was designed to establish adequate techniques for faecal collection and the validity of digestibility markers for *A. japonicus*. It also documents digestibility information for novel feedstuffs, which could partially or wholly replace fishmeal in diets for *A. japonicus*. In addition, this study establishes the optimal lysine requirement for *A. japonicus*, which was used to calculate each of the essential amino acid requirements by using the ideal protein concept.

1.9. Aims and objectives

The current study should be viewed as a continuation of a broader research project initiated by Rhodes University to the development of least-cost, nutritionally balanced diets for dusky kob using locally available feedstuffs. The aims of this study were to investigate the amino acid requirements of South African dusky kob *Argyrosomus japonicus* and to provide information on the potential of alternative feed ingredients to reduce or replace fishmeal in the diet for this species.

The objectives were to:

1. identify a suitable faecal collection technique in the digestibility of *A. japonicus*
2. identify a suitable marker for digestibility studies in *A. japonicus*
3. evaluate protein and amino acid digestibility of some feed ingredients in *A. japonicus*
4. compare two methods used to determine apparent protein and amino acid digestibility values for *A. japonicus*
5. determine the optimal dietary lysine requirement for juveniles of *A. japonicus*
6. determine the whole body amino acid composition of juvenile dusky kob and evaluate the optimum dietary essential amino acid profile for dusky kob, *A. japonicus.*

CHAPTER 2

A COMPARISON OF DIETARY MARKERS AND FAECAL COLLECTION METHODS TO ESTIMATE APPARENT DIGESTIBILITY

2.1. INTRODUCTION

The measurement of apparent digestibility coefficients (ADCs) of nutrients and energy is essential for effective animal nutrition research and for establishing the nutritional value of feed ingredients (Glencross *et al.*, 2007). Before any ingredients can be effectively evaluated in terms of their nutrient digestibility, a reliable method for measuring nutrient digestibility coefficients must be in place (Kim *et al.*, 2011). The direct method of faecal collection in aquatic environments is time consuming, as it requires an accurate quantitative assessment of feeds fed and faecal production, which is problematic in an aquatic environment (Hajen *et al.*, 1993). Due to the inherent difficulties encountered with quantitative collection of faeces over an extended period of time, the most widely employed method for the estimation of nutrient digestibility is the indirect approach, involving the use of an inert dietary marker to estimate digestibility (Hajen *et al.*, 1993). The use of markers enables the digestibility coefficients to be calculated by assessing the changing ratio of the marker concentration within the faeces relative to the dietary marker concentration in the nutrient (Maynard and Loosli, 1969). Using the indirect method, the quantitative total collection of all faeces is unnecessary as the digestibility can be calculated from the ratio of nutrient to marker in the feed and faeces (Maynard and Loosli, 1969).

A marker is an indigestible reference compound that may be used to assess physical and chemical aspects of digestion (Owens and Hanson, 1992; Pozza *et al.*, 2013). Markers

provide a means of calculating the digestibility when complete collection of faeces from a known quantity of feed consumed cannot be undertaken (Pozza *et al.*, 2013).

Chromic oxide (Cr_2O_3) is the most commonly used marker in digestibility studies in fish. It has been used extensively in studies with sunshine bass *Morone chrysops x M. saxatilis* (Rawles *et al.*, 2010); largemouth bass *Micropterus salmoides* and bluegill *Lepomis macrochirus* (Masagounder *et al.*, 2009); gilthead seabream *Sparus aurata* (Lupatsch *et al.*, 1997; Couto *et al.*, 2016); mulloway *A. japonicus* (Booth *et al.*, 2013); European seabass *Dicentrarchus labrax* and meagre *Argyrosomus regius* (Magalhães *et al.*, 2015); post-juvenile chinook salmon *Oncorhynchus tshanytscha* in sea water (Hajen *et al.*, 1993) and tilapia *Oreochromis niloticus* (Falaye and Jauncey, 1999; Vidal *et al.*, 2015). However, some results have brought into question the validity of using chromic oxide (De Silva *et al.*, 1997). In tilapia, it has been reported that chromic oxide (Cr_2O_3) may partially separate from the food during ingestion and passage through the gut resulting in unreliable digestibility estimates. In addition, studies with tilapia hybrids have shown that carbohydrate utilization is affected by chromium supplementation, indicating that chromium may not be entirely inert (Hanley, 1987).

Several alternative markers have been proposed as suitable as chromic oxide. For example, titanium dioxide (TiO_2) has been used as a marker with excellent recovery rates in Atlantic cod *Gadus morhua* (Lied *et al.*, 1982) and rainbow trout *Oncorhynchus mykiss* (Weatherup and McCracken, 1998). Richter *et al.* (2003) concluded that TiO_2 is an acceptable marker for Nile tilapia *Oreochromis niloticus.* Trivalent oxides of yttrium have been preferred to chromic oxide because they are more easily dissolved for analysis (Austreng *et al.*, 2000). In salmonid fish, oxides of yttrium and lanthanides have been used in digestibility studies (Hillestad *et al.*, 1999; Sugiura *et al.*, 1998).

Acid-insoluble ash (AIA), primarily comprising silica, occurs naturally in diets and is the most common internal marker used in digestibility studies (Sales and Janssens, 2003). It has been validated for use in mammals (Van Keulen and Young, 1977), poultry (Vogtmann *et al.,* 1975) and penaeid shrimp *Penaeus monodon* (Deering *et al.,* 1996). Similarly, Sales and Britz (2002) and Goddard and McLean (2001) identified AIA as a reliable, replicable and safe internal marker for use in nutrients digestibility studies with *Haliotis midae* and *Oreochromis aureus* respectively. The most suitable marker for *A. japonicus* has not been determined (based on scientific laboratory findings). Where internal AIA is not present in sufficient quantities for accurate measurement in fish samples, it has been supplemented with external sources. Celite, an acid-washed, diatomaceous silica powder (Atkinson *et al.,* 1984; Morales *et al.,* 1999) and acid-washed sand (Tacon and Rodrigues, 1984) have successfully been used as external sources of AIA in digestibility studies of various fish species (Goddard and Mclean, 2001, Li *et al.,* 2008 and Tibaldi *et al.,* 2015).

In rainbow trout, Tacon and Rodriques (1984) compared Cr_2O_3, crude fibre, polyethylene and AIA, and they concluded that Cr_2O_3 and crude fibre were the most reliable dietary markers for the estimation of digestibility. Chromic oxide was also the most suitable marker for red drum *Sciaenops ocellatus* (Rossi *et al.,* 2016). Atkinson *et al.* (1984) evaluated AIA and Cr_2O_3 as dietary markers for rainbow trout, they reported AIA as a suitable dietary marker. Morales *et al.* (1999) compared AIA, crude fibre and Cr_2O_3 as dietary markers and concluded that crude fibre was an effective marker for digestibility trials with rainbow trout.

To date, there have not been any comparative studies to determine the most appropriate dietary marker for *A. japonicus.* Therefore there is need to evaluate dietary markers in order to select the most suitable one for this species.

The most common faecal collection techniques in fish include the collection of faeces from the lower part of the intestine by stripping (Nose, 1960), suction (Windell *et al.,* 1978) and intestinal dissection (Smith and Lovell, 1971). Although, dissection, stripping and anal suction methods of faecal collection avoid leaching problems, the assumption that digestion and absorption are completed when the digesta has reached the distal end of the large intestine may prove unfounded and lead to an underestimation of nutrient digestibility (Allan *et al.,* 1999). In addition, faecal samples are easily contaminated with blood, slime, semen or eggs. During stripping, there is a requirement to anaesthetize the fish prior to handling and this appears to induce sudden defecation and acceleration of intestinal transit (Spyridakis *et al.,* 1989; Amirkolaie *et al.,* 2005). Furthermore, intestinal sampling techniques are not applicable to small fish because of the insufficient sample size that can be collected on any single occasion (Glencross *et al.,* 2007; Blyth *et al.,* 2015).

Another alternative technique for assessing the digestibility without manipulating the animals has been developed. Ogino *et al.* (1973) developed a method in which faeces are collected by passing the effluent water from the fish tank through a filtration column (TUF-System). Faeces were siphoned out of the base of the tank into the collection column, from where they were gathered and settled at the base of the column. The system was easy to use and could be adapted to any tank system. However, it was difficult to use with larger fish in big tanks as the angle of the tank needed to be tilted.

Choubert *et al.* (1982) built a separate system in which a conveyor belt was used to collect faeces from the effluent and then dry them. In this process, collection occurs within six to fifteen seconds after expulsion of faeces, with positive effects on reduction of leaching since faeces are in contact with water for a minimal period of time. However, the cost of the equipment and the system maintenance, in particular when working with salt water, which speeds up deterioration of the material, makes the use of this system unpopular.

Cho and Slinger (1979) developed the Guelph system as a method of faecal collection. Special conical tanks with a sloping bottom and a long, narrow drain are required for this system. The bottom of the column includes a control device for collecting faeces and uneaten feed, and for emptying the tank. The velocity of the water flow is adjusted to maximize the recovery of the faeces in the settling column. The advantage of this method is that fish do not need to be killed (by dissection) or excessively handled (by stripping) in order to collect faeces. Fish are maintained in tanks and the faecal material is collected in a settling column. The disadvantage associated with this method is that soluble material could be lost from faeces due to leaching. However, Cho and Slinger (1979) showed that results obtained by intestinal dissection and by anal suction in a digestibility experiment with rainbow trout were not significantly different from the results obtained using the Guelph system.

Given that the faecal collection method clearly influences the accuracy of nutrient digestibility coefficients, there is a need to establish and validate the most appropriate collection technique for *A. japonicus*.

The overall aim of this study was to determine the most suitable faecal collection methods for dusky kob *A. japonicus* and to determine the most suitable marker for use in digestibility studies.

The objectives of this study were: (1) to validate the gut evacuation period in dusky kob, (2) to evaluate the suitability of different markers (chromic oxide, titanium dioxide and acid-insoluble ash) for use in digestibility studies with *A. japonicus* and (3) to establish the most suitable faecal collection methods for determining apparent nutrient digestibility in *A. japonicus*.

2.2. MATERIALS AND METHODS

2.2.1. Study site

The digestibility trials were carried out at the Rhodes University Marine Research Laboratory in Port Alfred (33°45′ S, 26°00′ E) South Africa.

2.2.2. Experimental facilities and faecal collection

Approximately 10 % of the 10 000 L recirculating system's water was replaced daily with seawater pumped from the Kowie River Estuary. The recirculating system also includes a solids removal settlement tank (350 L) and a biological filter (1000 L; Ecotao South Africa). Water was circulated using four submerged, electrical pumps (Resun/P8500ECO, AC 220-240V~50Hz 90W) in series that delivered water at 20 L min^{-1} $tank^{-1}$. The systems comprised 18 circular tanks, each with 350 L water capacity (Figure 2.1). These tanks were used to hold the fish used for stripping and those used in the dissection method (Section 2.2.7). Each tank was aerated using air stones and water temperature was controlled using a heat pump (SIRAC LSQ20R, 3.7 kW).

A modified Guelph system (settlement tank) was constructed to hold the fish used to collect data for the settlement treatments, and consisted of fifteen fiberglass, sloping-bottom tanks (volume: 90 L) configured to promote rapid faecal collection. These tanks were connected to the same recirculating system as the fish holding system and received water at a rate of ten litres per minute (Figure 2.2). A pipe with a small opening (diameter of five millimetres) was attached to the base of the collection cylinder. The hydrodynamic properties of the system allowed faeces to settle at the base of the column and accumulate in the collection cylinder from where they could be collected. The tanks were flushed within one hour after feeding to remove any residual particulate matter such as residual faeces or uneaten feed. The following

morning, prior to feeding, faecal matter, which had settled overnight, was collected in plastic bottles and frozen at -20 ^{o}C for analysis.

2.2.3. Environmental variables

The mean (± standard deviation) temperature (23.2±0.2 ^{o}C) dissolved oxygen (4.7±0.3 mg L^{-1}) and pH (7.6) during the trial were measured daily with a hand-held electronic probe (Hanna Instruments HI 98128, Rhode Island, USA). Total ammonia nitrogen (0.2±0.2 mg L^{-1}) was measured once a week using a spectrophotometer (Spectroquant Pharo 100, Darmstadt Germany) and commercially available test kits (Merck KGaA, Darmstadt, Germany). Salinity (34±0.7 g L^{-1}) was measured daily with a refractometer (Atago S/Mill-E, Tokyo, Japan). A natural photoperiod was maintained throughout the experimental period (13 h light : 11 h darkness).

Figure 2.1. Circular tank in which dusky kob were kept when fed the trial diets which formed part of the recirculating system that was described in detail in Section 2.2.2.

Figure 2.2. Settlement tanks (modified Guelph system) were connected to the recirculating system.

2.2.4. Experimental fish

Hatchery-reared dusky kob were supplied by Pure Ocean Pty Ltd (East London, South Africa) and acclimated to laboratory conditions for a one-month period. Prior to the trial, during the acclimation period, they were fed a commercial finfish diet once daily to apparent satiation (45 % protein and 18 % lipid; Marifeed Pty Ltd, South Africa). Fish were distributed to their respective tanks seven days prior to the faecal-collection period to allow for acclimation to experimental diets. Nineteen juvenile *A. japonicus* (mean weight: 70±0.2 g)

were randomly assigned to each of the eighteen (350 L) circular and fifteen (90 L) conical digestibility tanks, for stripping, dissection and settlement faecal collection methods. For all treatment groups, no fish was used more than once in any treatment.

2.2.5. Experiment 1: Gut evacuation period

The fish were stocked into each of the settlement tanks (Section 2.2.2, Figure 2.2) and were fed to satiation between 08:00 h and 10:30 h. Routine tank cleaning followed the same procedure as described above. The faeces were collected from each tank every two hours for 24 h after feeding and the masses of recovered faeces were recorded.

2.2.6. Experiment 2: Comparison of dietary markers

Diets were formulated to include 0.5 % of each dietary marker, i.e. celite for AIA, Cr_2O_3 and TiO_2 (Table 2.1). Each treatment combination was randomly assigned to the fish that were stocked into three of the settlement tanks (Figure 2.2), to make three replicates per treatment. The duration of the digestibility trial was twenty-one days: seven days to acclimate to the diets and fourteen days for faecal collection. Diets were fed to apparent satiation between 10:30 h and 13:30 h (maximum faecal collection time was estimated from Section 2.2.5). Tank cleaning, faecal collection and storage followed the same procedure as outlined in Section 2.2.2. Daily faecal collections from each tank were pooled over consecutive days, oven dried at 60 ^{o}C for 24 h, and stored at -20 ^{o}C for analysis.

Three experimental diets were formulated containing three dietary markers. Diet 1 contained 0.5 % chromic oxide, Diet 2 contained 0.5 % Celtic-diatomaceous earth and Diet 3 contained 0.5 % titanium dioxide. Each diet was analysed separately. Three replicate groups of fish were used to test each dietary formulation (Table 2.1). Sources and compositions of all the feed ingredients used in the formulation of experimental diets are presented in Appendix 1.

The dry ingredients were weighed and mixed with an industrial food mixer (Macadam's Baking Systems, SM-201, Cape Town, South Africa). The fish oil and water were subsequently introduced to the dry ingredients and mixed to a homogenous dough. The dough was cold-extruded (ICME Motor electric Bologna, Italy), cut into pellets (2-mm), placed on trays and dried at 40 °C for 16 h and then frozen at -20 °C until used.

Table 2.1. Diet formulation and proximate analysis of the experimental diet.

Ingredients	Acid insoluble ash-diet (g/100g feed)	Chromic oxide diet-diet (g/100g feed)	Titanium dioxide-diet (g/100g feed)
Fishmeal standard	50.0	50.0	50.0
Soybean meal	14.0	14.0	14.0
Corn starch	23.0	23.0	23.0
Carboxymethylcellulose	2.5	2.5	2.5
Marine fish oil	8.0	8.0	8.0
Vitamin/mineral premix	2.0	2.0	2.0
Acid insoluble ash	0.5	-	-
Chromic oxide	-	0.5	-
Titanium oxide	-	-	0.5
Proximate analysis of the experimental diet (dry matter basis)			
Dry matter (%)	93.8	95.0	95.1
Protein (%)	43.0	41.7	45.5
Lipid (%)	10.7	11.6	9.6
Energy (MJ/kg)	20.0	18.1	22.4
Acid insoluble ash	0.3	-	-
Chromic oxide	-	0.49	-
Titanium oxide	-	-	0.53

2.2.7. Experiment 3: Comparison of faecal collection methods

Fish were stocked in both the circular tanks and settlement tanks (Section 2.2.2). Using the most effective marker established in Section 2.2.6, tests were carried out to compare apparent digestibility of feed when faeces were collected by stripping, dissection and collection from

the settlement tanks. Each treatment combination was randomly assigned to three experimental tanks. Daily faecal collections for each individual tank were pooled, oven dried at 60 °C for 24 h, and stored at -20 °C for chemical analysis.

Stripping method

The stripping was performed once every three days to minimize stress due to handling, six hours after the morning feeding before defaecation. Feaces were collected before defaecation, which in dusky kob starts 8-h post feeding (Section 2.3.1). Fish were removed from their respective tanks and placed in a smaller aerated tank containing the anaesthetic 2-phenoxy-ethanol (0.2 ml L^{-1}). Once anaesthetized, the fish were removed and blotted dry. To extract faeces, wet paper was placed over the head region of the fish to restrict movement and secure the fish, and gentle pressure was applied just above the anus with the thumb and forefinger, extracting faeces from the distal intestinal tract. Care was maintained to ensure that the faeces were not contaminated by urine, mucus or other contaminants from the collection vessels (Glencross *et al.*, 2005; Rawles *et al.*, 2010).

Dissection method

Digesta were collected by dissecting six fish from each tank at the end of the experiment. Fish were euthanized using anaesthetic 2-phenoxy-ethanol (8.0 ml L^{-1} overdose) and faecal material contained in the terminal end of the intestine was removed (Austreng, 1978).

Settlement method

Nine of the tanks that formed part of the system described in Section 2.2.2 were used in this experiment. Faeces were collected from all the nine settlement tanks using the same method described in Section 2.2.2.

2.2.8. Chemical and digestibility analysis

All laboratory proximate composition analyses (crude protein, lipid and energy) followed procedures recommended by the Association of Official Analytical Chemists (AOAC, 2003). Dry matter was calculated by gravimetric analysis following oven drying at 100 °C for 24 h. Chromic oxide was determined by spectrophotometric analysis following digestion in a Kjeldhal system with nitric and perchloric acid (Lied *et al.*, 1982). Titanium was measured spectrophotometrically after digestion using atomic absorption spectrophotometry according to Short *et al.* (1996). Acid-insoluble ash was determined by digesting the feed or faeces for 30 min in 200 mL 4HCL, filtering through ashless filter paper (Whatman no. 41) with boiling water until free of acid and ashing for six hours at 650 °C. Crude protein was determined using the Dumas combustion method in a LECO FP2000 Nitrogen analyser (AOAC, 2003). Lipid was extracted from the samples by solvent petroleum ether using a Buchi 810 Soxhlet fat extractor (AOAC, 2003). The lipid percentage was calculated by gravimetric analysis. Gross energy was determined using a bomb calorimeter (DDS Isothermal CP500) (AOAC, 2003). Apparent digestibility coefficients (ADCs) for protein and amino acid contents were determined using Equation 2.1 (Maynard and Loosli, 1969):

$$\text{ADC (\%)} = 100 - \left\{100 \times \left(\frac{\text{\% marker in diet}}{\text{\% marker in faeces}} \times \frac{\text{\% nutrient in faeces}}{\text{\% nutrient in diet}}\right)\right\} \qquad \text{Equation 2.1}$$

2.2.9. Statistical analysis

Results were presented as mean (± standard deviation) of the data collected from the three replicate tanks that were used for each treatment. The Kolmogorov-Smirnov test was used to determine if the residuals of the data were normally distributed and the Levene test was used to check for homogeneity of variances. Treatment means were compared using a one-way analysis of variance (ANOVA), and Tukey's multiple comparison test was used to evaluate

the difference among individual diets at $p<0.05$. All statistical analyses were conducted using STATISTICA™ Version 16.0.

2.3. RESULTS

2.3.1. Gut evacuation period

Faecal quantities increased between 10 and 18 h after feeding, and maximum faecal production occurred at 18 h post feeding (Figure 2.3). After 18 h post feeding, the faecal quantities collected declined. Therefore, faeces were collected 18 h post feeding in settlement facecal collection method. This is the optimum time period to collect sufficient faecal quantity for chemical analysis.

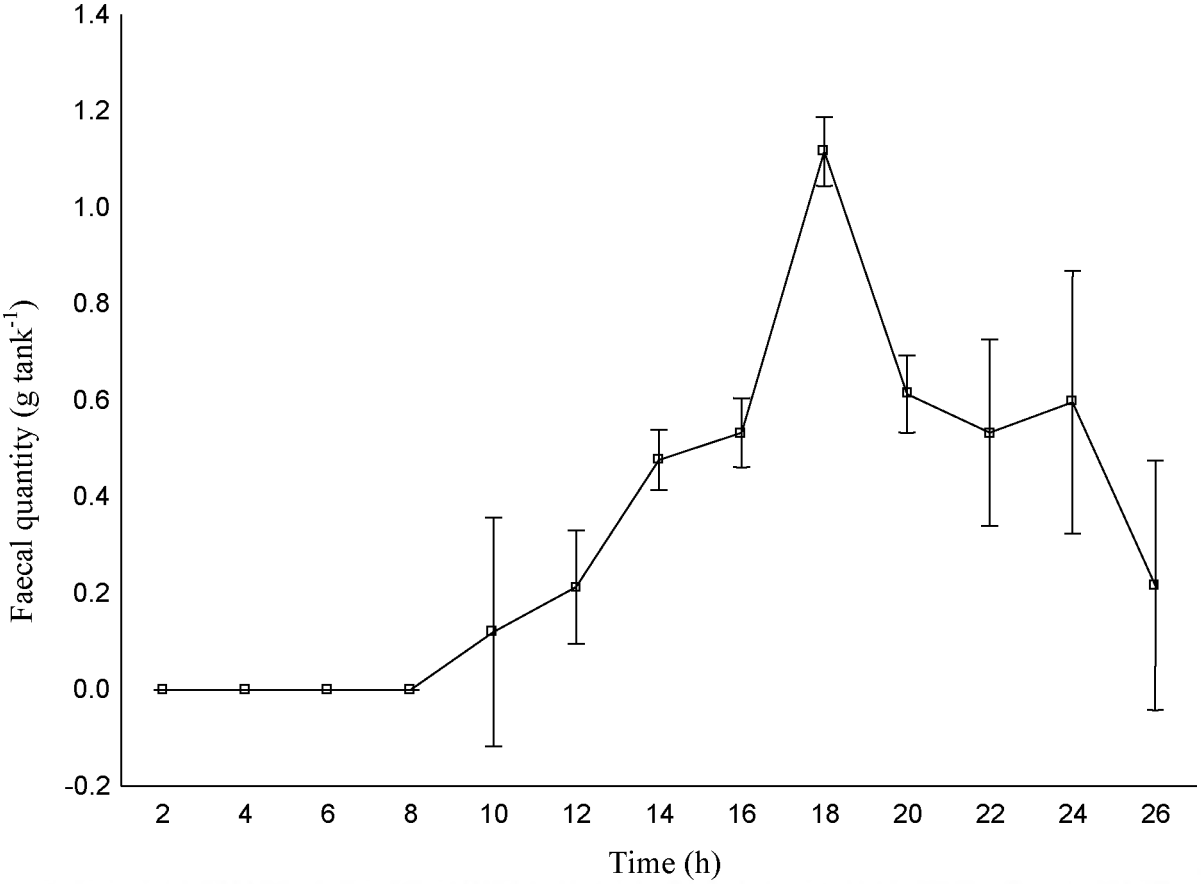

Figure 2.3. Mean (± standard deviation) quantities of faeces retrieved over varying periods post feeding.

2.3.2. Comparison of dietary marker techniques

Apparent digestibility coefficients for dry matter, protein, lipid and energy were significantly affected by the digestibility markers ($p<0.05$). The mean apparent protein digestibility values were 93.2±0.9 %, 79.1±3.8 % and 79.7±4.9 % for AIA, Cr_2O_3 and TiO_2, respectively ($p=0.005$). Lipid digestibility coefficients were 97.0±0.9 %, 90.5±3.5 % and 83.7±6.1 % for AIA, Cr_2O_3 and TiO_2 respectively ($p=0.021$). Energy digestibility coefficients were 93.0±0.8 %, 78.2±2.9 % and 80.5±2.7 % for AIA, Cr_2O_3 and TiO_2, respectively ($p<0.005$).

In all instances, the estimated values of protein, energy and dry matter digestibility, AIA resulted in significantly higher ADCs. With the exception of the lipid digestibility coefficients, there were no significant differences between all the estimated digestibility coefficients using either the Cr_2O_3 or the TiO_2 markers (Figure 2.4). The standard deviation of the mean protein, lipid and energy ADCs were all less than one percent for AIA, whereas they ranged from close to three to more than six percent for Cr_2O_3 and TiO_2 (Figure 2.4).

2.3.3. Comparison of faecal collection methods

An insufficient volume of faeces was collected when using the dissection and stripping method, and this made it impossible to carry out the protein, lipid and energy analyses required to determine ADC using these parameters. As such, ADC in this experiment was limited to that calculated using dry matter only. The ADCs calculated using dry matter were not significantly different between the different faecal collection methods (Table 2.2). Of the markers examined, AIA by settelement method exhibited good faecal recovery (Table 2.3).

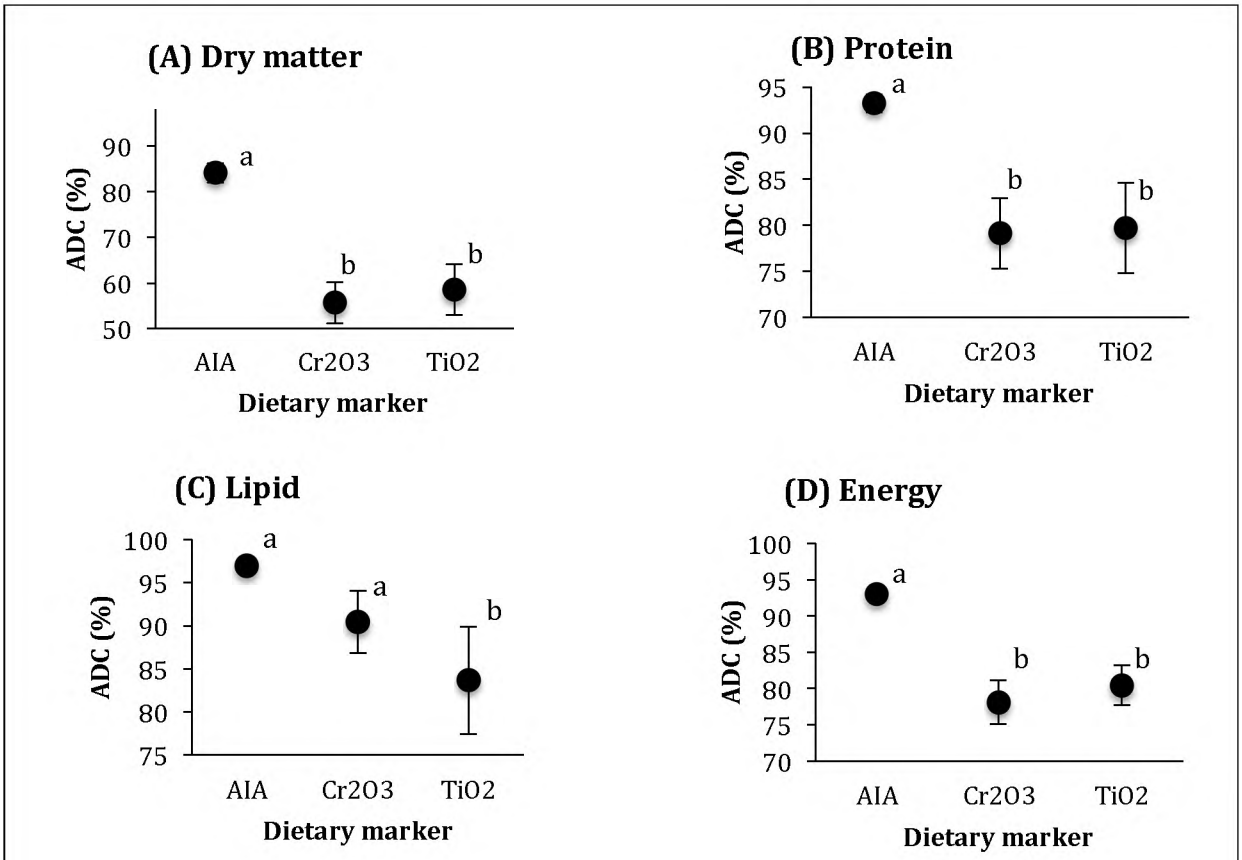

Figure 2.4. Mean (± standard deviation) apparent digestibility coefficient (ADC, %) of (A) dry matter, (B) protein, (C) lipid and (D) energy calculated using either acid-insoluble ash (AIA), chromic oxide (Cr_2O_3) or titanium dioxide (TiO_2) as the dietary marker. Means with different alphabetical letters, on each graph, were significantly different (ANOVA, $p<0.05$).

Table 2.2. Mean (± standard deviation) apparent digestibility of dry matter using acid-insoluble ash and three methods of faecal collection (ANOVA, p=0.05).

Faecal collection methods	Dry matter (%)
Stripping	77.0±10.7
Settlement	83.5±2.2
Dissection	80.1±4.9

Table 2.3 Marker content of dusky kob faeces (mean value of three sample replicate expressed in % dry matter, ±standard deviation).

	Marker type		
	Acid-insoluble ash	Chromic oxide	Titanium dioxide
Stripping	1.48±0.45	0.92±0.04	1.19±0.06
Settlement	1.84±0.24	1.07±0.11	1.23±0.17
Dissection	1.57±0.40	-	-

2.4. DISCUSSION

Gut evacuation period

The highest evacuation rate was recorded at 18 h post feeding. This was in agreement with the study of Hien *et al.* (2010), who established that the optimal faecal collection time was between 12 to 18 h post feeding for Tra catfish *Pangasinodon hypothalamus,* and that the ADCs calculated using faeces collected for each separate two-hour period showed no significant differences across time. In a recent study with *A. japonicus*, Booth *et al.* (2013) collected faecal samples from a settlement tank at 18 h post feeding. They reported that faeces from *A. japonicus* were reasonably intact and that the collection by settlement tank results in only minor losses in dry matter. In this study, faeces were settled gently at the base of the settlement tank by gravity and handled with care to avoid causing loss of nutrient. It was established that the optimal faecal output in *A. japonicus* was 18 h post feeding.

Dietary marker techniques

In the present study, it was evident that the digestibility coefficients estimated, using AIA as the marker, were consistently higher than those using either Cr_2O_3 or TiO_2 as the marker. Chromic oxide showed lower digestibility coefficients among replicate tanks of fish. The magnitude of variation in digestibility coefficients obtained using AIA was always lower than those obtained with either of the alternative markers.

The use of supplementary celite to increase total AIA levels may have reduced the variability of AIA determination in faecal samples in this study. These results are generally consistent with the findings of Atkinson *et al.* (1984) from studies on rainbow trout using celite-supplemented AIA. Acid-insoluble ash from supplemented celite, a form of diatomaceous silica, has been reported to be an effective marker for digestibility studies in humans (Rowan

et al., 1991), chickens (Cheng and Coon, 1990) and some fish species including rainbow trout *Oncorhynchus mykiss* (Atkinson *et al.,* 1984; Norambuena *et al.,* 2013; Saravanan *et al.,* 2012 and Turchini *et al.,* 2013), hybrid stripped bass *Morone chrysops x M. saxatilis* (Li *et al.,* 2008), *Apostichopus japonicus* (Xia *et al.,* 2013), European seabass *Dicentrarchus labrax* (Tibaldi *et al.,* 2015), tilapia *Oreochromis aureus* (Goddard and Mclean, 2001), and Nile tilapia *Oreochromis niloticus* (Jimoh *et al.,* 2010; Keramat and Schrama, 2015). However, AIA has been reported to be an inappropriate marker for use with lemon shark, *Negaprion brevirostris* (Wetherbee and Gruber, 1993). The reason for this contradiction could be attributed to sources of AIA (Vandenberg and De la Noüe, 2001).

Some studies with various species have reported that Cr_2O_3 passes through the digestive tract at a different rate from that of the nutrient under study (Kaushik and de Oliva Teles, 1985) and can influence nutrient digestibility (Shiau and Liang, 1995). Shiau and Lin (1993) also reported that small amounts of Cr_2O_3 could be absorbed into tissue, which can cause underestimation of digestibility. The reason for the difference in results obtained between Cr_2O_3 nor TiO_2 and those obtained using AIA in digestibility studies with *A. japonicus* needs to be investigated in future research.

Faecal collection method

Stripping was not considered an effective method of obtaining faeces from *A. japonicus*. This was because of the difficulties associated with obtaining sufficient samples from the fish due to induced spontaneous defaecation by the fish following the use of anaesthetics to minimize handling stress. Attempts to obtain faeces by stripping were abandoned by Allan *et al.* (1999), who were unable to collect sufficient samples from silver perch *Bidyanus bidyanus*. Faecal stripping is associated with repeated handling of the fish, which increases the level of stress in fish and may result in an underestimation of digestibility (Hajen *et al.,* 1993). The

use of anaesthetics to minimize stress has also been reported to induce spontaneous defaecation (Spyridakis *et al.,* 1989), thus accelerating intestinal transit, which will further alter nutrient absorption dynamics. Cho and Kaushik (1990) suggested that forced evacuation of faeces by stripping could result in the addition of intestinal epithelium to faecal contents.

In this study, dissection was abandoned for economic reasons. Dissection may result in some under-estimation of digestibility values following euthanasia, faeces may redistribute within the gut, with less digested material passing into the hindgut before dissection (Percival *et al.,* 2001). However, sufficient samples collected using AIA as the marker for the three collection methods, revealed that dry matter ADCs calculated using faeces obtained by dissection and stripping were not significantly different from those calculated by the settlement method. This was consistent with the findings of Austreng (1978) who reported that ADC values obtained from stripping are highly correlated with those obtained by dissection in a digestibility experiment with rainbow trout. Vandenberg and De la Noüe (2001) reported that ADCs for rainbow trout were consistently higher using the settlement method (Guelph system) and lowest using the stripping method. Hajen *et al.* (1993) concluded that the settlement method (Guelph system) of faecal collection is a reliable digestibility procedure for post-juvenile chinook salmon *Oncorhynchus tshanytscha* in seawater. The ADCs calculated using faeces obtained by dissection were lower than those calculated using faeces collected by the settlement method (Spyridakis *et al.,* 1989). Similarly, Amirkolaie *et al.* (2005) reported that the recovery percentage of faeces was higher with the settlement method compared with other faecal collection methods.

In this study, settlement collection was used as the preferred method primarily due to the ability to collect a larger faecal sample size within a short time, without stressing or sacrificing fish. However, collecting faeces after they have been voided can result in leaching of dry matter and nutrients, and lead to the overestimation of digestibility values. Collection

facilities that ensure rapid settlement of faecal material from the water column (Spyridakis *et al.*, 1989; Cho and Kaushik, 1990; Hajen *et al.*, 1993) have been used to reduce this problem (Allan *et al.*, 1999). *A. japonicus* faecal material settled at the terminal end of the settlement tank within a few minutes of being voided.

2.5. CONCLUSION

The results obtained in the present study have shown that 18 h post feeding is considered as the optimum faecal collection period for *A. japonicus*. Faecal collection by the settlement method was recommended to obtain the most reliable digestibility data. The key parameter that was considered here was the level of variability, as noted in the magnitude of the standard deviation for other methods examined. In this study, highly variable ADCs for Cr_2O_3 and TiO_2 were found in faecal samples, suggesting loss of markers relative to nutrient content. This observation may be because Cr_2O_3 and TiO_2 are heavier and might aggregate on the bottom of the feed during the mixing process. It is possible that there was a lack of uniform dispersion of Cr_2O_3 and TiO_2 in the feed as well as in the faecal matter and thus a larger devaiation around the mean might have occurred. Further work is warranted to determine possible analytical errors. Acid insoluble-ash had higher ADC values with lower standard deviation in most cases. Therefore, AIA supplemented with celite is preferred for routine dusky kob *A. japonicus* feed digestibility studies using diets formulated from practical ingredients.

CHAPTER 3

APPARENT PROTEIN AND AMINO ACID DIGESTIBILITY OF FEED INGREDIENTS

3.1. INTRODUCTION

In many respects the culture of carnivorous fish relies on the availability of fishmeal, which is a palatable and highly digestible feedstuff with balanced essential amino acids that is ideal for feeding fish (Wang *et al.,* 2010). However, the increasing cost of fishmeal and projected shortages as requirements for aquafeeds increase; create problems for cost-effective feed formulation (Tacon and Metian, 2008; Hertrampf and Piedad-Pascual, 2012). The development of a nutritionally complete cost-effective diet for carnivorous fishes, such as dusky kob *Argyrosomus japonicus*, in which the fishmeal (FM) is partially replaced with alternative ingredients, is needed. However, the availability of the nutrients in these alternative ingredients needs to be established in order to formulate diets that do not compromise fish growth and health.

The determination of nutrient digestibility is one of the most important aspects in the evaluation of a potential feed ingredient for use in dietary formulations (Allan *et al.,* 2000; Glencross *et al.,* 2007). Ingredient digestibility provides a measurement of the proportion of nutrients, which an animal can obtain from a particular ingredient through its digestive and absorptive processes (Glencross *et al.,* 2007). As nutrients are not available to an animal before they are absorbed, the nutritional value of ingredients depends not only on their chemical composition but also on the digestibility of these nutrients.

Generally, the amino acid (AA) digestibility of ingredients is rarely estimated (Wilson *et al.,* 1981; Anderson *et al.,* 1992; Sadiku and Jauncey, 1995), with most researchers opting for

developing the apparent protein digestibility (APD) of ingredients because it is easier and less expensive than amino acid digestibility. Previous research that aimed to determine the digestibility of various feed ingredients in *A. japonicus* is limited to methods that included apparent dry matter and protein digestibility only (Booth *et al.*, 2013). Research into the digestibility of amino acids has not been conducted for this species. High protein digestibility by itself does not give a good indication of the digestibility of essential amino acids (EAAs), which need to be available in sufficient quantities and in adequate balance for the fish to support optimal feed conversion and somatic growth (De Silva *et al.*, 2000). In order to provide adequate but not excessive levels of EAAs, it is important to formulate diets based on available EAAs rather than gross EAA levels. The nutritive value of protein is determined not simply by its amino acid composition but also by its ability to supply biologically available EAAs for protein synthesis (Tacon and Cowey, 1985). Knowledge of the EAA digestibility of feedstuffs is particularly important in formulating fish diets since the minimum quantity of high-protein feedstuffs can be included to meet the requirement for EAAs. This will aid the selection of appropriate ingredients and the formulation of cost-effective diets (Hajen *et al.*, 1993).

Apparent digestibility of feed ingredients for aquatic animals has been determined with an individual test ingredient (Masagounder *et al.*, 2009). Most researchers evaluate the digestibility of feed ingredients in combination with other ingredients in the test diet (Allan *et al.*, 2000; Lu *et al.*, 2015; Heinitz *et al.*, 2015; Hernández *et al.*, 2015). The assumption is that the digestibility of nutrients is additive, i.e., digestibility of a nutrient in one ingredient does not affect the digestibility of the same nutrient in another ingredient (Cho *et al.*, 1982). In this method, the test ingredient replaces part of a reference diet to obtain the test diet. Cho *et al.* (1982) developed this method for digestibility studies in fish with test diets comprising 30 % of the test ingredient and 70 % of the reference diet. An advantage of this method over testing

ingredients singly is that the test ingredients may be more palatable to the animal when fed with other ingredients especially when evaluating plant protein sources or other less palatable protein sources.

Additivity of digestibility coefficients determined for individual feed ingredients in compound and fishmeal based diets has been demonstrated in fish using a wide range of ingredients in fish (Watanabe *et al.,* 1996; Lupatsch *et al.,* 1997; Allan *et al.,* 1999; Kissil *et al.,* 2000; Tibbetts *et al.,* 2006). Non-additivity of individually determined apparent digestibility coefficients (ADCs) in compound diets has also been reported in crayfish (Reigh *et al.,* 1990) and abalone *Haliotis midae* (Sales and Britz, 2002). However, there is no information on additivity of ingredients in *A. japonicus* and this will be investigated here for the first time.

The present study was conducted to evaluate:

1) apparent protein and amino acid digestibility of four feed ingredients including two fishmeals, pork meal and poultry meal as a single protein source in the diets for *A. japonicus*;

2) apparent protein and amino acid digestibility of five ingredients comprising canola meal, corn gluten meal, soybean meal, sunflower meal and blood meal substituted into a reference diet; and

3) additivity of apparent protein and amino acid digestibility values of poultry meal and pork meal determined either with single protein diets or by means of substitution in reference diets.

3.2. MATERIALS AND METHODS

3.2.1. Experimental animals

Dusky kob *A. japonicus* were supplied by a commercial hatchery (Pure Ocean Pty. Ltd, East London, South Africa) and acclimated to laboratory conditions for one month prior to the experiment, during which time they were fed a commercial finfish diet once daily to apparent satiation (45 % protein and 18 % lipid; Marifeed Pty Ltd, South Africa). Twelve fish (mean weight: 133±1.6 g standard deviation) were randomly assigned to each of the experimental tanks and fed the test diets for seven days prior to the faecal-collection period to allow for acclimation to experimental conditions.

3.2.2. Environmental variables

As described in Section 2.2.3.

3.2.3. Experimental systems

All the experiments were carried out in the 18 holding tanks (Figure 2.1, Chapter 2) and 15 faecal collection tanks (Figure 2.2, Chapter 2). Approximately 10 % of the 10 000 L recirculating system's water was replaced daily with seawater pumped from the Kowie River Estuary.

3.2.4. Experiment 1: Apparent protein and amino acid digestibility of animal feed ingredients using a single protein source

Feed ingredients were selected based on their local availability and potential for use in dietary formulations. The four test feeds were prepared as single protein diets for the fish and included: fishmeal-prime (crude protein 64.8 %; a low fishmeal temperature made of whole

fish pelagic species), fishmeal-standard (crude protein 57.2 %; drying phase is direct hot air made from a whole fish pelagic species), poultry meal (PLM) and pork meal (PKM) (Table 3.1). Each diet was supplemented with 0.5 % celite (acid-insoluble ash) as an indicator for determining the digestibility, the test ingredient and a starch binder (Table 3.2). All the diets were isonitrogenous (43 % protein) and isoenergetic (17.8 MJ kg^{-1}) (Table 3.2). The inclusion level of starch varied between diets (Table 3.2), but the maximum inclusion level remained within a range found to have no detrimental effects for dusky kob growth and health (Mabasa, 2017). Sources and compositions of all the feed ingredients used in the formulation of experimental diets are presented in Appendix 1.

3.2.5. Experiment 2: Apparent nutrient digestibility of protein ingredients substituted into a practical reference diet

Apparent protein and amino acid (excluding tryptophan and cysteine) digestibility of canola meal (CNM), corn gluten meal (CGM), soybean meal (SBM), sunflower meal (SFM) and blood meal, spray dried (BLM) (Table 3.3) were determined by including these ingredients at 30 % into a practical reference diet (Table 3.4). Amino acid digestibility in the reference diet are presented in Appendix 2. Fishmeal was chosen as the protein source in the reference diet. Each diet contained 0.5 % celite (acid-insoluble ash) used as an indicator for determining the digestibility (Table 3.4).

3.2.6. Diet preparation

The feed ingredients were weighed and mixed with an industrial food mixer (Macadam's Baking Systems, SM-201, Cape Town, South Africa). The fish oil and water fractions were subsequently introduced to the dry ingredients and mixed to a homogenous dough. The dough was cold-extruded (ICME Motor electric Bologna, Italy) and cut into pellets (four

millimeters), placed on trays and dried at 40 °C for 16 h in a dryer (9000 Series Scientific Oven, South Africa). The dried pellets were stored in plastic packets at -20 °C until used.

3.2.7. Feeding and faecal collection

Dusky kob was purged of feed for 24 h prior to receiving the test feed. The first seven days were used for acclimation to the feed and no faeces were collected. Fish were fed to satiation between 10:00 h and 13:30 h. Within one hour after feeding, the rearing tank and collection column were flushed to remove any faeces, uneaten feed or bacterial matter. The following morning, at 8:00 h, faecal matter was collected, dewatered, and stored in plastic bottles. Faecal collection was undertaken over a 15 d period and the faeces collected over this period were combined for each replicate tank. For all treatment groups, faecal collections were preserved at -20 °C until analyses were carried out. Prior to analyses, faeces were oven dried at 60 °C for 24 h, and stored at -20 °C.

3.2.8. Experiment 3: Comparison of two methods used to determine apparent protein and amino acid digestibility values

The apparent protein and amino acid (excluding tryptophan and cysteine) coefficients for poultry meal (PLM) and pork meal (PKM) were compared by using (a) reference diets-substitution method (Section 3.2.5) and (b) single protein source method (Section 3.2.4). The data were used to predict additivity of these feed ingredients in kob diets.

Table 3.1. Chemical composition (%, on dry matter basis) of the feedstuffs.

Components	Fishmeal-prime[3]	Fishmeal-standard[3]	Poultry meal[4]	Pork meal[4]
Crude protein[1]	66.9	57.4	65.6	49.2
Amino acid (% AA)[2]				
Arginine	4.7	4.9	4.7	5.1
Histidine	2.5	1.3	2.4	1.8
Isoleucine	2.4	2.4	2.9	1.5
Leucine	4.2	3.8	4.9	3.1
Lysine	4.3	3.5	6.2	3.5
Methionine	1.2	1.6	2.1	0.9
Phenylalanine	2.3	2.2	2.7	1.9
Threonine	2.6	2.6	3.1	1.8
Valine	2.8	3.0	3.1	2.2
Aspartic acid	5.3	4.4	6.2	1.3
Glutamic acid	8.8	7.6	9.2	3.3
Alanine	4.1	3.8	3.8	2.9
Glycine	6.4	4.3	4.0	7.7
Serine	2.7	2.6	2.9	2.3
Proline	4.3	2.7	2.7	4.7
Tyrosine	1.3	1.0	2.1	0.9

Method used to determine crude protein (CP): [1] Dumas combustion method in a LECO FP2000 Nitrogen analyser (AOAC, 2003).
Method used to determine amino acid content: [2] Chromatographic and ion exchange analyses.
[3] Marifeed Pty Ltd, Hermanus, South Africa.
[4] Ingredients sourced and provided by Montego Pty Ltd, Eastern Cape, South Africa.

Table 3.2. Composition of diets used for the determination of digestibility of single protein source in Experiment 1.

Ingredients (%)	Fishmeal-prime	Fishmeal-standard	Poultry meal	Pork meal
Fishmeal-prime	72.90			
Fishmeal-standard		64.10		
Poultry meal			65.60	
Pork meal				86.00
Maize starch	20.58	22.80	4.50	2.80
Fish oil	5.90	6.40	11.50	8.70
Carboxymethyl-cellulose	0.02	6.10	17.80	1.90
Vitamin mixture	0.10	0.10	0.10	0.10
Acid insoluble ash (celite)	0.50	0.50	0.50	0.50

Table 3.3. Chemical composition of feed ingredients tested (% dry matter basis).

Components	CNM[2]	CGM[1]	SBM[1]	SFM[1]	BLM[1]
Crude protein	33.4	41.3	45.6	34.9	89.5
EAA					
Arginine	2.8	1.8	4.2	3.5	5.8
Histidine	0.8	0.9	0.9	0.9	3.0
Isoleucine	1.4	1.5	2.1	1.4	3.4
Leucine	2.4	5.6	3.2	2.0	8.6
Lysine	2.3	0.7	2.5	1.4	8.1
Methionine	0.6	0.7	0.5	0.5	1.2
Phenylalanine	1.4	2.2	2.2	1.6	5.0
Threonine	1.5	1.4	1.8	1.1	4.4
Val	1.7	1.7	2.1	1.7	5.4
NEAA					
Aspartic acid	2.4	2.4	5.0	2.9	7.5
Glutamic acid	6.1	8.6	8.4	6.5	8.9
Alanine	1.5	3.3	1.9	1.4	6.4
Glycine	1.8	2.2	1.5	2.1	5.5
Serine	1.6	2.1	2.2	1.4	3.5
Proline	2.1	3.4	2.2	1.4	3.3
Tyrosine	1.0	1.0	1.1	0.9	2.7

Abbreviations: AA, amino acid; EAA, essential amino acid; NEAA, non-essential amino acid; CNM, canola meal; CGM, corn gluten meal, SBM, soybean meal; SFM, sunflower meal and BLM, blood meal.

[1] Marifeed Pty Ltd, Hermanus, South Africa.

[2] Ingredients sourced and provided by Montego Pty Ltd, Eastern Cape, South Africa.

Table 3.4. Composition of the reference diet.

Ingredient	g 100 g^{-1} (dry weight basis)
Fishmeal [1]	64.0
Maize starch [1]	25.0
Carboxymethyl cellulose (CMC) [2]	3.0
Fish oil [1]	5.0
Vitamin/mineral mixture [1]	2.5
Celite (acid insoluble ash) [2]	0.5
Analysis (dry matter basis)	
Crude protein (%)	41.5
Gross energy (MJ/kg)	17.1
Essentail amino acids (g 100 g^{-1} dry matter)	
Arginine	3.61
Histidine	1.86
Isoleucine	1.96
Leucine	3.41
Lysine	3.70
Methionine	1.37
Phenylalanine	1.89
Threonine	1.85
Valine	2.20

[1] Fishmeal (prime 66.9 % CP), Marifeed Pty Ltd, Hermanus, South Africa.
[1] Fish oil, Marifeed Pty Ltd, Hermanus, South Africa.
[2] Sigma Aldrich Pty Ltd, Johannesburg, South Africa.

3.2.9. Chemical and digestibility analyses

Diet and faecal samples were analysed for acid-insoluble ash (AIA), protein and amino acid content. Acid-insoluble ash content was determined by digesting the feed or faeces for 30 min in 200 mL 4HCL, filtering through ashless filter paper (Whatman no. 41) with boiling water until free of acid and ashing for six hours at 650 °C. Crude protein was determined using the Dumas combustion method in a LECO FP2000 Nitrogen analyser (AOAC, 2003). Amino acid content was determined after acidic and basic digestion for chromatographic and ion exchange analyses (high-performance liquid chromatography) performed in sealed glass tubes under a nitrogen atmosphere at 110 °C. Methionine was determined by acid hydrolysis in 6N HCl after oxidation with performic acid. After hydrolysis, the solutions were vacuum

filtered, diluted to 0.25 M with 0.02 N HCl and adjusted to pH 8.5, and filtered through a Millipore membrane (0.45 mm). Tryptophan and cysteine were not measures and analysed.

3.2.10. Calculation of apparent digestibility

Apparent digestibility coefficients (ADCs) for protein and amino acid content were determined using Equation 2.1 (Section 2.2.8)

Apparent digestibility coefficients of test ingredients ($ADC_{test\ ingredient}$) and individual amino acids were calculated based on the digestibility of the reference diet and the test diets using the equation proposed by Forster (1999) and mathematically simplified by the recommendation of Bureau and Hua (2006) using Equation 3.1:

$$ADC_{test\ ingredients}\ (\%) =$$

$$ADC_{test\ diet} + (0.7D_{ref}/0.3D_{ingr})(ADC_{test\ diet} - ADC_{ref.\ diet}) \qquad \text{Equation 3.1}$$

where, $D_{ref.}$ is the nutrient content (%) of the reference diet and D_{ingr} represents the nutrient content (%) of the test ingredients.

3.2.11. Statistical analyses

Results were presented as mean (± standard deviation) of the data collected from the three replicate tanks that were used for each treatment. The Kolmogorov-Smirnov test was used to determine if the residuals of the data were normally distributed and the Levene test was used to check for homogeneity of variances. Treatment means were compared using a one-way analysis of variance (ANOVA), and Tukey's multiple comparison test was used to evaluate the difference among individual diets at $p<0.05$. Differences between the single protein diets and reference diets-substitution methods were determined by independence *t*-test. All statistical analyses were conducted using STATISTICA™ Version 16.0.

3.3. RESULTS

3.3.1. Experiment 1: Apparent protein and amino acid digestibility of animal feed ingredients using a single protein source

Protein digestibility

Protein digestibility ranged from 83.1 to 85.8 % for all test ingredients (Table 3.5). There were no significant differences for protein digestibility among FM-prime, FM-standard, PLM and PKM ($p=0.86$).

Essential amino acid digestibility

Apparent digestibility of arginine, isoleucine, leucine, phenylalanine, threonine and valine were not significantly different between the feed ingredients ($p>0.05$, Table 3.5). However, digestibility of histidine, lysine and methionine differed significantly between all treatments ($p<0.05$, Table 3.5). Apparent digestibility of histidine was highest (93.0±1.9 %) for FM-standard, followed by PKM (74.4±7.2 %), PLM (70.9±4.0 %), which did not differ significantly and was lowest (63.6 %) for FM-prime. Apparent digestibility of lysine in FM-standard (90.5±2.6 %) and PLM (89.9±1.1 %) were similar and higher compared with all other feedstuffs tested. This was followed by PKM (83.6±4.0 %). The apparent digestibility of lysine in FM-prime (73.3±9.8 %) was the lowest in comparison to other feedstuffs.

Methionine digestibility in FM-prime was higher (94.0±2.2 %) than in other feedstuffs tested. This was followed by PKM (87.7±4.4 %) and PLM (85.6±1.1 %), which did not differ significantly from each other, and its digestibility in FM-standard (83.0±1.4 %) was significantly lower than that in all other treatments.

Table 3.5. The mean (± standard deviation) apparent digestibility coefficient (ADC) for crude protein and amino acids (AA) of the test ingredients in single protein source dusky kob diets. Means with different superscripts within a row were significantly different (ANOVA, $p<0.05$, n=3).

ADC (%)	FM-prime	FM-standard	Poultry meal	Pork meal	Pvalue
Crude protein	84.5±4.1	83.8±3.9	85.8±1.4	83.1±5.5	0.86
EAA					
Arginine	92.7±2.5	92.8±1.9	92.1±0.8	88.8±4.7	0.33
Histidine	63.6±15.1^{c}	93.0±1.9^{a}	70.9±4.0^{b}	74.4±7.2^{b}	0.01
Isoleucine	85.8±5.2	87.0±3.7	87.2±1.8	87.7±5.1	0.95
Leucine	86.8±4.2	88.6±2.9	87.9±1.9	87.4±4.6	0.93
Lysine	73.3±9.8^{c}	90.5±2.6^{a}	89.9±1.1^{a}	83.6±4.0^{b}	0.02
Methionine	94.0±2.2^{a}	83.0±1.4^{c}	85.6±1.1^{b}	87.7±4.4^{b}	0.01
Phenylalanine	86.0±4.4	86.4±2.9	85.1±1.7	84.3±4.4	0.89
Threonine	92.0±2.7	87.5±3.9	85.4±2.0	85.6±4.8	0.16
Valine	88.8±3.7	86.2±4.3	85.6±2.3	87.4±5.1	0.76
Mean EAA	84.7	87.9	85.5	85.0	
NEAA					
Aspartic acid	68.1±9.9	85.9±9.0	83.4±2.9	77.8±6.1	0.08
Glutamic acid	81.5±6.0	88.6±3.7	89.1±1.5	85.0±4.9	0.19
Alanine	85.6±4.9	88.3±3.3	88.8±1.3	86.8±4.8	0.73
Glycine	82.6±6.2	84.1±4.7	87.2±1.7	84.1±5.3	0.69
Serine	89.9±3.5	91.4±3.0	86.0±0.9	86.4±4.6	0.20
Proline	86.0±4.7	84.0±4.6	86.3±2.4	84.9±5.5	0.92
Tyrosine	97.2±1.2^{a}	88.1±7.6^{b}	85.4±3.5^{c}	78.2±4.9^{d}	0.01
Mean NEAA	84.4	87.2	86.5	83.3	
Mean AA	84.6	87.6	86.0	84.3	

Abbreviations: AA, amino acid; FM, fishmeal; EAA, essential amino acid; NEAA, non-essential amino acid.

3.3.2. Experiment 2: Apparent nutrient digestibility of protein ingredients substituted into a practical reference diet

Protein digestibility

The apparent protein digestibility (APD) was significantly different from each of the feed ingredients evaluated ($p<0.04$, Table 3.6). That of sunflower meal (92.4±1.5 %) and soybean (92.0±0.6 %) was higher than all of the remaining test ingredients. This was followed by

canola meal (90.7±1.2 %) and blood meal (89.7±2.1 %), which did not differ significantly from each other. Corn gluten meal had the lowest APD (85.5±90.7 %, Table 3.6).

Amino acid digestibility

The mean amino acid digestibility for all the test ingredients ranged from 86.3 to 92.1 % (Table 3.6).

Essential amino acid digestibility

Apparent digestibility of histidine differed between treatments (p=0.04) with a mean ADC of 86.3±3.0 % for blood meal, which was significantly higher than that of all the plant ingredients (Table 3.6). Of the plants, the ADC for histidine was similar and higher for CNM (76.1±4.2 %) and CGM (75.4±3.8 %) compared with all others. Soybean meal and sunflower meal had the lowest ADC for histidine, both with an ADC of about 72 % (Table 3.6). All other EAA digestibility coefficients were similar (p>0.05, Table 3.6).

3.3.3. Experiment 3: Comparison of two methods used to determine apparent protein and amino acid digestibility values

Apparent protein digestibility did not differ significantly between treatments that used the single protein source diets and reference diets-substitution (fishmeal based diets) for both ingredients that were tested (poultry meal and pork meal). Similar results were observed for EAA using single protein diets and the reference diets-substitution values except for arginine and histidine in PLM (p=0.02) and lysine in PKM (p=0.04, Table 3.7). Acid insoluble-ash content of dusky kob feed and faeces are presented in Appendix 3.

Table 3.6. Apparent protein and amino acid digestibility (%) in the test ingredients (mean ± standard deviation, n=3).

Diet	CNM	CGM	SBM	SFM	BLM	Pvalue
Crude protein	90.7±1.2[b]	85.5±4.6[c]	92.0±0.6[a]	92.4±1.5[a]	89.7±2.1[b]	0.04
EAA						
Arginine	94.1±1.1	92.5±3.4	96.2±1.5	93.8±5.0	94.6±3.0	0.69
Histidine	76.1±4.2[b]	75.4±3.8[b]	72.4±5.4[c]	72.6±4.2[c]	86.3±6.7[a]	0.04
Isoleucine	92.0±1.3	87.8±4.2	93.2±1.9	92.2±6.0	92.5±4.5	0.51
Leucine	93.0±1.3	84.1±5.6	93.9±1.4	93.3±4.8	92.3±4.5	0.06
Lysine	95.1±1.6	95.8±2.8	95.9±0.9	94.1±3.3	95.0±2.5	0.89
Methionine	92.4±2.9	90.3±3.3	94.4±1.1	92.1±3.2	94.8±2.6	0.32
Phenylalanine	91.7±1.5	85.7±4.6	92.4±1.7	90.1±5.8	92.3±4.6	0.27
Threonine	91.5±1.6	88.9±3.6	93.8±1.1	91.0±6.5	93.6±3.7	0.53
Valine	91.8±1.5	87.9±3.8	92.9±1.7	91.6±6.4	92.3±4.6	0.56
Mean EAA	90.5	87.1	91.4	89.6	92.5	
NEAA						
Aspartic acid	91.6±1.4	88.8±3.3	93.2±1.1	95.0±2.8	87.5±7.0	0.16
Glutamic acid	94.3±1.0	85.8±4.1	94.4±1.1	92.4±4.8	92.1±4.6	0.08
Alanine	91.3±1.8	84.2±4.8	93.0±1.3	90.1±6.3	91.3±5.3	0.21
Glycine	87.8±2.2	85.2±5.2	92.9±0.9	81.4±9.2	91.3±4.4	0.13
Serine	88.6±3.4	85.6±4.9	93.0±1.3	86.0±11.0	92.1±4.5	0.46
Proline	88.3±2.5	81.7±5.0	91.1±1.2	86.0±8.2	91.9±4.3	0.15
Tyrosine	93.8±2.9	87.9±9.3	94.7±1.4	90.4±5.1	89.9±4.5	0.52
Mean NEAA	90.7	85.4	93.1	88.7	90.8	
Mean AA	90.6	86.3	92.1	89.2	91.8	

Means in the same row with different superscript (a, b, c) are statistically different ($p<0.05$)
Abbreviations: CNM, canola meal; CGM, corn gluten meal; SBM, soybean meal; SFM, sunflower meal; BLM, blood meal; AA, amino acid; EAA, essential amino acid; NEAA, non-essential amino acid.

Table 3.7. Comparison of single protein source diets vs. reference diets-substitution digestibility and amino acid digestibility values in pork meal and poultry meal for determination of additivity. Values are means ± standard deviation of three replicate analyses. Means with a different superscript within each row were significantly different (*t*-test, $p<0.05$).

	Poultry meal (%)			Pork meal (%)		
	Single protein diets method	Reference diets-substitution method	Pvalue	Single protein diets method	Reference diets-substitution method	Pvalue
Protein	85.8±1.4	77.3±6.5	0.09	83.1±5.5	78.2±5.0	0.32
EAA						
Arginine	92.1±0.8[a]	82.3±4.6[b]	0.02	88.8±4.7	86.8±7.7	0.72
Histidine	70.9±4.0[b]	87.0±6.0[a]	0.02	74.4±7.2	78.4±6.0	0.51
Isoleucine	87.2±1.8	84.4±3.4	0.27	87.7±5.1	88.4±5.5	0.88
Leucine	87.9±1.9	84.8±5.3	0.40	87.4±4.6	91.4±0.7	0.21
Lysine	89.9±1.1	90.3±3.2	0.83	83.6±4.0[b]	90.7±1.2[a]	0.04
Methionine	85.6±1.1	79.5±5.3	0.12	87.7±4.4	87.6±2.9	0.97
Phenylalanine	85.1±1.7	80.0±6.0	0.23	84.3±4.4	87.4±2.6	0.36
Threonine	85.4±2.0	87.9±7.1	0.59	85.6±4.8	87.0±4.8	0.73
Valine	85.6±2.3	80.3±7.4	0.31	87.4±5.1	89.3±1.2	0.57

EAA, essential amino acid.

3.4. DISCUSSION

This study evaluated the suitability of animal and plant protein ingredients for use in diets for *A. japonicus*. This is the first report of the essential amino acid digestibility for a variety of potential feed ingredients for inclusion in compound feeds for the species. High apparent protein digestibility and essential amino acid digestibility were observed for all ingredients tested.

Protein digestibility

Fishmeal was very well digested by *A. japonicus*. Apparent protein digestibility coefficients for the two fishmeals (84.5 and 83.8 %) examined were similar to the ADCs found for gilthead seabream *Sparus aurata,* spotted rose snapper *Lutjanus guttstus*, European seabass *Dicentrarchus labrax* and turbot *Scophthalamus maxima* (Table 3.8), although higher values

have been reported in Ecuador-FM (97 %) for *Argyrosomus japonicus* (Booth *et al.,* 2013) in comparison to the ADCs estimated for the two fishmeal tested in this study (Table 3.8).

With reference to protein digestibility of the fishmeal/soybean diet in Chapter two that was 93 %, whereas ADP for both fishmeal in Chapter three were lower than this, at about 84 %. Soybean exhibited a high digestibility value of 92 %, which could have increase the protein digestibility values in Chapter two of this study.

Poultry meal has been widely studied as an alternative protein source for FM in fish diets (Yigit *et al.,* 2006; Davies *et al.,* 2009). In this study, crude protein ADCs for FM (83.8-84.5 %) was similar to PLM (85.8 %). These results were similar to PLM values of other fish species (Table 3.8). The chemical composition and quality of animal product meals have been shown to differ considerably depending on raw materials and processing methods (Dong *et al.,* 1993). Forster *et al.* (2003) reported that meat and bone meal of porcine origin had lower nutrient digestibility than beef meat and bone meal. In this study, protein ADCs of PLM and PKM were comparable to that of fishmeal.

Soybean meal exhibited a high apparent protein digestibility coefficient of 92.0 %, which was similar to data obtained for meagre *Argyrosomus regius* (Olim, 2012; Feij *et al.,* 2012), mulloway *Argyrosomus japonicus* (Booth *et al.,* 2013) and other fish species (Table 3.8). Soybean meal appears to be a good protein source for *A. japonicus* according to its high protein digestibility in this study.

The apparent protein digestibility for CGM was 85.5 % and this value was similar to APD reported in other fish species (Table 3.8). The value obtained in this study for CGM protein digestibility was higher than the values for yellowtail *Seriola quinqueradiata* and turbot

Scophthalmus maxima as reported by Masumoto *et al.* (1996) and Wei *et al.* (2015) respectively (Table 3.8).

The apparent protein digestibility of blood meal in this study was 89.7 %. Similar digestibility coefficients were reported for other fish species (Table 3.8).

This study showed that sunflower meal exhibited a high apparent protein digestibility value of 92.4 %. This result was similar to the value of 95.5 % indicated by Booth *et al.* (2013) for sunflower meal by *A. japonicus*. These values were higher than the 69 % reported by Gaylord *et al.* (2004) for hybrid striped bass digestibility studies and 75.8 % APD for rainbow trout as reported by Smith *et al.* (1995).

The apparent protein digestibility of canola meal was 90.7 %. This result was similar to a range of apparent digestibility values as reported for different fish species (Table 3.8). The value was higher than values reported for rainbow trout, spotted rose snapper *Lutjanus guttatus* and Asian seabass *Lates calcaarifer* (Table 3.8). In this study, canola meal appears to be a good protein source for *A. japonicus*.

Table 3.8. Ingredients digestibility coefficients (%) for different fish species.

Feed ingredients	Crude protein (%)	Species	Reference
Fishmeal	83-84.5	Dusky kob, *Argyrosomus japonicus*	This study
	83.0	Gilthead seabream, *Sparus aurata*	Lupatsch *et al.*, 1997
	84.0	Spotted rose snapper, *Lutjanus guttstus*	Hernández *et al.*, 2015
	89.5	European seabass, *Dicentrarchus labrax*	Da Silva and Oliva-Teles, 1998
	87.68	Turbot, *Scophthalamus maxima*	Wei *et al.*, 2015.
	97.0	Mulloway, *Argyrosomus japonicus*	Booth *et al.*, 2013
Poultry meal	85.8	Dusky kob, *Argyrosomus japonicus*	This study
	82.5	Spotted rose snapper, *Lutjanus guttatus*	Hernández *et al.*, 2015
	87.1	Rainbow trout, *Oncorhynchus mykiss*	Cheng and Hardy, 2002
	89.5	Gilthead seabream, *Sparus aurata*	Lupatsch *et al.*, 1997
	88.0	Largemouth bass, *Micropterus salmoides*	Masagounder *et al.*, 2009
	91.0	Bluegill, *Lepomis macrochirus*	Masagounder *et al.*, 2009
	75.7	Mulloway, *Argyrosomus japonicus*	Booth *et al.*, 2013
Blood meal	89.7	Dusky kob, *Argyrosomus japonicus*	This study
	88.0	Rainbow trout, *Oncorhynchus mykiss*	Gaylord *et al.*, 2010
	86-87	Rockfish, *Sebastes schlegeli*.	Lee, 2002
	86.0	Hybrid striped bass, Morone saxatilis♀ X Morone chrysops♂.	Sullivan and Reigh, 1995
	90.4	Mulloway, *Argyrosomus japonicus*	Booth *et al.*, 2013
	90.0	Gilthead seabream, *Sparus aurata*	Lupatsch *et al.*, 1997
Soybean meal	92.0	Dusky kob, *Argyrosomus japonicus*	This study
	92.6	Mulloway, *Argyrosomus japonicus*	Booth *et al.*, 2013
	92.3	Atlantic cod, *Gadus morhua*	Tibbetts *et al.*, 2006
	83.2	Chinese sucker, *Myxocyprinus asiaticus*	Yuan *et al.*, 2010
	81.6	Rohu, *Labeo rohita*.	Hossain *et al.*, 1997
	95.9	Rainbow trout Oncorhynchus mykiss	Cho and Slinger, 1979

Table 3.8. Continued

Feed ingredients	Crude protein (%)	Species	Reference
Corn gluten	85.5	Dusky kob, Argyrosomus japonicus	This study
	88.9	Mulloway, *Argyrosomus japonicus*	Booth *et al.*, 2013
	86.3	Atlantic cod, *Gadus morhua*	Tibbetts *et al.*, 2006
	89.0	Rainbow trout, *Oncorhynchus mykiss*	Gaylord *et al.*, 2010
	89.0	Rainbow trout, *Oncorhynchus mykiss*	Watanabe *et al.*, 1996
	49.7	Yellowtail, *Seriola quinqueradiata*	Masumoto *et al.*, 1996
	48.5	Turbot *Scophthalmus maxima*	Wei *et al.*, 2015
Sunflower meal	92.4	Dusky kob, *Argyrosomus japonicus*	This study
	95.5	Mulloway, *Argyrosomus japonicus*	Booth *et al.*, 2013
	69.0	Hybrid striped bass, Morone saxatilis♀ X Morone chrysops♂.	Gaylord *et al.*, 2004
	75.8	Rainbow trout, *Oncorhynchus mykiss*	Smith *et al.*, 1995
Canola meal	90.7	Dusky kob, *Argyrosomus japonicus*	This study
	95.9	Mulloway *Argyrosomus japonicus*	Booth *et al.*, 2013
	81.5	Spotted rose snapper, *Lutjanus guttatus*	Hernandez *et al.*, 2015
	79.7-83.8	Asian seabass, *Lates calcaarifer*	Ngo *et al.*, 2015
	79.0	Rainbow trout, *Oncorhynchus mykiss*	Gaylord *et al.*, 2010

The differences in nutrient digestibility are associated with a number of factors including processing and drying of the ingredient, physiological character of fish and differences in experimental procedure used by various laboratories including faecal collection methods (Allan *et al.*, 2000; Lee, 2002; NRC, 2011).

Essential amino acid digestibility

High apparent digestibility of EAA from animal protein sources has been established for many fish species including spotted rose snapper (Hernández *et al.*, 2015), Atlantic salmon (Anderson *et al.*, 1995), bluegill and largemouth bass (Masagounder *et al.*, 2009). Poultry meal demonstrated a good EAA digestibility in this study (mean EAA 85.5 %). Similar to the findings of the present study, Wei *et al.* (2015), Lupatsch *et al.* (1997) and Masagounder *et al.* (2009) also observed comparable digestibility of EAA from PLM for turbot, gilthead seabream and largemouth bass respectively. Poultry meal is one of the most promising aqua feed ingredient due to the improvement in manufacturing technique applied to meal, which in turn improved its digestibility and nutritive quality. In this study, poultry meal presented good amino acid profile content and there were similarities in the EAA digestibility between FM and PLM, which indicated that PLM could be used as a potential protein source for *A. japonicus*.

All EAAs, with the exception of histidine, were available in sufficient quantities based on data obtained from this study. Despite the low methionine content in all the ingredients tested, this amino acid presented a high digestibility in all the ingredients tested for dusky kob. In this study all the protein examined showed very good and high digestibility values for these two EAAs.

In this study, EAAs availability in soybean meal was favourable. Essential amino acids digestibility values recorded for SBM in this study were comparable to values reported for other fish species. Lupatsch *et al.* (1997) reported high essential amino acid digestibility in SBM for arginine (96 %), lysine (92 %) and threonine (91 %), whereas histidine, valine, methionine, isoleucine and phenylalanine were 89 % for gilthead seabream. Masumoto *et al.* (1996) reported a high apparent EAA values in SBM for arginine (85.4 %) and lower

histidine digestibility value (53 %) for yellowtail *Seriola quinqueradiata*. Yuan *et al.* (2010) reported maximum essential amino acid digestibility in SBM for arginine (85.26 %) for Chinese sucker *Myxocyprinus asiaticus*, while the rest ranged from histidine (77.41 %) to threonine (59.88 %). Hernandez *et al.* (2015) reported the essential amino acid digestibility for arginine (90.7 %) and lysine (92 %), whereas, threonine, histidine, valine, methionine, isoleucine and phenylalanine were between 82.1-89.8 % for spotted rose snapper *Lutjanus guttatus*. This result showed that *A. japonicus* has a similar capacity to digest SBM as spotted rose snapper (Hernandez *et al.,* 2015), yellow tail seabream *Sparus latus* (Masumoto *et al.,* 1996; Wu *et al.,* 2006) and gilthead seabream (Lupatsch *et al.* (1997). In this study soybean meal has a high protein digestibility and essential amino acid digestibility, indicating that it is a potential plant protein source for *A. japonicus* diets.

Essential amino acids digestibility for CGM ranged from 95.8 % (lysine) to 75.4 % (histidine). Masumoto *et al.* (1996) found histidine digestibility in CGM to be 50.8 % for yellowtail. For turbot, amino acid digestibility in CGM ranged from lysine (64.12 %) to threonine (27 %) (Wei *et al.,* 2015). Gaylord *et al.* (2010) found essential amino acid digestibility in CGM for arginine to be 99 % and all others ranged from 96 % for histidine to 91 % for isoleucine. Anatomical and physiological differences in the digestive systems between species, different pH of CGM and processing might be the factors related to differences in digestibility between species (Masumoto *et al.,* 1996). Results from this study showed that CGM had the lowest protein digestibility coefficient and mean EAA digestibility in all the plant protein tested. This could be as a result of its amino acid contents especially for lysine and methionine (Table 3.3). If there is a very little content of AA in a diet and there is a high digestibility, it may not be of use to the animal.

The range of values for blood meal EAA digestibility in this study was between 95.0 % (lysine) to 86.3 % (histidine). The results of the present study are similar to the findings of Lupatsch *et al.* (1997), who reported that essential amino acid digestibility in BLM ranged from arginine (96 %) to methionine (89 %) for gilthead seabream. Gaylord *et al.* (2010) recorded essential amino acid digestibility for all the EAAs ranging from arginine (90 %) to isoleucine (87 %) for rainbow trout. Most animal products undergo heating and drying processes. This may be the factor contributing to differences among essential amino acid digestibility (Opstvedt *et al.,* 1984). Temperature treatment also has an influence on the essential amino acid digestibility of blood meal and other feed ingredients (Opstvedt *et al.,* 1984; Lupatsch *et al.*, 1997). Spray dried blood meal used in this experiment has a greater content of total and available amino acid. High apparent digestibility coefficients in this study suggested that blood meal might not be damaged by heat during the processing and that the drying process did not affect the digestibility of its essential amino acids.

In the present study, canola meal exhibited a favourable EAA digestibility, ranging from 95.1 % (lysine) to 91.5 % (threonine) with the exception of histidine, which was 76.1 %. This result is similar to the findings of Gaylord *et al.* (2010) who reported apparent amino acid digestibility value of 92 % for arginine in rainbow trout. Ngo *et al. (*2015) reported a maximum value of 92.1 % for arginine, followed by lysine and the lowest value was found in histidine (86 %). Canola meals have been shown to have considerable potential for FM replacement as they contain high protein content (Burel *et al.,* 2000) with a good amino acid profile, and high lysine digestibility. Many fish species have been shown to have good growth when fed diets containing CNM. These include rainbow trout (Gomes *et al.,* 1995), gilthead seabream (Kissil *et al.,* 2000), Japanese seabass *Lateolabrax japonicus* (Cheng *et al.,* 2010) and cobia *Rachycentron canadum* (Luo *et al.,* 2012). However, growth performance is restricted to 20-30 % of CNM in the diets due to the presence of anti-nutritional factors

(Burel *et al.,* 2000; Ngo *et al.,* 2015). This study suggests that CNMs are well digested by *A. japonicus* and could be used as a potential protein source for this species.

Histidine was found to be least available to *A. japonicus* among all EAAs in the ingredients tested. Similarly, a lower digestibility value of histidine from SBM was recorded for Chinese sucker *Myxocyprinus asiaticus* (77.4 %; Yuan *et al.,* 2010), and yellowtail (50.8 %, CGM; 53 %, SBM) (Masumoto *et al.,* 1996).

It is interesting to note that dusky kob *A. japonicus* was able to digest plant protein efficiently. It is often observed that sensitivity to anti-nutritional factors varies between species of animal (Kumar, 1992). This indicates that *A. japonicus* might not be as sensitive to the anti-nutritional factors that are present in the plant protein tested.

Comparison of two methods used to determine apparent protein and amino acid digestibility values

For digestibility data of a single protein source ingredient to be useful in least-cost formulation, it is assumed that the digestibility values of the single protein source ingredient would be similar to data obtained from the reference diets-substitution method. This assumption has been tested and validated for a number of fish species including rainbow trout, gilthead seabream, Atlantic cod *Gadus morhua* and seabass (Cho and Kaushik, 1990; Lupatsch *et al.,* 1997; Da Silva and Oliva-Teles, 1998; Allan *et al.,* 1999; Tibbetts *et al.,* 2006) but yet to be validated for *A. japonicus.* This study compared the single protein source diets and reference diets-substitution in order to test this assumption using poultry meal and pork meal. The results confirm that apparent digestibility is additive and that digestible EAA supply in a complete diet can be predicted based on EAA digestibility determined for individual feed ingredients. This was in agreement with the findings of Lupatsch *et al.* (1997)

and Tibbetts *et al.* (2006), who concluded that ingredient digestibility in gilthead seabream was additive for protein and essential amino acids. However, these results do not exclude possible interaction between the feed ingredients, particularly those containing a high level of fibre or anti-nutritive factors or carbohydrate. Lupatsch *et al.* (1997) reported that digestibility of carbohydrate in compound feeds was slightly lower than the individual feed ingredients. Apparent digestibility of mixed feeds containing such ingredients will be lowered by the influence of increased endogenous amino acid secretion, and true rather than apparent digestibility values may be more accurate in reflecting the additivity of digestible EAA supply (Angkanaporn *et al.,* 1996). Our results suggested that the apparent digestibility coefficient of animal protein ingredients could be calculated from compound diets to accurately determine protein and amino acid digestibility in *A. japonicus*, and possibly other carnivorous marine fish species.

Higher values obtained in digestibility studies may be attributed to faceal suoluble nitrogen compounds, which is always associated with the settlement faecal collection method (Sugiura, 1998). Comparison of digestibility coefficients for any ingredients between species is compromised because of differences in methodology used (Allan, 2000). In digestibility studies there are close agreement in digestibility coefficients derived using different methods of obtaining faeces (Cho *et al.,* 1982; Hajen *et al.,* 1993), others have found large discrepancies (Smith *et al.*, 1980). Provided the same method is used for different ingredients for a single species, the comparison between ingredients is valid and these data are critical if diets are to be formulated from a variety of ingredients with balanced digestible energy and digestible nutrient contents (Cho and Kaushik, 1990; Gomes *et al.,* 1995).

3.5. CONCLUSION

Blood meal showed high digestibility compared to the other animal protein sources tested. However, imbalance of leucine and isoleucine content in blood meal is known to be a cause of antagonism between amino acids and limits potential use of this ingredient in diets. Therefore, high inclusion levels of blood meal in practical diet formulation can negatively affect feed palatability. *Argyrosomus japonicus* is competent at digesting animal protein sources as well as plant protein feedstuffs based on high apparent digestibility coefficients obtained from this study. This is in agreement with research by Olim (2012) and Booth *et al.* (2013), who reported that *Argyrosomus species* are efficient at digesting protein from a variety of plant protein sources.

The assumption that digestibility of a nutrient in one ingredient does not interact with the digestibility of the same nutrient in another ingredient is true for animal protein in *A. japonicus* in this study. The variation between APD replicates in animal protein sources tested for additivity were large; this indicates the need for amino acid digestibility data when formulating diets, which contain a range of ingredients. The digestible essential amino acid values for individual ingredients can be used to estimate the essential amino acid digestibility in a complete diet.

In this study, poultry meal demonstrated a good EAA profile and digestibility, which indicated that it could potentially partially replace fishmeal diets for *A. japonicus.* Soybean meal EAA was more fully digested than any of the other ingredients tested. It is well known that soybean meal is a promising feed ingredient in the aquaculture industry compared to other plant ingredients. Adequate palatability of soybean meal and its excellent nutritional profile including high level of protein, complementary amino acid profile and relatively high nutritional digestibility proved it to be a valuable food for fish. Thus, SBM had a greater

potential to be used as a dietary replacement for FM for kob diets. Among protein sources tested, canola meal (mean EAA 90.5 %) and blood meal (mean EAA 92.5 %) were also effectively digested, so these locally available ingredients could be utilized in the diet of *A. japonicus*.

Data established in the present study could be used as a starting point for more accurate and least-cost formulations to find the optimum combination of ingredients to stimulate a balanced pattern of amino acids in compound diets for maximum growth of *A. japonicus*. This will be achieved by using relevant data on both EAA dietary requirements of dusky kob and the EAA supplied by the feed ingredients.

CHAPTER 4

LYSINE REQUIREMENT AND ESSENTIAL AMINO ACID ESTIMATES FOR *ARGYROSOMUS JAPONICUS*

4.1. INTRODUCTION

Amino acids play important and versatile roles in protein metabolism (Wu *et al.,* 2013; Wright and Fyhn, 2001). Lysine is often the first limiting EAA in protein sources commonly used in fish feeds, especially in feeds that include plant feedstuffs (NRC, 2011; Hauler and Carter, 2001; Small and Soares, 2000) that are typically low in lysine and methionine. Based on the ideal protein concept, the lysine requirement can be used to predict the requirements of other EAAs in a fish species (Akiyama *et al.,* 1997). Where dietary lysine is limiting, lysine supplementation could reduce the oxidation of other amino acids by improving the use of other EAAs for protein synthesis and anabolism (Kerr and Easter, 1995). Dietary lysine supplementation is also related to improved feed efficiency, weight gain, feed conversion, nitrogen retention and a reduction in the body lipid contents (Furuya *et al.,* 2006; Marcouli *et al.,* 2006). However, lower growth rate due to excess dietary lysine has been observed in some species (Mai *et al.,* 2006; Zhou *et al.,* 2007; Deng *et al.,* 2010). Therefore, an optimal inclusion of lysine is required to formulate nutritionally complete and cost effective fish feeds.

Dietary lysine requirements have been established through a range of methods. The most commonly applied method is the dose-response method. For a growing fish, the quantitative lysine requirement comprises the lysine required for both somatic growth and lysine losses, which include obligatory and regulatory losses (Hauler and Carter, 2001). Dose-response estimates are truly quantitative because they include the lysine required for growth and both types of losses (Hauler and Carter, 2001). Others have estimated the lysine requirement from

the daily deposition of amino acids in the body (Ogino, 1980). This method assumes the daily deposition of lysine per unit of body weight is equivalent to the daily requirement per unit of body weight. The quantitative lysine requirement is then calculated in relation to a nominal weight-specific feed or protein intake. The use of this method is problematic as it ignores the maintenance component of quantitative dietary lysine requirement (Cowey, 1995; Ng and Hung, 1995; Wilson and Poe, 1985). In addition, this method does not account for regulatory losses, which alone can be up to 40 % of the quantitative dietary requirement (NRC, 1993; Cowey, 1995). Comparison of other methods with dose-response experiments has confirmed that lower quantitative dietary lysine requirements are estimated by studies investigating the daily deposition of amino acids in the body (Jauncey *et al.,* 1983). In addition, Griffin *et al.* (1992) used serum lysine concentrations to confirm lysine requirement in channel catfish. Of all these methods, dose-response experiments provide the most accurate quantitative requirements of fish (Hauler and Carter, 2001).

Dietary amino acid requirements of fish are usually determined through a dose-response study, which is costly and time-consuming, especially when determining the requirement for all the ten EAAs (Akiyama *et al.,* 1997). The essential amino acid requirement patterns of fish have been shown to correlate well with the EAA patterns of the whole body tissue of that fish (Wilson and Poe, 1985). If the requirement of one of the EAAs is known, the requirements of the remaining nine EAAs can be accurately estimated from the ratio of the whole body amino acid pattern of the species (Tacon, 1989; Akiyama *et al.,* 1997). Based on lysine requirement, other EAA requirements can be estimated from the ratio of the fish body EAAs composition. Such estimations have been carried out on colliroja *Astyanax fasciatus* (Furuya *et al.,* 2015), bluegill *Lepomis macrochirus* (Masagounder *et al.,* 2011), Eurasian perch *Perca fluviatalis* (Langeland *et al.,* 2014), pacu *Piaractus mesopotamicus* (Abimorad *et al.,* 2010) and largemouth bass *Micropterus salmoides* (Portz and Cyrino, 2003). Amino acid

requirements determined through this approach have been found not to differ significantly from those determined through dose-response methods in fish (Wilson, 2002). Knowing the EAA requirements in cultured fish species will allow a more appropriate diet formulation so as to reduce the diet cost, nutrient waste and environmental impact.

In the recent years there has been a growing interest in the study of biochemical parameters of fish for aquaculture purposes. Blood chemistry parameters can provide predictive information on fish health conditions (Vázquez and Guerrero, 2007). They have also been used to monitor fish responses to stressors, and thus their health status under adverse conditions (Vázquez and Guerrero, 2007).

Dusky kob *A. japonicus* is an economically important fish in South Africa and in other parts of the world (PIRSA, 2001). However, the information on the dietary essential amino acid requirements of this species is not available. Therefore, this study aimed to determine the lysine requirement of juvenile dusky kob through the dose-response method by using diets containing crystalline lysine, fishmeal and soybean as protein sources, and also to estimate other essential amino acid requirements by using the ideal protein concept.

4.2. MATERIALS AND METHODS

4.2.1. Determination of dietary lysine requirement level

Experimental diets

Six isonitrogenous (431 g kg^{-1} CP) and isoenergetic (20 kJ kg^{-1}) diets containing fishmeal and soybean meal as protein sources were formulated and supplemented with graded levels of L-lysine and a mixture of essential and non-essential amino acids were added to simulate the amino acid body profile of *A.japonicus* (Table 4.1). The crystalline L-lysine was added to the experimental diets at increments of 6.6 g kg^{-1}, ranging from 0 to 33 g kg^{-1} of the dietary

ingredients and a crystalline glutamic acid/aspartic acid (1:1) mixture was used to standardise dietary protein levels. The level of protein in the diets was formulated to be about 431 g kg^{-1}, which is slightly lower than the dietary protein requirement for dusky kob to ensure utilization of lysine for growth. The dry ingredients were weighed and mixed in an industrial food mixer (Macadam's Baking Systems, SM-201, Cape Town, South Africa). The fish oil, sunflower oil and water were subsequently introduced to the dry ingredients and mixed to a homogenous dough. The dough was cold-extruded (ICME Motor electric Bologna, Italy), cut into pellets (2 mm), placed on trays and oven dried at 40 ^{o}C for 16 hours. The dietary lysine levels in the six experimental diets were confirmed by amino acid analysis (Waters AccQ Tag kits analyser) and the values were 18, 21, 29, 33, 36 and 42 g kg^{-1} dry diet, designated as DL_1, DL_2, DL_3, DL_4, DL_5 and DL_6 respectively. The analysed EAA content for each diet is presented in Table 4.2. The feed was placed into sealed packets, and stored at -20 ^{o}C until required. Sources and compositions of all the feed ingredients used in the formulation of experimental diets are presented in Appendix 1.

Table 4.1. Formulation and proximate composition of experimental diets (g kg^{-1}).

Ingredients	DL_1 (18)	DL_2 (21)	DL_3 (29)	DL_4 (33)	DL_5 (36)	DL_6 (42)
Formulation (g kg^{-1} dry matter)						
Fishmeal standard[1]	350.0	350.0	350.0	350.0	350.0	350.0
Soybean meal[1]	90.0	90.0	90.0	90.0	90.0	90.0
Corn starch[1]	263.9	263.9	263.9	263.9	263.9	263.9
Fish oil	60.0	60.0	60.0	60.0	60.0	60.0
Sunflower oil	25.0	25.0	25.0	25.0	25.0	25.0
CAA[2]	41.7	41.7	41.7	41.7	41.7	41.7
L-Lysine-HCl[2]	00.0	6.6	13.2	19.8	26.4	33.0
L-asp/L-glu (1:1)[2]	33.0	26.4	19.8	13.2	6.6	0.0
CMC[2]	26.0	26.0	26.0	26.0	26.0	26.0
Vitamin/mineral mixture[1]	30.0	30.0	30.0	30.0	30.0	30.0
Cellulose[2]	80.0	80.0	80.0	80.0	80.0	80.0
Analysed composition (g kg^{-1} dry matter)						
Crude protein	410.6	420.6	426.9	428.4	430.3	432.5
Crude lipid	103.7	101.6	99.3	103.1	100.1	96.7
Ash	68.6	70.5	74.6	64.7	71.4	65.4
Gross energy (MJ kg^{-1})	19.78	20.16	20.25	20.31	20.31	20.35
Lysine	18.0	21.0	29.0	33.0	36.0	42.0

Crystalline amino acids (CAA); Carboxymethyl cellulose (CMC); aspartic acid (asp); glutamic acid (glu)

CAA mixture (g kg^{-1} DM): L-arginine 2; L-histidine 0.3; L-isoleucine 5.5; L-leucine 8.9; L-methionine 7.4; phenylalanine 4.4; L-threonine 6.0; L-valine 7.2.

[1] Marifeed (Pty) Ltd, South Africa.

[2] Sigma Aldrich, South Africa.

Table 4.2 Analysed essential amino acid composition (EAA, g kg^{-1}) in the experimental diets (excluding tryptophan)

EAAs (g kg^{-1})	Diets DL_1 (18)	DL_2 (21)	DL_3 (29)	DL_4 (33)	DL_5 (36)	DL_6 (42)
Arginine	26	24	26	25	24	25
Histidine	03	04	05	05	06	07
Isoleucine	23	22	24	23	22	22
Leucine	41	39	43	41	40	42
Methionine	16	15	17	16	15	16
Phenylalanine	34	33	39	34	35	35
Threonine	24	22	24	22	21	22
Valine	28	26	29	28	27	28
Lysine	18	21	29	33	36	42

Experimental conditions

The feeding trial was carried out at the Rhodes University Marine Research Laboratory in Port Alfred (33°45′ S, 26°00′ E), South Africa. Hatchery-reared dusky kob were supplied by Pure Ocean Pty Ltd (East London, South Africa) and acclimated to laboratory conditions for one month, prior to the experiment, during which time they were fed a commercial finfish feed thrice daily to apparent satiation (450 g kg^{-1} protein and 180 g kg^{-1} lipid; Marifeed Pty Ltd, South Africa). The experiment was carried out in a 3200 L recirculating system. Approximately 10 % of the system's water was replaced daily with seawater pumped from the Kowie River Estuary. Each tank was aerated using air stones and water temperature was thermostatically controlled by a heater in the reservoir tank (500 L) and distributed to the 90 L experimental tanks, which were cleaned of faeces by siphoning every morning. Water flow rate in each tank was maintained at 4 L/min. Water quality was measured daily, except ammonia nitrogen, which was measured once a week. The mean (± standard deviation) temperature, total ammonia nitrogen, dissolved oxygen and salinity during the trial was 24.2±0.2 ^{o}C, 0.2±0.2 mg L^{-1}, 4.7±0.3 mg L^{-1} and 34±0.7 L^{-1} respectively. A light regime of 12 h light : 12 h dark was maintained throughout the experimental period. Two hundred and sixteen fish (mean weight: 4.5±0.2 g.$fish^{-1}$) were randomly assigned to the 18 experimental tanks (12 fish.$tank^{-1}$). Three replicate groups of fish were used to test each dietary formulation. Fish were fed to satiation three times daily. Satiation was assessed by their feeding behaviour; fish would swim to the water surface to ingest the feeds. As long as fish were fed to satiation, they would not come up to the water surface again. The experiment was undertaken over a 12-week period.

Data collection

Prior to the trial, the fish were purged for 24 h and 12 fish were randomly taken from the original population and euthanized (8 ml L^{-1} of the anaesthetic 2-phenoxy-ethanol) and frozen at -20°C for whole body proximate composition analysis. At the start of the experiment the fish were anesthetized (2-phenoxyethanol: 0.2 ml L^{-1}) and individual fish weight (4.5±0.2 g) and total length (66.50±1.1 mm) were recorded using an electronic scale (Highlander, Adam Equipment, HCB 1002, UK) and a metre rule. The process was repeated every 21 days. At the end of the experiment, three fish per replicate were individually weighed, and the weights of the viscera and liver were recorded for the calculation of viscerosomatic index and hepatosomatic index. To establish the amino acid profile of the fish, three fish from each replicate were combined to form one composite sample for each treatment. The whole body of each fish was then sealed in a plastic bag and frozen at -20 °C for the analysis. Blood samples were collected from the caudal vein of three fish taken from each tank using a disposable hypodermic syringe fitted with a 26-gauge needle and expelled into separate heparinized tubes on ice, which were sent for analysis immediately. These analyses were undertaken 24 h after the last feeding.

Laboratory analyses, comprising estimations of crude protein, lipid, ash and energy, followed procedures recommended by the Association of Official Analytical Chemists (AOAC, 2003). Crude protein was determined using the Dumas combustion method in a LECO FP2000 Nitrogen analyser (AOAC, 2003). Lipid was extracted from the samples by solvent petroleum ether using a Buchi 810 Soxhlet fat extractor (AOAC, 2003). The lipid percentage was calculated by gravimetric analysis. Ash content was determined by combustion at 550 °C in a muffle furnace for 24 h. Gross energy was determined using a bomb calorimeter (DDS Isothermal CP500) (AOAC, 2003). The amino acid content of the whole body tissue and feed formulation was determined after acidic and basic digestion for chromatographic and ion

exchange analyses (high-performance liquid chromatography) performed in sealed glass tubes under a nitrogen atmosphere at 110 °C. Cysteine and methionine were determined by hydrolysis after oxidation with performic acid. After hydrolysis, the solutions were vacuum filtered, diluted to 0.25 M with 0.02 N HCl for adjusting the pH to 8.5, and filtered through a Millipore membrane (0.45 mm). Plasma protein estimations were carried out according to the method of Lowry *et al.* (1951). Blood glucose was determined by the glucose oxidase and peroxidase method using the glucose-GOD/POD kit and cholesterol level was determined by using the cholesterol oxidase/phenol + aminophenazone Kit (Triander, 1969). The analyses were carried out in a clinical laboratory.

Calculations

The following indices were determined:

- Growth rate was calculated as specific growth rate (SGR, % day^{-1}) = (ln FBW – ln IBW) x 100/t, where FBW is the final body weight (g); IBW is the initial body weight (g); t is the duration of the feeding trial (d);
- Feed intake (%, BW day^{-1}) = 100 x I/[(IBW+FBW)/2 x t], where I (g) is the total dry feed fed; IBW is the initial body weight; FBW is the final body weight; t is the duration of the feeding trial;
- Feed conversion ratio (FCR) = total dry feed fed (g) / wet weight gain (g);
- Essential amino acid (EAA) retention (%) = (body EAA_{final} x body $weight_{final}$)-(body $EAA_{initial}$ x body weight $_{initial}$)/EAA intake x 100;
- Protein retention = (body $protein_{final}$ x body $weight_{final}$)-(body $protein_{initial}$ x body weight $_{initial}$)/protein intake x 100;
- Hepatosomatic index (HSI) = 100 x (liver weight, g) / (body weight, g);
- Condition factor (CF) = 100 x whole body weight (g) / [body length (cm)]^3; and
- Viscerosomatic index (VSI) = 100 x Viscera weight (g) / whole body weight (g)

4.2.2. Determination of dietary requirements for other essential amino acids using ideal protein concept

Eighty-seven juvenile dusky kob (*Argyrosomus japonicus*) with an average weight of 1.2±0.1 g were collected from a commercial fish farm, euthanized, placed in crushed ice and transported to the laboratory for amino acid analysis.

The requirement of the EAAs were calculated using Equations 4.1 and 4.2 (Akiyama *et al.*, 1997):

Essential amino acids ratio (A/E) was calculated according to Equation 4.1

$$\text{A/E ratio} = \left\{ \frac{\text{individual EAA content in the whole body}}{\text{total EAA content including cysteine and tyrosine}} \times 1000 \right\} \qquad \text{Equation 4.1}$$

The A/E ratio was then used to calculate the dietary requirements of amino acids based on the determined lysine requirement levels according to Equation 4.2

$$\text{EAA requirement} = \left\{ \frac{\text{requirement for lysine x specific A/E ratio}}{\text{A/E ratio for lysine}} \right\} \qquad \text{Equation 4.2}$$

4.2.3. Statistical analysis

Individual fish data for each replicate tank were pooled and the replicate mean was used in further analyses. Results were presented as means ± standard deviation. The Kolmogorov-Smirnov test was used to determine if the residuals of the data were normally distributed and the Levene test was used to check for homogeneity of variances. Treatment means were compared using a one-way analysis of variance (ANOVA), and Tukey's multiple comparison test was used to evaluate the difference among individual diets at $p<0.05$.

All statistical analyses were conducted using STATISTICA™ Version 16.0.

The optimum dietary lysine requirement, based on SGR, FCR and protein retention, was estimated using a 'segmented' broken line analysis [y = b1*x + b2*x(x-c) + gI(x>c)] that was determined using R Version 3.3.0 (Muggeo, 2008).

4.3. RESULTS

4.3.1. Growth performance

Growth performance was significantly affected by dietary lysine level (Table 4.3). No evidence of external outward pathological signs was noted in fish fed the low levels of dietary lysine. Feed intake was similar for all the treatments ($p=0.078$). Mean specific growth rate (SGR) ranged from 1.51 to 2.12 for fish fed higher levels of dietary lysine ($\geq$3.6 g kg^{-1} dry diet), showing significantly lower SGR than fish fed lysine at 18, 21, 29 and 33 g kg^{-1} dry diet (Table 4.3). Fish fed with diets containing lysine at 21, 29 and 33 g kg^{-1} dry diet had similar growth rate and showed a higher growth rate (SGR) than fish fed with diets containing lysine at 18 and 36 g kg^{-1} dry diet, while fish fed the diet containing the highest level of lysine at 42 g kg^{-1} dry diet (DL_6), had lower SGR (Table 4.3). Final mean body weights differed significantly among dietary treatments, with fish fed the lysine 21 g kg^{-1} (DL_2) and 33 g kg^{-1} dry diets (DL_4) recording the highest final mean body weights, while fish fed the highest lysine level (42 g kg^{-1} dry diet) recorded the lowest final mean body weight. The final mean body weight (FBW) ~~and LWG~~ of fish fed the highest inclusion of lysine level (42 g kg^{-1} dry diet) were unexpectedly low (Table 4.3). Whole body protein retention was similar in fish fed lysine at 21, 29 and 33 g kg^{-1} dry diet and these values were significantly higher than those of fish fed the other diets ($p<0.001$). The most efficient feed conversion ratio (FCR) was observed in groups fed lysine at 29 g kg^{-1} dry diet. Fish fed at lysine 42 g kg^{-1} dry diet (DL_6) showed a higher FCR than the fish fed the other experimental diets.

Similarly, fish fed a diet exceeding 33 g kg^{-1} dry feed did not show any improvement in any of the growth parameters measured (Table 4.3).

4.3.2. Estimation of lysine requirement

Based on the SGR, the optimal lysine requirement for juvenile dusky kob was estimated to be 31.7 g kg^{-1} dry diet (Figure 4.1), corresponding to 73.5 g kg^{-1} dietary protein. Similarly, values of 31.2±1.6 g kg^{-1} and 31.4±1.5 g kg^{-1} were estimated as optimal dietary lysine levels, using protein retention and FCR, respectively (Table 4.4).

4.3.3. Condition factor, hepatosomatic index and viscerosomatic index

Dietary lysine levels significantly affected the viscerosomatic index (VSI) and Hepatosomatic index (HIS) ($p<0.05$, Table 4.3). The HSI and VSI were higher in fish fed the lower level lysine diets than in those fish fed the higher lysine levels. The condition factor (CF) showed no significant differences among the treatments ($p=0.22$; Table 4.3).

Table 4.3. Growth performance and feed utilization of juvenile dusky kob fed diets containing graded levels of lysine for 12 weeks. Means with a different superscript within each row were significantly different (ANOVA, $p<0.05$).

	Dietary lysine levels (g kg^{-1} dry diet)						
Varia-bles	DL_1 (18)	DL_2 (21)	DL_3 (29)	DL_4 (33)	DL_5 (36)	DL_6 (42)	P value
IBW	4.42±0.2	4.50±0.0	4.55±0.2	4.63±0.3	4.51±0.2	4.46±0.0	0.840
FBW	$22.83±2.5^{c}$	$26.72±1.5^{a}$	$25.24±0.7^{b}$	$26.72±1.8^{a}$	$20.71±3.1^{d}$	$15.90±1.5^{e}$	0.001
SGR	$1.95±0.1^{b}$	$2.12±0.1^{a}$	$2.08±0.0^{a}$	$2.08±0.1^{a}$	$1.80±0.2^{c}$	$1.51±0.1^{d}$	<0.001
FI	2.30±0.1	2.17±0.1	2.05±0.0	2.11±0.2	2.20±0.2	2.45±0.2	0.078
FCR	$1.44±0.1^{b}$	$1.28±0.1^{c}$	$1.23±0.1^{e}$	$1.27±0.1^{d}$	$1.45±0.2^{b}$	$1.85±0.2^{a}$	0.000
PR	$7.47±0.9^{b}$	$8.69±0.6^{a}$	$8.72±0.7^{a}$	$8.45±1.5^{a}$	$6.48±1.2^{c}$	$4.11±0.6^{d}$	<0.001
CF	1.10±0.0	1.16±0.1	1.13±0.1	1.16±0.0	1.18±0.0	1.19±0.1	0.220
HSI	$2.95±0.1^{a}$	$2.42±0.0^{b}$	$2.13±0.3^{c}$	$2.41±0.5^{b}$	$1.87±0.1^{d}$	$1.33±0.1^{e}$	<0.001
VSI	$8.59±0.2^{a}$	$8.38±0.6^{b}$	$7.94±0.5^{c}$	$7.90±0.5^{c}$	$6.85±0.5^{d}$	$5.82±0.4^{e}$	<0.001

IBW (g $fish^{-1}$) initial mean body weight; FBW (g $fish^{-1}$) final mean body weight; SGR, specific growth rate (% day^{-1}); FI (% BW day^{-1}) feed intake; PR, protein retention (%); FCR, feed conversion ratio; CF, condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index

Table 4.4. The modled mean weight of juvenile dusky kob (g $fish^{-1}$), measured every 21 days for 12 weeks, when fed different levels of dietary lysine (Regression Analysis); and the estimated optimal dietary lysine level using protein retention (%) and feed conversion ratio (Segmented, Broken Line Analysis).

Model		r^2	Break point	n
Regression analysis (mean body weight over 84 days):				
DL_1 18 g kg^{-1} Lysine	y = 0.223 x + 3.299	0.963	-	15
DL_2 21 g kg^{-1} Lysine	y = 0.266 x + 3.023	0.963	-	15
DL_3 29 g kg^{-1} Lysine	y = 0.261 x + 3.024	0.964	-	15
DL_4 33 g kg^{-1} Lysine	y = 0.267 x + 3.048	0.930	-	15
DL_5 36 g kg^{-1} Lysine	y = 0.194 x + 3.341	0.910	-	15
DL_6 42 g kg^{-1} Lysine	y = 0.139 x + 3.902	0.953	-	15
Broken-line analysis (18-42 g kg^{-1} dietary lysine):				
Protein retention (%)	y = 0.091 x -0.470 x (x -31.21) + gI(x >31.21)	0.731	31.21±1.61	18
Food conversion ratio	y = -0.017 x 0.062 x (x -31.44) + gI(x >31.44)	0.703	31.44±1.52	18

4.3.4. Plasma biochemistry parameters

The plasma biochemistry parameters of the juvenile dusky kob fed experimental diets containing different dietary lysine levels are presented in Table 4.5. Dietary lysine concentrations had no significant effect on plasma protein (p=0.68), total cholesterol (p=0.88), triglyceride (p=0.49) or glucose (p=0.26) levels.

Table 4.5. Determination of plasma protein, total cholesterol, triglyceride and glucose concentration in juvenile dusky kob fed with graded levels of lysine for 12 weeks (ANOVA, $p<0.05$).

Parameters	Dietary lysine levels (g kg^{-1} dry diet) DL_1 (18)	DL_2 (21)	DL_3 (29)	DL_4 (33)	DL_5 (36)	DL_6 (42)	P value
Total protein (g L^{-1})	42.50±3.5	44.50±3.3	50.50±2.1	41.50±4.2	51.00±3.6	51.50±3.6	0.68
Total cholesterol (mmol L^{-1})	1.60±0.1	1.55±0.1	1.55±0.1	1.55±0.1	1.60±0.1	1.65±0.1	0.88
Triglyceride (mmol L^{-1})	0.85±0.1	0.95±0.1	0.65±0.3	0.80±0.2	1.00±0.1	1.15±0.3	0.49
Glucose (mmol L^{-1})	3.95±0.6	3.80±1.1	4.90±1.5	2.40±0.9	3.20±0.1	3.00±0.3	0.26

4.3.5. Essential amino acid retention in the whole body tissue

Dietary lysine had a significant affect on the whole body retention of all the essential amino acids ($p<0.001$; Table 4.6). With the exception of histidine, amino acid retention increaded significantly with an increase in dietary amino acid from 18 to 21 g kg^{-1} (Table 4.6). In most cases retention reached a maximum between 21 and 33 g kg^{-1}, and dropped significantly with further increases in dietary lysine (Table 4.6). Whole body histidine levels were highest when dieary lysine was fed at 18 g kg^{-1} and this decreased significnatly with increasing dietary lysine ($p<0.0001$; Table 4.6).

Table 4.6. Essential amino acid (EAA) retention (%) in the whole body tissue of juvenile dusky kob fed experimental diets with different dietary lysine levels (g kg^{-1} dry diet).

EAAs	Diets (g kg^{-1} dry diet) DL_1 (18)	DL_2 (21)	DL_3 (29)	DL_4 (33)	DL_5 (36)	DL_6 (42)	P value
Arginine	32.9±4.1^{b}	43.0±2.9^{a}	42.7±3.5^{a}	36.7±6.6^{b}	28.0±5.2^{d}	23.9±3.1^{e}	0.0009
Histidine	63.5±9.2^{a}	33.1±3.5^{b}	25.4±3.4^{c}	4.5±3.7^{e}	9.6±3.7^{d}	5.1±2.0^{e}	<0.0001
Isoleucine	21.3±2.9^{b}	28.7±2.1^{a}	27.7±2.5^{a}	23.7±4.7^{b}	16.4±3.6^{c}	13.5±2.2^{d}	0.0004
Leucine	21.1±2.9^{b}	28.7±2.2^{a}	27.1±2.5^{a}	23.1±4.6^{b}	16.6±3.6^{c}	12.5±2.0^{d}	0.0002
Meth	20.84±2.9^{c}	29.29±2.2^{a}	28.20±2.6^{a}	22.18±4.6^{b}	17.68±3.9^{d}	12.66±2.1^{e}	0.0003
Phenyl	17.1±2.2^{b}	21.5±1.6^{a}	18.4±1.6^{b}	16.8±3.3^{b}	12.1±2.5^{c}	9.4±1.5^{d}	0.0003
Threonine	26.7±3.0^{d}	31.8±2.1^{a}	30.4±2.4^{b}	28.4±5.1^{c}	25.0±4.1^{d}	19.8±2.1^{e}	0.0014
Valine	21.9±2.6^{c}	27.2±2.1^{a}	25.0±2.4^{b}	20.0±4.4^{d}	16.8±3.4^{e}	13.4±2.0^{f}	0.0002
Lysine	44.7±6.2^{b}	53.9±4.1^{a}	41.56±3.8^{b}	31.6±6.2^{c}	19.18±4.1^{d}	13.4±2.1^{e}	<0.0001

Means with a different superscript within each row were significantly different (ANOVA, $p<0.05$).
Meth, methionine; Phenyl, phenylalanine.

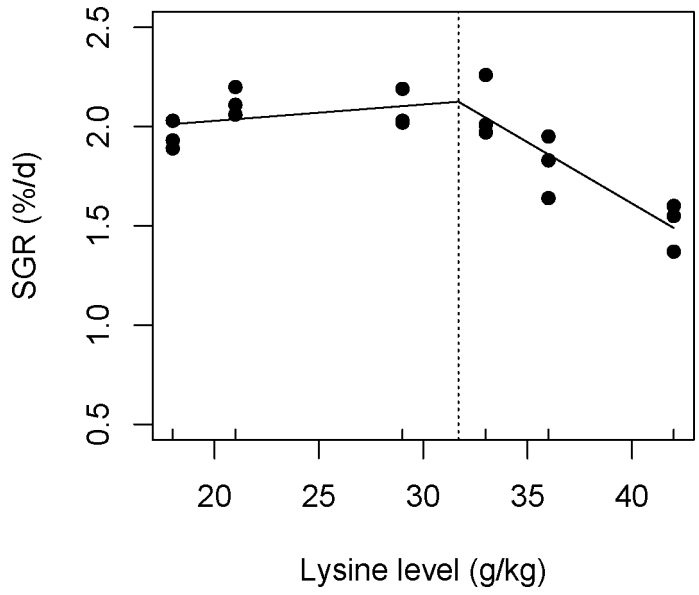

Figure 4.1. The relationship between specific growth rate (SGR) and dietary lysine levels (g kg^{-1} dry diet) in juvenile dusky kob (y = 0.0083x – 0.0618x(x-31.67) + gI(x>31.67).

4.3.6. Determination of dietary requirements for other essential amino acids

The mean whole body amino acid level of juvenile dusky kob ranged from 0.2 % (cysteine) to 16.1 % (glutamic acid) (Table 4.7). These data, together with a dietary lysine level of 73.5 g kg^{-1} dry diet (which was determined in Section 4.3.2, Figure 4.1), were used to estimate the dietary requirements of the remaining nine essential amino acids. The dietary requirements of these amino acids ranged from 22 g kg^{-1} for histidine to 71 g kg^{-1} for leucine (Table 4.8).

Table 4.7. Whole body composition of essential and non-essential amino acids of dusky kob *A. japonicus* (g/100 protein). Values are means ± standard deviation of three replicate analyses.

Composition	%
Essential amino acid	
Arginine	4.4±0.1
Histidine	2.4±0.2
Isoleucine	4.4±0.1
Leucine	7.8±0.1
Lysine	8.1±0.1
Methionine	3.3±0.0
Phenylalanine	4.4±0.1
Threonine	4.9±0.1
Valine	5.4±0.1
Non essential amino acid	
Serine	4.7±0.1
Glycine	8.3±0.2
Aspartic acid	10.2±0.1
Glutamic acid	16.1±0.2
Alanine	6.7±0.1
Proline	5.1±0.2
Cysteine	0.2±0.1
Tyrosine	3.7±0.1

Table 4.8. Dietary quantitative essential amino acid (EAA) requirement (including cysteine and tyrosine) of *A. japonicus* (as feed basis).

EAA	EAA body composition (g kg^{-1} dry matter)	A/E ratio	Dietary EAA (g kg^{-1} dietary protein)
Histidine	24	49.0	22
Arginine	44	89.8	40
Threonine	49	100.0	44
Lysine	81	165.3	73
Methionine	33	67.3	30
Methionine + Cys	35	71.4	32
Valine	54	110.2	49
Isoleucine	44	89.8	40
Leucine	78	159.2	71
Phenylalanine	44	89.8	40
Phenylalanine + Tyr	81	166.3	73

Cys-cysteine; Tyr-tyrosine.

4.4. DISCUSSION

Supplementing dietary lysine significantly improved the growth performance of juvenile *A. japonicus*, which indicates that lysine is essential for the growth of the species, and further, that crystalline amino acids including lysine, can be utilised by this species. Broken line 'segmented' analysis of the SGR indicates that to maximise the growth performance, juvenile dusky kob have a dietary lysine requirement of 31.7 g kg^{-1} dry diet. The dietary requirement of 31.7 g kg^{-1} dry diet equates to 73.5 g kg^{-1} dietary protein, which was lower than the reported value for black sea bream *Sparus macrocephalus,* 87.1 g kg^{-1} dietary protein (Zhou *et al.,* 2010) and yellow catfish *Pelteobagrus fulvidraco,* 83.2 g kg^{-1} dietary protein (Cao *et al.,* 2012). However, this level is higher than the range of values reported for other commonly cultured fishes (Wilson, 2002). Examples include rainbow trout *Oncorhynchus mykiss*, 60.1 and 61.0 g kg^{-1} dietary protein (Yun *et al.,* 2016; Ketola, 1983), stinging catfish *Heteropneustes fossilis*, 61 g kg^{-1} dietary protein (Khan, 2013), catla *Catla catla,* 62 g kg^{-1} dietary protein (Ravi and Devaraj, 1991), Japanese seabass *Lateolabrax japonicus* 58-61 g kg^{-1} dietary protein (Mai *et al.,* 2006), grouper *Ephinephelus coioides* 55.6 g kg^{-1} dietary protein (Luo *et al.,* 2006), Indian major carp *Cirrhinus mrigala* 57.5 g kg^{-1} dietary protein (Ahmed and Khan, 2004) and beluga *Huso huso* 55 g kg^{-1} dietary protein (Hoseini *et al.,* 2013). The above-mentioned results suggest that juvenile dusky kob have a higher lysine requirement than that of other fish species. The explanation for this might be that dusky kob is a carnivorous fish with a concomitant high dietary protein requirement. Carnivorous fish species usually need higher lysine content in their diet than fish demanding low dietary protein (Zhou *et al.,* 2007). In addition, the variation in the estimated lysine requirements reported for different fish species is probably due to a number of factors including dietary protein source, age, genetic strain, dietary energy content and essential amino acid profile of the animal and the protein source (De Silva *et al.,* 2000; Forster and Ogata, 1998). Fish size,

levels of other nutrients and experimental conditions (Kim *et al.,* 1992), and the mathematical model used to estimate the requirement value (Mai *et al.,* 2006; Zhou *et al.,* 2007) have also been shown to influence the estimated requirements. The lysine in the control feed was relatively high due to the higher fishmeal content, so the increase in growth might have been more substantial with a lower base of lysine.

A number of researchers have suggested that fish do not utilise dietary crystalline amino acids (CAAs) as effectively as intact proteins (Wilson and Halver, 1986). It has been hypothesised that crystalline amino acids added to the diet in free-form are more readily absorbed than protein-bound amino acids (Yamada *et al.,* 1981 and Murai *et al.,* 1982), causing an imbalance in amino acid profile in the tissues, resulting in the diversion of amino acids into catabolic rather than anabolic processes (Cowey and Sargent, 1979). To avoid this metabolic effect, this study used a feeding frequency of three times per day. Yamada *et al.* (1981) showed that more frequent feeding intervals increase weight gain and utilisation of crystalline amino acids in common carp *Cyprinus carpio.* In the present study, feeds were offered by hand slowly and care was taken to avoid feed wastage, and to ensure that all the feed supplied was consumed. Thus, loss of feed and crystalline lysine due to leaching was avoided or minimised.

Depending on the level of dietary lysine deficiency, responses observed in fish have ranged from reduced growth rates to poor survival. Fin erosion and poor growth rate were observed in rainbow trout fed lysine deficient diets (Ketola, 1983). However, in this study, growth rates remained positive for fish fed at the lowest lysine level (18 g kg^{-1} dry diet) indicating that as little as 18 g kg^{-1} of dietary lysine was adequate, not only for meeting maintenance requirements, but to elicit some limited growth. However, this growth was indeed limited, so this level of lysine was evidently below optimum.

In the present study, fish fed with diets containing 21, 29 and 33 g kg^{-1} lysine showed better growth performance and FCR than fish fed with diets containing 18 and 42 g kg^{-1} lysine, respectively. The same observations were made for protein retention, which was highest in fish fed the dietary treatments with a lysine level of 21, 29 and 33 g kg^{-1}. This trend of weight gain is consistent with the results reported by earlier researchers (Ahmed and Khan, 2004; Furuya *et al.,* 2012; Xie *et al.,* 2012). The reduction in the growth of fish fed higher than the required levels of dietary lysine may be due to the stress caused by excess amino acids in the body of the fish leading to extra energy expenditure towards deamination and excretion (Abidi and Khan, 2010). Dietary lysine-arginine antagonism is a well-known phenomenon in poultry and rats (Jones, 1964; Harper *et al.,* 1970; Fico *et al.,* 1982), resulting in lower growth rate but few reports exists of this occurring in fish. Kaushik and Fauconneau (1984) have offered some biochemical evidence to indicate that some metabolic antagonism may exist between lysine and arginine in rainbow trout. The excessive dietary lysine of 3 % diet considerably affected the catabolism of dietary arginine. Similarly, some studies showed that a metabolic interaction occurred between arginine and lysine when elevated levels of either were fed to Atlantic salmon (Berge *et al.,* 1998). Mai *et al.* (2006) also reported lower growth rate of Japanese seabass fed high levels of lysine (36.6 and 42.5 g kg^{-1} diet). Lysine retention was significantly affected by the dietary lysine level. This could be due to efficient utilization when dietary lysine was deficient, and poor utilization when it was in excess. This was consistent with the findings of Finke *et al.* (1987) and Gahl *et al.* (1991) who reported that decrease lysine retention at an excessive level of dietary lysine might develop due to diminishing returns in a dose-response approach in which efficiency decreases as the growth response approached the maximum.

A lower HSI in fish fed diets with higher levels of lysine compared to fish fed lysine-deficient diets, has been reported for different fish species, for example, stinging catfish

Heteropneustes fossilis (Khan, 2013), European seabass *Dicentrarchus labrax* (Tibaldi *et al.*, 1994), gilthead sea bream (Marcouli *et al.*, 2006), turbot (Peres and Oliva-Teles, 2008) and silver perch (Yang *et al.*, 2011). Probably due to deficiencies of dietary lysine for protein synthesis in fish fed diets with lower levels of dietary lysine at 18 g kg^{-1} dry diet, the dietary amino acids used to deposit muscle protein may have been converted to lipid or glycogen, and they consequently may have contributed to the decrease in CF and increase in HSI. Some reports have also suggested that the liver lipid content of fish is negatively correlated with dietary lysine levels (Luo *et al.*, 2006; Zhou *et al.*, 2010). In this study, dusky kob fed low dietary lysine diets expressed higher HSI values. The same trend was also observed for VSI.

It is well known that plasma glucose, triglyceride, cholesterol and total protein are related to fish health (Zhou *et al.*, 2007) and they are important in assessing stress responses and nutritional condition in fish (Vázquez and Guerrero, 2007). Total protein can be used as a diagnostic tool and a valuable test for evaluating the general physiological state of the fish (De Pedro *et al.*, 2005). Understanding the effect of lysine levels on health indices of dusky kob will contribute to a better comprehension of the dietary lysine requirement of this species. In this experiment total protein, cholesterol, triacylglycerol and glucose concentrations in plasma were not affected by the level of dietary lysine, which indicates that dietary lysine did not influence this aspect of *A. japonicus* health. This was in contrast to previous studies (Wang *et al.*, 2005; Zhou *et al.*, 2007). Wang *et al.* (2005) studying grass carp *Ctenopharyngodon idella*, observed that total protein, cholesterol, triacylglycerol and glucose in plasma increased with increasing dietary lysine up to 2.18 % (dry diet). After that, total protein reached a plateau, cholesterol and triacylglycerol showed a slight decline whereas glucose continued to increase. Zhou *et al.* (2007) showed that lysine concentration in serum significantly increased with the increased dietary lysine from 1.15 % to 2.38 % dry diet fed to cobia *Rachycentron canadum*. Lou *et al.* (2006) suggested that, in groupers,

glucose was not affected by dietary lysine. There is little information available on the effect of lysine on blood characteristics and further investigation is needed.

The ideal protein concept has been used as a method to estimate the other EAA requirements when only one EAA requirement is known (Wilson, 2002). Recently, the dietary requirements for all EAAs have been determined from whole body amino acid profiles in bluegill (Masagounder *et al.*, 2011), pacu (Abimorad *et al.*, 2010), Chinese sucker (Lin *et al.*, 2012) and colliroja *Astyanax fasciatus* (Furuya *et al.*, 2015). Determining EAA requirements by using the ideal protein concept is considered effective for establishing an ideal dietary protein profile for an emerging aquaculture species. However, the accuracy of this method has been questioned (Mambrini and Kaushik, 1995). The method ignores individual differences in maintenance requirements among EAAs (Mambrini and Kaushik, 1995) and assumes that the maintenance requirement of lysine and those of other EAAs is similar (Green and Hardy, 2002). Some AAs are needed in greater quantities for maintenance, while others are needed in greater quantities for growth (Wang and Fuller, 1989; Ng and Hung, 1995). Amino acids needed in higher quantities for maintenance tend to be under-represented, and those needed more for protein growth over-represented, if whole body protein is used as an estimate for dietary AA pattern (Akiyama *et al.*, 1997).

The deletion method, originally developed for pigs (Wang and Fuller, 1989), is based on the concept that if an AA is present in the diet in excess of the optimum level relative to other AAs, then deletion of that excess portion will have no effect on nitrogen retention (Wang and Fuller, 1989). This approach assumes nitrogen retention to be linearly correlated to dietary EAA content, when a particular amino acid is limiting. The deletion method could produce erroneous results if there is substantial deviation in the linear relationship between any EAA levels and nitrogen gain (Green and Hardy, 2002). Few studies have been conducted to

determine dietary requirements of EAAs for fish using the deletion method (Green and Hardy, 2002; Rollin *et al.*, 2003; Peres and Oliva-Teles, 2009).

Peres and Oliva-Teles (2009) found a strong correlation between EAA requirements determined by whole body amino acid composition (ideal protein concept) and those determined by the deletion method in gilthead seabream. Furthermore, Green and Hardy (2002) compared the deletion method and whole body amino acid pattern and concluded that the EAA pattern of whole body protein provides a good first approximation of the optimum dietary amino acid pattern, and could be used on an interim basis for fish species which have not been studied with respect to amino acid requirements, as has been suggested by Wilson and Poe (1985). The dietary requirement of fish is more precisely obtained by dose-response experiments compared to estimates from whole body amino acid profiles (NRC, 2011). It would be beneficial to compare estimates of EAA requirements in the current study with values determined from future dose-response studies.

At present *A. japonicus* in commercial South African farms are fed seabass diets. Dietary requirements of EAAs for dusky kob versus those for seabass (Kaushik, 1998) showed that dietary requirements for most EAAs appear to be lower for seabass than for dusky kob, suggesting that seabass diets may be deficient in certain EAAs needed for *A. japonicus*.

4.5. CONCLUSION

The result of the present study showed that lysine is essential for the growth of *A. japonicus*, and the species is able to efficiently utilize crystalline lysine. The optimum dietary lysine level for growth performance of dusky kob was estimated to be 31.7 g kg^{-1} on a dry diet basis corresponding to 73.5 g kg^{-1} protein. The lysine level and the whole body amino acid profile were successfully used to estimate the dietary requirements of the other essential amino acids.

CHAPTER 5

CONCLUSION

At the start of this research, knowledge on the dietary essential amino acids requirement of *A. japonicus* and essential amino acid digestibility of feed ingredients did not exist for this species, and this information is crucial to formulating species-specific diets. To address this, a comprehensive series of trials were conducted. This research followed a seven-step approach: (i) identifying a suitable marker for digestibility studies in *Argyrosomus japonicus,* (ii) identifying suitable faecal collection techniques for digestibility studies in *A. japonicus,* (iii) evaluating the apparent protein and amino acid digestibility from common animal feed ingredients for *A. japonicus* using single protein source, (iv) evaluating a reference diet substitution method for determination of apparent protein and amino acid digestibility coefficients of feed ingredients (plant and animal) for *A. japonicus*, (v) determining the additivity of the apparent protein and amino acid digestibility in compound diets for *A. japonicus*, (vi) determining the optimal dietary lysine requirement for juvenile *Argyrosomus japonicus,* and (vii) determining the whole body amino acid composition and using this to evaluate the optimum dietary essential amino acid profile for juvenile *A. japonicus*. It is important to recognize that nutritional research programs should follow a stepwise approach before the conclusions that are drawn in this field of research can be considered reliable. Therefore, this study has established a seven-step approach to fish nutritional research programs (Figure 5.1).

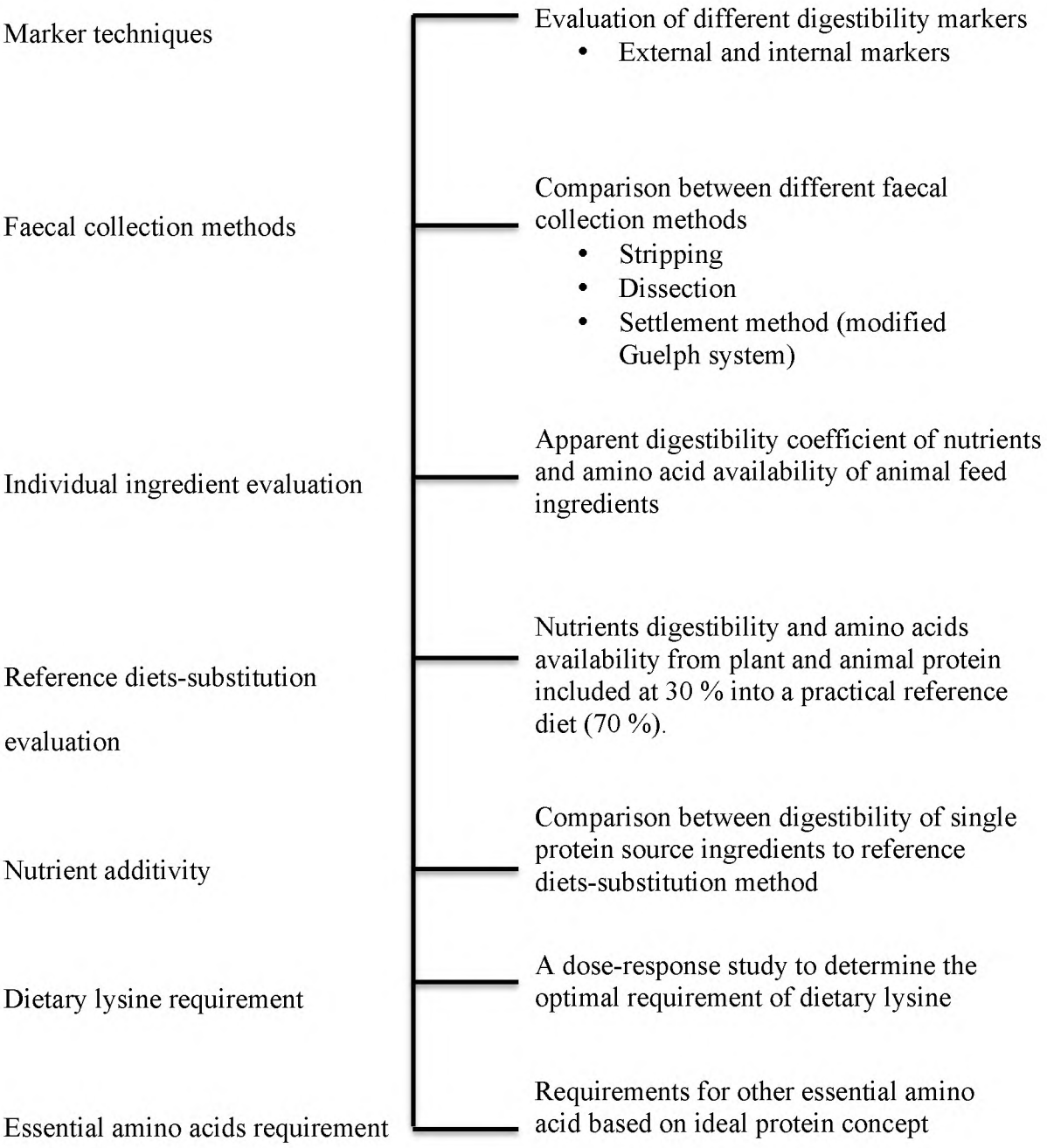

Figure 5.1. A seven-step approach to fish nutritional research programs

Prior to the start of trials to determine the nutrient digestibility of feed ingredients by dusky kob, a suitable protocol was established in order to produce valid digestibility values. The determination of accurate digestible nutrient values of feed ingredients depends on having a viable method to measure the digestibility of these parameters in the diet (Sugiura *et al.*, 1998; Blyth *et al.*, 2015). It should be noted that the digestibility values of aquafeeds may vary significantly depending on the different analytical methods used (Glencross *et al.*, 2007). In order to determine the nutrient digestibility of feed ingredients, an accurate faeces-collecting method and marker technique should be used to provide reliable data on the digestive capacity of the species under study. Therefore, the first step in a nutritional research program is to evaluate faecal collection methods for a selected fish species. It is well known that faecal collection methods can affect the determination of the digestibility values of diets. In the present study, various faecal collection methods ranging from the stripping method, intestinal dissection and faecal sample collection from the water by using the settlement tank (modified Guelph system) were evaluated. This study demonstrated that *A. japonicus* is easily stressed as observed during stripping. Based on the level of variability, as noted by the magnitude of the standard deviations for stripping in the data collected, the settlement method was found to be the preferred technique for collecting faeces for the determination of ingredient digestibility in *A. japonicus*.

Although, there are many concerns with regard to the degree of nutrient leaching, which may occur with settlement method, research has shown that nutrient loss is minimal when using this method. Similarly, Cho and Slinger (1979), and Cho *et al.* (1982) showed that the results obtained by intestinal dissection and by anal suction were not significantly different from the results obtained by the Guelph system for the digestibility of dry matter, crude protein and lipid. This indicates that nutrient leaching is an unlikely source of error when using this method.

A wide range of markers has been used in aquaculture nutrition for different fish species. In this study, chromic oxide, titanium oxide and acid-insoluble ash were evaluated. The performance of chromic oxide as a dietary marker during this investigation was unexpected. It is possible that there was a lack of uniform dispersion of Cr_2O_3 in the feed as well as in the faecal matter and thus a larger devaiation around the mean might have occurred. Further work is warranted to determine possible analytical errors.

In the current investigation, acid-insoluble ash is chosen as a dietary marker. In comparison to values obtained from chromic oxide and titanium dioxide, the variability between the samples in acid-insoluble ash results was reduced. Based on the present results acid-insoluble ash, a natural component of feed is a preferred marker for use in *A. japonicus* digestibility studies. The advantages of using acid-insoluble ash, as a digestibility marker is its low cost and ease of measurement using basic laboratory equipment (Goddard and McLean, 2001).

The above nutritional protocol is essential for establishing a reliable method for digestibility estimates. Other researchers have followed this same protocol before conducting digestibility trials for different fish species (Tacon and Rodrigues, 1984; Goddard and McLean, 2001; Atkinson *et al.*, 1984; Morales *et al.*, 1999; Shipton and Britz, 2001; Blyth *et al.*, 2015; Hien *et al.*, 2010; Vandenberg and De la Noüe, 2001; Mota *et al.*, 2015).

Evaluating a method in nutritional studies will certainly yield the most accurate results. This appoach is more costly, nevertheless it is practicable. Successful validation of these methods will assist in digestibility estimates.

However, many reserachers tend not to follow these protocols before embarking on digestibility studies. They randomly assumed a particular digestibility marker or collection method without evaluation. Clearly, the inappropriate use of digestibility markers or faecal

collection methods in fish nutritional studies may lead to fictitious digestibility estimates. Until this study, there have been no comparative studies to determine the most suitable dietary marker for *A. japonicus*. Booth *et al.* (2013) collected faeces by the settlement method using chromic oxide in a digestibility study with *A. japonicus*, while Pirozzi *et al.* (2010) used diatomaceous earth to calculate apparent digestibility in *A. japonicus*.

By establishing an appropriate digestibility protocol (marker techniques and faecal collection method), it was possible to evaluate protein and amino acid digestibility of feed ingredients. Protein and amino acid digestibility are the most important task in evaluating the suitability of feed ingredients for a fish species (De Silva *et al.*, 2000). The apparent protein and amino acid digestibility values of various feed ingredients (fishmeal-prime, fishmeal-standard, poultry meal, pork meal, blood meal, canola meal, soybean meal, sunflower meal and corn gluten meal) were determined. This is the first report on the amino acid digestibility coefficients for a variety of potential ingredients for *A. japonicus* diets.

In the present study, high apparent digestibility values of protein and essential amino acid digestibility were observed for all the experimental diets. Histidine was least available for *A. japonicus* in all the protein sources tested. The variation for fishmeal-prime histidine was higher than expected, although it was significantly lower than other treatments.

This study showed that poultry meal and soybean meal appear to be good ingredients for *A. japonicus* diets in term of the digestibility of crude protein and essential amino acid. Therefore, these ingredients could be incorporated in diets for *A. japonicus* and partially replace fishmeal. Among other ingredients tested, canola meal, sunflower meal and blood meal were also effectively digested, and also show potential for use in the diet of this species.

In general, *A. japonicus* appears to have a high capacity to digest crude protein and essential amino acids from a range of feed ingredients, including animal and plant protein origin.

A fundamental assumption used to formulate nutritionally complete feeds is that the nutrient digestibility in individual feedstuffs are additive in order to meet the nutrient specifications of the diets. Additivity is assumed to be applicable under most practical situations but has rarely been tested experimentally. In this study, a trial was conducted to evaluate the accuracy of the prediction of apparent protein and amino acid digestibility in compound formulations with the use of digestibility values estimated from individual feed ingredients. Additivity was tested using poultry meal and pork meal. It was established that protein and amino acid digestibility in animal protein ingredients are additive in *A. japonicus* and values determined for single protein source ingredients may be used to estimate digestibility in formulated compound diets. This is similar to the findings of Lupatsch *et al.* (1997), who concluded that nutrients digestibility in gilthead seabream were additive. However, non-additivity, due to interactions among dietary ingredients, is common in terrestrial herbivores' nutrition and is also found with some aquatic species (Sales and Britz, 2002; Sales, 2009).

Data obtained describing essential amino acid digestibility of feed ingredients would remain of limited value until the dietary essential amino acid requirements have been established. Once, the dietary essential amino acid requirements have been established, it becomes possible to use the essential amino acid digestibility data obtained from this study to design diets that supply optimal balance and adequate levels of essential amino acids to dusky kob. It is noteworthy that this is the first research work on essential amino acid digestibility, additivity and essential amino acid requirements in *A. japonicus.*

This research has established a species-specific seven-step approach to a nutritional research program; based on digestibility protocols, feed ingredients digestibility and essential amino

acid requirements of fish species. A stepwise nutritional approach in fish nutrition provides an extremely useful tool in development of diets or nutritional studies for a fish species. This approach should be followed before reliable conclusions can be drawn in fish nutrition. Furthermore, the species-specific seven-step nutritional research approach suggested in this study is highly adaptive and could be applied to other carnivorous finfish species that are being explored as potential aquaculture candidates.

A framework for evaluation of the species-specific seven-step approach is provided in Figure 5.1. It can be concluded that by applying this approach in nutritional research programs, an understanding of basic nutrient requirements that can be used to formulate nutritional, least-cost balanced diets that will optimise growth and reduce pollution could be established.

As dusky kob aquaculture is still new in South Africa, there will be an increasing need to formulate diets based on amino acids rather than protein requirement. Thus, data provided in this study could be used to develop nutritionally, least-cost diets for *A. japonicus*. In addition, studies should be conducted on grow-out fish to confirm the present findings and to quantify the impact on fish performance and on environmental water quality.

REFERENCES

Abidi, S.F., Khan, M.A., 2010. Growth, protein retention, and body composition of fingerling Indian major carp, *rohu labeo rohita* (hamilton), fed diets with various levels of lysine. *Journal of the World Aquaculture Society* 41, 791-799.

Abimorad, E.G., Favero, G., Squassoni, G., Carneiro, D., 2010. Dietary digestible lysine requirement and essential amino acid to lysine ratio for pacu *Piaractus mesopotamicus*. *Aquaculture Nutrition* 16, 370-377.

Ahmed, I., Khan, M.A., 2004. Dietary lysine requirement of fingerling Indian major carp, *cirrhinus mrigala*. *Aquaculture* 235, 499-511.

Akiyama, T., Oohara, I., Yamamoto, T., 1997. Comparison of essential amino acid requirement with A/E ratio among fish species (review paper). *Fisheries Science* 63, 963-970.

Akpinar, Z., Sevgili, H., Özgen, T., Demir, A., Emre, Y., 2012. Dietary protein requirement of juvenile shi drum, *Umbrina cirrosa* (L.). *Aquaculture Research* 43, 421-429.

Aksnes, A., Mundheim, H., Toppe, J., Albrektsen, S., 2008. The effect of dietary hydroxyproline supplementation on salmon (*Salmo salar L.*) fed high plant protein diets. *Aquaculture* 275, 242-249.

Alan Ward, D., Carter, C., Townsend, A., 2005. The use of yttrium oxide and the effect of faecal collection timing for determining the apparent digestibility of minerals and trace elements in Atlantic salmon (*Salmo salar, L.*) feed. *Aquaculture Nutrition* 11, 49-59.

Allan, G.L., Parkinson, S., Booth, M.A., Stone, D.A., Rowland, S.J., Frances, J., Warner Smith, R., 2000. Replacement of fishmeal in diets for Australian silver perch *Bidyanus bidyanus:* I. Digestibility of alternative ingredients. *Aquaculture* 186, 293-310.

Allan, G.L., Rowland, S.J., Parkinson, S., 1999. Nutrient digestibility for juvenile silver perch *Bidyanus bidyanus*: development of methods. *Aquaculture* 170, 131-141.

Ambardekar, A.A., Robert, C., Reigh, Millie, B.W., 2009. Absorption of amino acids from intact dietary proteins and purified amino acid supplements follows different time-courses in channel catfish (*Ictalurus punctatus*). *Aquaculture* 291, 179-189.

Amirkolaie, A.K., El-Shafai, S.A., Eding, E.H., Schrama, J.W., Verreth, J.A., 2005. Comparison of faecal collection method with high-and low-quality diets regarding digestibility and faeces characteristics measurements in Nile tilapia, *Aquaculture Research* 36, 578-585.

Anderson, J.S., Lall, S.P., Anderson, D.M., Chandrasoma, J., 1992. Apparent and true availability of amino acids from common feed ingredients for Atlantic salmon (*Salmo salar*) reared in seawater. *Aquaculture* 108, 111-124.

Anderson, J.S., Lall, S.P., Anderson, D.M., McNiven, M.A., 1995. Availability of amino acids from various fishmeal fed to Atlantic salmon (*Salmo salar*). *Aquaculture* 138, 291-301.

Anderson, P.M., Broderius, M.A., Fong, K.C., Tsui, K.N., Chew, S.F., Ip, Y.K., 2002. Glutamine synthetase expression in liver, muscle, stomach and intestine of *Bostrichthys sinensis* in response to exposure to a high exogenous ammonia concentration. *Journal of Experimental Biology* 205, 2053-2065.

Anderson, S., Lall, S.P., 2005. Amino acid nutrition of salmonids: Dietary requirements and bioavailability. *Cahiers Options Méditerranéennes* 63, 73-90.

Angkanaporn, K., Ravindran, V., Bryden, W.L., 1996. Additivity of apparent and true ileal amino acid digestibilities in soybean meal, sunflower meal, and meat and bone meal for broilers. *Poultry Science* 75, 1098-1103.

Association of Official Analytical Chemist (AOAC), 2003. Official methods of analysis. 18th Edition. Gaithersburg Maryland, U.S.A.

Atkinson, J., Hilton, J., Slinger, S., 1984. Evaluation of acid-insoluble ash as an indicator of feed digestibility in rainbow trout (*Salmo gairdneri*). *Canadian Journal of Fisheries and Aquatic Sciences* 41, 1384-1386.

Austreng, E., 1978. Digestibility determination in fish using chromic oxide marking and analysis of contents from different segments of the gastrointestinal tract. *Aquaculture* 13, 265-272.

Austreng, E., Storebakken, T., Thomassen, M.S., Refstie, S., Thomassen, Y., 2000. Evaluation of selected trivalent metal oxides as inert markers used to estimate apparent digestibility in salmonids. *Aquaculture* 188, 65-78.

Aya, F.A., Cuvin-Aralar, M.L., Coloso, R.M., 2015. Potential of cowpea (*Vigna unguiculata* L.) meal as an alternative protein source in diets for giant freshwater prawn (*Macrobrachium rosenbergii*, de Man 1879). In: Romana-Eguia, M.R.R., Parado-Estepa, F.D., Salayo, N.D., Lebata-Ramos, M.J.H. (Eds.). Proceedings of the International Workshop on Resource Enhancement and Sustainable Aquaculture Practices in Southeast Asia 2014, 231-237. Aquaculture Department, Southeast Asian Fisheries Development Center.

Baker, D.H., Han, Y., 1994. Ideal amino acid profile for chicks during the first three weeks post hatching. *Poultry Science* 73, 1441-1447.

Barrows, F.T., Frost, J.B., 2014. Evaluation of the nutritional quality of co-products from the nut industry, algae and an invertebrate meal for rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 434, 315-324.

Battaglene, S.C., Talbot, R.B., 1994. Hormone induction and larval rearing of mulloway, *Argyrosomus hololepidotus* (Pisces: Sciaenidae). *Aquaculture* 126, 73-81.

Bendiksen, E., Berg, O., Jobling, M., Arnesen, A., Måsøval, K., 2003. Digestibility, growth and nutrient utilization of Atlantic salmon parr (*Salmo salar L.*) in relation to temperature, feed fat content and oil source. *Aquaculture* 224, 283-299.

Berge, G.E., Sveier, H., Lied, E., 1998. Nutrition of Atlantic salmon (salmo salar): The requirement and metabolic effect of lysine. *Comparative Biochemistry and Physiology* 120A, 447-485.

Bernard, J.B., Ullrey, D.E., Wolff, P.L., 1995. Selecting appropriate markers for digestibility studies. Proceedings of the 11th Conference of Nutrition Advisory Group of AZA, Toronto, Ontario, Canada 1, 203–206.

Blyth, D., Tabrett, S., Bourne, N., Glencross, B., 2015. Comparison of faecal collection methods and diet acclimation times for the measurement of digestibility coefficients in barramundi (*Lates calcarifer*). *Aquaculture Nutrition* 21, 248-255.

Booth, M., Allan, G., Smullen, R., 2013. Digestibility of common feed ingredients by juvenile mulloway *Argyrosomus japonicus*. *Aquaculture* 414, 140-148.

Buentello, J.A., Gatlin, D.M., 1999. Nitric oxide production in activated macrophages from channel catfish (*Ictalurus punctatus*): influence of dietary arginine and culture media. *Aquaculture* 179, 513-521.

Bureau, D.P., Hua, K., 2006. Letter to the Editor of Aquaculture. *Aquaculture* 252, 103-105.

Burel, C., Boujard, T., Tulli, F., Kaushik, S.J., 2000. Digestibility of extruded peas, extruded lupin, and rapeseed meal in rainbow trout (*Oncorhynchus mykiss*) and turbot (*Psetta maxima)*. *Aquaculture* 188, 285-298.

Cao, J., Chen, Y., Zhu, X., Huang, Y., Zhao, H., Li, G., Lan, H., Chen, B., Pan, Q., 2012. A study on dietary l-lysine requirement of juvenile yellow catfish Pelteobagrus fulvidraco. *Aquaculture Nutrition 18*, 35-45.

Catacutan, M.R., Eusebio, P.S., Teshima, S., 2003. Apparent digestibility of selected feedstuffs by mud crab, *Scylla serrata*. *Aquaculture* 216, 253-261.

Cheng, T.K., Coon, C.N., 1990. Effect of calcium source, particle size, limestone solubility in vitro, and calcium intake level on layer bone status and performance. *Poultry Scien*ce 69, 2214-2219.

Cheng, Z., Ai, Q., Mai, K., Xu, W., Ma, H., Li, Y., Zhang, J., 2010. Effects of dietary canola meal on growth performance, digestion and metabolism of Japanese seabass, *Lateolabrax japonicus*. *Aquaculture* 305, 102-108.

Cheng, Z.J., Hardy, R.W., 2002. Apparent Digestibility Coefficients of Nutrients and Nutritional Value of Poultry By-product Meals for Rainbow Trout *Oncorhynchus mykiss* Measured in vivo Using Settlement. *Journal of the World Aquaculture Society* 33, 458-465.

Childs, A. R., Fennessy, S. T., 2013. Dusky kob (*Argyrosomus japonicus*). Mann, B.Q. (Ed), In Southern African Marine Linefish Species Profiles. Special Publication. 154-156. Oceanographic Research Institute: Durban, South Africa.

Cho, C., Bureau, D., 2001. A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. *Aquaculture Resources* 32, 349-360.

Cho, C., Kaushik, S., 1990. Nutritional energetics in fish: energy and protein utilization in rainbow trout (Salmo gairdneri). *World Review of Nutrition and Dietetics* 61, 132-172.

Cho, C., Slinger, S., 1979. Apparent digestibility measurement in feedstuffs for rainbow trout. *Finfish Nutrition and Fish Feed Technology* 2, 239-247.

Cho, C., Slinger, S., Bayley, H., 1982. Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. Comparative Biochemistry and Physiology Part B: *Comparative Biochemistry* 73, 25-41.

Chor, W., Lim, L., Chong, M., Lu, K., Sade, A., Shapawi, R., 2015. Evaluation of tempeh as a potential alternative protein source in the diets for juvenile tiger grouper, epinephelus. *Malaysian Journal of Science* 34, 58-68.

Choubert Jr, G., De La Noüe, J., Luquet, P., 1979. Continuous quantitative automatic collector for fish feces. *The Progressive Fish-Culturist* 41, 64-67.

Choubert, G., De la Noüe, J., Luquet, P., 1982. Digestibility in fish: improved device for the automatic collection of feces. *Aquaculture* 29, 185-189.

Chowdhury, M.K., Tacon, A.G., Bureau, D.P., 2012. Digestibility of amino acids in Indian mustard protein concentrate and Indian mustard meal compared to that of a soy protein concentrate in rainbow trout and Atlantic salmon. *Aquaculture* 356, 128-134.

Collett, P.D., Vine, N.G., Kaiser, H., 2008. The effect of light intensity on growth of juvenile dusky kob *Argyrosomus japonicus* (Temminck & Schlegel 1843). *Aquaculture Research* 39, 526-531.

Couto, A., Peres, H., Oliva-Teles, A., Enes, P., 2016. Screening of nutrient digestibility, glycaemic response and gut morphology alterations in gilthead sea bream (Sparus aurata) fed whole cereal meals. *Aquaculture* 450, 31-37.

Cowey, C. B., 1995. Protein and amino acid requirements: A critique of methods. *Journal of Applied Ichthyology* 11, 199-204.

Cowey, C., 2013. Protein metabolism in fish. Protein Deposition in Animals: *Proceedings of Previous Easter Schools in Agricultural Science*, 271.

Cowey, C.B., Sargent, J.R., 1979. In: [Anonymous] *Nutrition*. In: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds), Fish Physiology, Vol. VIII. Bioenergetic and Growth. Academic Press, New York, NY, 1-69.

Da Silva J. G., Oliva-Teles A, 1998. Apparent digestibility coefficients of feedstuffs in seabass seabass (*Dicentrarchus labrax*) juveniles. *Aquatic Living Resources* 11, 187-191.

DAFF, 2014. South Africa's aquaculture yearbook. Department of Agriculture, Forestry and Fisheries, Republic of South Africa.

Dairiki, J.K., 2007. Lysine requirements of largemouth bass, *Micropterus salmoides:* A comparison of methods of analysis of dose-response trials data. *Journal of Applied Aquaculture* 19, 1-27.

Davidson, J., Barrows, F.T., Kenney, P.B., Good, C., Schroyer, K., Summerfelt, S.T., 2016. Effects of feeding a fishmeal-free versus a fishmeal-based diet on post-smolt Atlantic salmon *Salmo salar* performance, water quality, and waste production in recirculation aquaculture systems. *Aquaculture Engineering* 74, 38-51.

Davies, S.J., Gouveia, A., Laporte, J., Woodgate, S.L., Nates, S., 2009. Nutrient digestibility profile of premium (category III grade) animal protein by-products for temperate marine fish species (European sea bass, gilthead sea bream and turbot). *Aquaculture Research* 40, 1759-1769.

De Francesco, M., Parisi, G., Pérez-Sanchez, J., Gomez-Réqueni, P., Medale, F., Kaushik, S., Mecatti, M., Poli, B., 2007. Effect of high-level fishmeal replacement by plant proteins in gilthead sea bream (*Sparus aurata*) on growth and body/fillet quality traits. *Aquaculture Nutrition* 13, 361-372.

De Pedro, N., Guijarro, A.I., López-Patiño, M.A., Martínez-Álvarez, R., Delgado, M.J., 2005. Daily and seasonal variations in haematological and blood biochemical parameters in the tench, *Tinca tinca* Linnaeus, 1758. *Aquaculture Research* 36, 1185–1196.

De Silva, S., Deng, D., Rajendram, V., 1997. Digestibility in goldfish fed diets with and without chromic oxide and exposed to sub lethal concentrations of cadmium. *Aquaculture Nutrition* 3, 109-114.

De Silva, S.S., Gunasekera, R.M., Gooley, G., 2000. Digestibility and amino acid availability of three protein-rich ingredient-incorporated diets by Murray cod *Maccullochella peelii peelii* (Mitchell) and the Australian shortfin eel Anguilla australis Richardson. *Aquaculture Research* 31, 195-205.

De Silva, S.S., Hasan, M.R., 2007. Feeds and fertilizers: the key to long-term sustainability of Asian aquaculture. In: Study and Analysis of Feeds and Fertilizers for Sustainable Aquaculture Development, FAO Fisheries Technical Paper 497 (ed. by M.R. Hasan, Hecht, T, De Silva, S.S., Tacon, A.G.J.), 19–47. Food and Agriculture. Organization (FAO) of the United Nations, Rome, Italy.

Deering, M., Hewitt, D., Sarac, H., 1996. A comparison of inert markers used to estimate protein digestibility in the leader prawn *Penaeus monodon. Journal of the World Aquaculture Society* 27, 103-106.

Deng, D., Dominy, W., Ju, Z.Y., Koshio, S., Murashige, R., Wilson, R.P., 2010. Dietary lysine requirement of juvenile pacific threadfin (*polydactylus sexfilis*). *Aquaculture* 308, 44-48.

Divakaran S., Leonard, G.O, Ian, P.F., 2002. Note on the methods for determination of chromic oxide in shrimp feeds. *Journal of Agriculture and Food Chemistry* 50, 44-467.

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

El-Sayed, A.M., 1999. Alternative dietary protein sources for farmed tilapia, *Oreochromis spp. Aquaculture* 179, 149-168.

Emmert, J.L., Baker, D.H., 1997. Use of the ideal protein concept for precision formulation of amino acid levels in broiler diets. *The Journal of Applied Poultry Research* 6, 462-470.

Fagbenro, O., Davies, S., 2001. Use of soybean flour (dehulled solvent-extracted soybean) as fishmeal substitute in practical diets for African catfish, *Clarias gariepinus* (Burchell 1822): growth, feed utilization and digestibility. *Journal of Applied Ichthyology* 17, 64-69.

Falaye, A., Jauncey, K., 1999. Acceptability and digestibility by tilapia *Oreochromis niloticus* of feeds containing cocoa husk. *Aquaculture Nutrition* 5,157-162.

FAO (Food and Agriculture Organization of the United Nations), 2014. The state of world fisheries and aquaculture.

FAO (Food and Agriculture Organization of the United Nations), 2016. The state of world fisheries and aquaculture.

Feij, P., Colen, R., Rodrigues, V., Pous Ferreira, P., Dias, L.E.C.C., 2012. Ability of Meagre (*Argyrosomus regius*) to Digest Vegetable Protein Sources. World Aquaculture Society AQUA, Prague (Abstract 1125).

Ferguson, G.J., Ward, T.M., Ivey, A., Barnes, T., 2014. Life history of *Argyrosomus japonicus,* a large sciaenid at the southern part of its global distribution: implications for fisheries management. *Fisheries Research* 151, 148-157.

Fico, M.E., Hassan, A.S., Milner, J.A., 1982. The influence of excess lysine on urea cycle operation and pyrimidine biosynthesis. *Journal of Nutrition* 112, 1854-1861.

Fielder, D., Bardsley, W., 1999. A preliminary study on the effects of salinity on growth and survival of mulloway *Argyrosomus japonicus* larvae and juveniles. *Journal of World Aquaculture Society* 30, 380-387.

Finke, M.D., DeFoliart, G.R., Benevenga, N.J., 1987. Use of a four-parameter logistic model to evaluate the protein quality of mixtures of Mormon cricket meal and corn gluten meal in rats. *Journal of Nutrition* 117, 1740-1750.

Firman, J.D., Boling, S.D., 1998. Lysine: Ideal protein in turkeys. *Poultry Science* 77, 105-110.

Forster, I., Dominy, W., Obaldo, L., Tacon, A., 2003. Rendered meat and bone meals as ingredients of diets for shrimp *Litopenaeus vannamei* (Boone, 1931). *Aquaculture* 219, 655-670.

Forster, I., Dominy, W., Tacon, A., 2002. The use of concentrates and other soy products in shrimp feeds. *Advaces en Nutricion Acuicola VI. Memorias del VI Simposium Internacional de Nutrcion Acuicola* 3–6 de Septiembre del 2002. Cancun, Quintana Roo, Mexico.

Forster, I., Ogata, H.Y., 1998. Lysine requirement of juvenile Japanese flounder paralichthys olivaceus and juvenile red sea bream *pagrus major*. *Aquaculture* 161, 131-142.

Francis, G., Makkar, H.P.S., Becker, K., 2001. Anti-nutritional factors present in plant–derived alternate fish feed ingredients and their effects in fish. *Aquaculture* 199, 197-227.

Furuya, W.M., Graciano, T.S., Vidal, L.V.O., Xavier, T.O., Gongora, L.D., Righetti, J.S., Furuya, V.R.B., 2012. Digestible lysine requirement of Nile tilapia fingerlings fed arginine-tolysine-balanced diets. *Revista Brasileira De Zootecnia* 41, 485-490.

Furuya, W.M., Michelato, M., Salaro, A.L., da Cruz, T.P., Barriviera-Furuya, V.R., 2015. Estimation of the dietary essential amino acid requirements of colliroja Astyanax fasciatus by using the ideal protein concept. *Latin American Journal of Aquaculture Research* 43, 888-894.

Furuya, W.M., Santos, V.G., Silva, L.C.R., Furuya, V.R.B., Sakaguti, E.S., 2006. Digestible lysine requirement of Nile tilapia juveniles. *Brazilian Journal of Animal Science* 35, 937-942.

Gahl, M.J., Finke, M.D., Crenshaw, T.D., Benevenga, A.J., 1991. Use of a Four-Parameter Logistic Equation to Evaluate the Response of Growing Rats to Ten Levels of Each Indispensable Amino Acid. *Animal Science Journal* 67, 240.

Gao, S., Yin, N., Zhou, F., Li, H., Zhou, J., Wang, R., Shao, Q., 2013. Evaluation of pea proteins and poultry protein as fishmeal alternatives in the diets for juvenile black sea bream, *Acanthopagrus schlegelii. Aquaculture Nutrition* 19, 278-288.

García-Ortega, A., Kissinger, K.R., Trushenski, J.T., 2016. Evaluation of fishmeal and fish oil replacement by soybean protein and algal meal from Schizochytrium limacinum in diets for giant grouper *Epinephelus lanceolatus. Aquaculture* 452, 1-8.

Gatlin III, D., 2010. Principles of fish nutrition. Southern Regional Aquaculture Center (SRAC) Publication 5003, 8. https://srac.tamu.edu/index.cfm/event/viewAllSheets/.

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Resources* 38, 551-579.

Gaylord, T., Barrows, F., Rawles, S., 2010. Apparent amino acid availability from feedstuffs in extruded diets for rainbow trout Oncorhynchus mykiss. *Aquaculture Nutrition* 16, 400-406.

Gaylord, T., Rawles, S., Gatlin, D., 2004. Amino acid availability from animal blended and plant feedstuffs for hybrid striped bass (*Morone chrysops*× *M. saxatilis*). *Aquaculture Nutrition*. 10, 345-352.

Glencross, B., Blyth, D., Irvin, S., Bourne, N., Campet, M., Boisot, P., Wade, N.M., 2016. An evaluation of the complete replacement of both fishmeal and fish oil in diets for juvenile Asian seabass, *Lates calcarifer*. *Aquaculture* 451, 298-309.

Glencross, B., Booth, M., Allan, G., 2007. A feed is only as good as its ingredients-a review of ingredient evaluation strategies for aquaculture feeds. *Aquaculture Nutrition* 13, 17-34.

Glencross, B., Evans, D., Dods, K., McCafferty, P., Hawkins, W., Maas, R., Sipsas, S., 2005. Evaluation of the digestible value of lupin and soybean protein concentrates and isolates when fed to rainbow trout, *Oncorhynchus mykiss,* using either stripping or settlement faecal collection methods. *Aquaculture* 245, 211-220.

Glencross, B., Hawkins, W., Curnow, J., 2004. Nutritional assessment of Australian canola meals. I. Evaluation of canola oil extraction method and meal processing conditions on the digestible value of canola meals fed to the red seabream (*Pagrus auratus, Paulin*). *Aquaculture Resources* 35, 15-24.

Goddard, J., McLean, E., 2001. Acid-insoluble ash as an inert reference material for digestibility studies in tilapia, *Oreochromis aureus*. *Aquaculture* 194, 93-98.

Gomes, E.F., Rema, P., Kaushik, S.J., 1995. Replacement of fishmeal by plant proteins in the diet of rainbow trout (*Oncorhynchus mykiss*): digestibility and growth performance. *Aquaculture* 130, 177-186.

Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J., Médale, F., Martin, S., Houlihan, D., Kaushik, S., Pérez-Sánchez, J., 2004. Protein growth performance, amino acid

utilization and somatotropic axis responsiveness to fishmeal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). *Aquaculture* 232, 493-510.

Green, J., Hardy, R., 2002. The optimum dietary essential amino acid pattern for rainbow trout (*Oncorhynchus mykiss*), to maximize nitrogen retention and minimize nitrogen excretion. *Fish Physiology Biochemistry* 27, 97-108.

Griffin, M.E., Brown, P.B., Grant, A.L., 1992. The dietary lysine requirement of juvenile hybrid striped bass. *Journal of Nutrition*, 122, 1332-1337.

Griffiths, M. H. and Heemstra, P. H. 1995. A contribution to the taxonomy of the marine fish genus *Argyrosomus* (Perciformes: Sciaenidae), with descriptions of two new species from southern Africa. J. L. B. Smith Institute of Ichthyology: *Ichthyological Bulletin* 45, 1-40.

Griffiths, M.H., 1997. Management of South African dusky kob *Argyrosomus japonicus* (Sciaenidae) based on per-recruit models. *South African Journal of Marine Science* 18, 213-228.

Hajen, W., Beames, R., Higgs, D., Dosanjh, B., 1993. Digestibility of various feedstuffs by post-juvenile chinook salmon (*Oncorhynchus tshawytscha*) in seawater. 1. Validation of technique. *Aquaculture* 112, 321-332.

Halver, J.E., Hardy, R.W., 2002. Fish Nutrition. 3rd Edition, Academic Press. California, USA, 15-110.

Hanley, F., 1987. The digestibility of foodstuffs and the effects of feeding selectivity on digestibility determinations in tilapia, *Oreochromis niloticus* (L). *Aquaculture* 66, 163-179.

Hardy, R.W., 1996. Alternate protein sources for salmon and trout diets. *Animal Feed Science Technology* 59, 71-80.

Hardy, R.W., 2008. Farmed fish diet requirements for the next decade and implications for global availability of nutrients. Lim, C., Webster, C.D., Lee, C.S. (Eds.). 1-16, In: *Alternate Protein Sources in Aquaculture Diets*, The Haworth Press, New York.

Hardy, R.W., 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Aquaculture Research* 41, 770-776.

Harpaz, S., 2005. L-carnitine and its attributed functions in fish culture and nutrition-a review. *Aquaculture* 249, 3-21.

Harper, A.E., Benevenga, N.G., Wohlhuetre, R.M., 1970. Effects of ingestion of disproportionate amount of amino acid. *Physiological Review* 50, 428-558.

Hasan, M.R., 2000. Nutrition and feeding for sustainable aquaculture development in the third millennium. In *Aquaculture in the third millennium*. Technical proceedings of the conference on aquaculture in the third millennium, Bangkok, Thailand, 20-25.

Hauler, R.C., Carter, C.G., 2001. Reevaluation of the quantitative dietary lysine requirements of fish. *Reviews in Fisheries Science* 9, 133-163.

Heinitz, M., Lemme, A., Schulz, C., 2015. Measurement of digestibility in agastric fish based on stripping method–apparent nutrient, energy and amino acid digestibility of common feed ingredients for carp diets (*Cyprinus carpio*). *Aquaculture Nutrition* 22, 1065-1078.

Hernández, A., Roman, D., 2016. Phosphorus and nitrogen utilization efficiency in rainbow trout (*Oncorhynchus mykiss*) fed diets with lupin (*Lupinus albus*) or soybean (Glycine max) meals as partial replacements to fishmeal. *Czech Journal of Animal Science* 61, 67-74.

Hernández, C., Hardy, R., Marquez Martinez, D., Dominguez-Jimenez, P., González-Rodríguez, B., 2015. Evaluation of apparent digestibility coefficients of individual

feed ingredients in spotted rose snapper *Lutjanus guttatus* (Steindachner, 1869). *Aquaculture Nutrition* 21, 835-842.

Hernández, C., Olvera-Novoa, M., Hardy, R., Hermosillo, A., Reyes, C., González, B., 2010. Complete replacement of fishmeal by porcine and poultry by-product meals in practical diets for fingerling Nile tilapia *Oreochromis niloticus:* digestibility and growth performance. *Aquaculture Nutrition* 16, 44-53.

Hertrampf, J.W., Piedad-Pascual, F., 2012. Handbook on Ingredients for Aquaculture Feeds. Kluwer Academic Publishers, Dordrecht, The Netherlands. 573.

Hien, T.T.T., Phuong, N., Le Tu, T., Glencross, B., 2010. Assessment of methods for the determination of digestibility of feed ingredients for Tra catfish, *Pangasinodon hypothalamus*. *Aquaculture Nutrition* 16, 351-358.

Hillestad, Marie, Torbjørn, Å., Gerd, M.B., 1999. Determination of digestibility of commercial salmon feeds. *Aquaculture* 179, 81-94.

Hoseini, S.M., Hosseini, S.A., Soudagar, M., 2013. Effect of dietary free L-lysine on growth performance and muscle composition of beluga *huso huso* (Linnaeus 1785) juveniles. *International Journal of Aquatic Biology* 1, 42-47.

Hossain, M., Nahar, N., Kamal, M., 1997. Nutrient digestibility coefficients of some plant and animal proteins for rohu (*Labeo rohita*). *Aquaculture* 151, 37-45.

Hutchings, K., Lamberth, S., 2003. Likely impacts of an eastward expansion of the inshore gill-net fishery in the Western Cape, South Africa: implications for management. *Marine and Freshwater Research* 54, 39-56.

Jauncey, K., Tacon, A., Jackson, A., 1983. The quantitative essential amino acid requirements of Oreochromis (*Sarotherodon mossambicus*). In: Fishelson, L., Yarron, Z. (Eds.), Proceedings of the First Symposium on Tilapia in Aquaculture, Nazareth, Israel, 328-337.

Jimoh, W., Fagbenro, O., Adeparusi, E., 2010. Digestibility coefficients of processed jackbean meal *Cannavalia ensiformis* (L.) DC for Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) diets. *International Journal of Fisheries and Aquaculture* 2, 102-107.

Jirsa, D., Barrows, F., Hardy, R., Drawbridge, M., 2015. Alternative protein blends as a replacement for fishmeal in diets for white seabass, *Atractoscion nobilis*. *Aquaculture Nutrition* 21, 861-867.

Jones, J.D., 1964. Lysine-arginine antagonism in the chick. *Journal of Nutrition* 84, 313-321.

Kaushik, S.J., 1998. Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psettamaxima*) with an estimation of their IAA requirement profiles. *Aquaculture Living Resources* 11, 355-358.

Kaushik, S.J., de Oliva Teles, A., 1985. Effect of digestible energy on nitrogen and energy balance in rainbow trout. *Aquaculture* 50, 89-101.

Kaushik, S.J., Fauconneau, B., 1984. Effects of lysine administration on plasma arginine and on some nitrogenous catabolites in rainbow trout. *Comparative Biochemistry and Physiology* 79A, 459-462.

Kaushik, S.J., Seiliez, I., 2010. Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. *Aquaculture Research* 41, 322-332.

Keramat Amirkolaie, A., Schrama, J., 2015. Time related alterations in digestibility and faecal characteristics as affected by dietary composition in the Nile tilapia (*Oreochromis niloticus L.*). *Aquaculture Research* 46, 1078-1086.

Kerr, B.J., Easter, R.A., 1995. Effect of feeding reduced protein, amino acid-supplemented diets on nitrogen and energy balance in grower pigs. *Journal Animal Science* 73, 3000-3008.

Ketola, H.G., 1983. Requirement for dietary lysine and arginine by fry of rainbow trout. *Journal of Animal Science* 56, 101-107.

Khan, M.A., 2013. Dietary l-lysine requirement of fingerling stinging catfish, heterppneustes fossilis (bloch) for optimizing growth, feed conversion, protein and lysine deposition. *Aquaculture Resources* 44, 523-533.

Kim, E.J., Utterback, P.L., Applegate, T.J., Parsons, C.M., 2011. Comparison of amino acid digestibility of feedstuffs determined with the precision-fed cecectomized rooster assay and the standardized ileal amino acid digestibility assay. *Poultry Science* 90, 2511-2519.

Kim, J., Lall, S.P., 2000. Amino acid composition of whole body tissue of Atlantic halibut (*Hippoglossus hippoglossus*), yellowtail flounder (*Pleuronectes ferruginea*) and Japanese flounder (*Paralichthys olivaceus*). *Aquaculture* 187, 367-373.

Kim, K.I., Kayes, T.B., Amundson, C.H., 1992. Requirements for lysine and arginine by rainbow trout, *oncorhynchus mykiss*. *Aquaculture* 106, 333-344.

Kiron, V., Phromkunthong, W., Huntley, M., Archibald, I., Scheemaker, G.d., 2012. Marine microalgae from bio refinery as a potential feed protein source for Atlantic salmon, common carp and white leg shrimp. *Aquaculture Nutrition* 18, 521-531.

Kissil, G.W., Lupatsch, I., Higgs, D., Hardy, R., 2000. Dietary substitution of soy and rapeseed protein concentrates for fishmeal, and their effects on growth and nutrient utilization in gilthead seabream *Sparus aurata* L. *Aquaculture Resources* 31, 595-601.

Krome, C., Focken, U., 2016. Effects of earthworm, *Perionyx excavatus* meal in practical diets on growth and body composition of common carp, *Cyprinus carpio*. *AACL Bioflux* 9, 340-344.

Krontveit, R.I., Bendiksen, E.Å., Aunsmo, A., 2014. Field monitoring of feed digestibility in Atlantic salmon farming using crude fiber as an inert marker. *Aquaculture* 426, 249-255.

Kumar, R., 1992. Anti-nutritional factors, the potential risks of toxicity and methods to alleviate them. Legume trees and other fodder trees as protein source for livestock. *FAO Animal Production and Health Paper* 102, 145-160.

Langeland, M., Andersson, K., Lundh, T., Lindberg, J.E., 2014. Dietary lysine requirement of juvenile Eurasian perch (*perca fluviatalis*). *Journal of Fisheries Sciences* 8, 161-175.

Lee, S., 2002. Apparent digestibility coefficients of various feed ingredients for juvenile and grower rockfish (*Sebastes schlegeli*). *Aquaculture* 207, 79-95.

Lepage, O., Tottmar, O., Winberg, S., 2002. Elevated dietary intake of L-tryptophan counteracts the stress-induced elevation of plasma cortisol in rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology* 205, 3679-3687.

Li, P., Gatlin III, D.M., 2007. Evaluation of dietary supplementation of graded levels of β-Hydroxy-β-Methylbutyrate for hybrid striped bass *Morone chrysops*× *M. saxatilis*. *Journal of Applied Aquaculture* 19, 77-88.

Li, P., Mai, K., Trushenski, J., Wu, G., 2009. New developments in fish amino acid nutrition: towards functional and environmentally oriented aqua feeds. *Amino Acids* 37, 43-53.

Li, P., Webb, K.A., Gatlin, D.M., 2008. Evaluation of Acid-insoluble Ash as an Indicator for Digestibility Determination with Red Drum, *Sciaenops ocellatus,* and Hybrid Striped Bass, *Morone chrysops*× *M. saxatilis. Journal of the World Aquaculture Society* 39, 120-125.

Lied, E., Julshamn, K., Braekkan, O., 1982. Determination of protein digestibility in Atlantic cod *(Gadus morhua*) with internal and external indicators. *Canadian Journal of Fisheries and Aquatic Sciences* 39, 854-861.

Lin, Y., Gong, Y., Yuan, Y., Gong, S., Yu, D., Li, Q., Luo, Z., 2012. Dietary L-Lysine requirement of juvenile Chinese sucker, *Myxocyprinus asiaticus*. *Aquaculture Research* 1-11.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193, 265-275.

Lu, F., Haga, Y., Satoh, S., 2015. Effects of replacing fishmeal with rendered animal protein and plant protein sources on growth response, biological indices, and amino acid availability for rainbow trout *Oncorhynchus mykiss*. *Fisheries Science* 81, 95-105.

Luo, Y., Ai, Q., Mai, K., Zhang, W., Xu, W., Zhang, Y., 2012. Effects of dietary rapeseed meal on growth performance, digestion and protein metabolism in relation to gene expression of juvenile cobia (*Rachycentron canadum*). *Aquaculture* 368, 109-116.

Luo, Z., Liu, Y.J., Mai, K.S., Tian, L.X., Yang, H.J., Liang, G.Y., Liu, D.H., 2006. Quantitative L-lysine requirement of juvenile grouper, epinephelus coioides. *Aquaculture* Nutrition 12, 165-172.

Lupatsch, I., Kissil, G.W., Sklan, D., Pfeffer, E., 1997. Apparent digestibility coefficients of feed ingredients and their predictability in compound diets for gilthead seabream, *Sparus aurata L. Aquaculture Nutrition* 3, 81-89.

Magalhães, R., Coutinho, F., Pousão-Ferreira, P., Aires, T., Oliva-Teles, A., Peres, H., 2015. Corn distiller's dried grains with solubles: Apparent digestibility and digestive enzymes activities in European seabass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*). *Aquaculture* 443, 90-97.

Mai, K., Zhang, L., Ai, Q., Duan, Q., Zhang, C., Li, H., Wan, J., Liufu, Z., 2006. Dietary lysine requirement of juvenile Japanese seabass, *Lateolabrax japonicus*. *Aquaculture* 258, 535-542.

Marais, J., 2000. Use of markers. Felix D'Mello, J.P. (Ed.), Farm animal metabolism and nutrition: critical reviews. Wallingford: CAB International, 255-277.

Marcouli, P.A., Alexis, M.N., Andriopoulou, A., Iliopulu- Georgudak, J., 2006. Dietary lysine requirement of juvenile gilthead seabream, *Sparus aurata. Aquaculture Nutrition* 12, 25-33.

Masagounder, K., Firman, J., Hayward, R., Sun, S., Brown, P., 2009. Apparent digestibility of common feedstuffs for bluegill Lepomis macrochirus and largemouth bass Micropterus salmoides using individual test ingredients. *Aquaculture Nutrition* 15, 29-37.

Masagounder, K., Hayward, R., Firman, J., 2011. Comparison of dietary essential amino acid requirements determined from group-housed versus individually-housed juvenile bluegill, *Lepomis macrochirus. Aquaculture Nutrition* 17, 559-571.

Masumoto, T., Ruchimat, T., Ito, Y., Hosokawa, H., Shimeno, S., 1996. Amino acid availability values for several protein sources for yellowtail (*Seriola quinqueradiata*). *Aquaculture* 146, 109-119.

Maynard, L.A., Loosli, J.K., 1969. Animal nutrition. McGraw-Hill, New York, 6th edition.

Mabasa, N., 2017. The effect of carbohydrate on dusky kob, *Argyrosomus japonicus,* fed pelleted diets. MSc Thesis. Rhodes University, Grahamstown, South Africa. 93.

Miles, R., Chapman, F., 2006. *The Benefits of Fish Meal in Aquaculture Diets.* University of Florida, IFAS Extension. FA122, 1-6.

Minjarez-Osorio, C., Castillo-Alvarado, S., Gatlin, D., González-Félix, M.L., Perez-Velazquez, M., Rossi, W., 2016. Plant protein sources in the diets of the sciaenid red drum (*Sciaenops ocellatus*) and shortfin corvina (*Cynoscion parvipinnis):* A comparative study. *Aquaculture* 453, 122-129.

Mommsen, T.P., French, C., Hochachka, P., 1980. Sites and patterns of protein and amino acid utilization during the spawning migration of salmon. *Canadian Journal of Zoology* 58, 1785-1799.

Morales, A., Cardenete, G., Sanz, A., De la Higuera, M., 1999. Re-evaluation of crude fibre and acid-insoluble ash as inert markers, alternative to chromic oxide, in digestibility studies with rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 179, 71-79.

Mota, C., Araújo, J., Pádua, D., Martins, G., Guimarães, I., 2015. Testing various faeces-collecting methods to improve digestibility studies with tambaqui, *Colossoma macropomum* (Cuvier, 1816). *Journal of Applied Ichthyology* 31, 102-109.

Muggeo, V.M., 2008. Segmented: an R package to fit regression models with broken-line relationships. *R news* 8, 20-25.

Murai, T., Akiyama, T., Nose, T., 1982. Effects of casein coating on utilization of dietary amino acids by fingerling carp and channel catfish. *Bulletin of the Japanese Society of Scientific Fisheries* 48, 787-792.

Murray, H.M., Lall, S.P., Rajaselvam, R., Boutilier, L.A., Blanchard, B., Flight, R.M., Colombo, S., Mohindra, V., Douglas, S.E., 2010. A nutrigenomic analysis of intestinal response to partial soybean meal replacement in diets for juvenile Atlantic halibut, *Hippoglossus hippoglossus*, L. *Aquaculture* 298, 282-293.

Ng, W., Hung, S., 1995. Estimating the ideal dietary indispensable amino acid pattern for growth of white sturgeon, Acipenser transmontanus (Richardson). *Aquaculture Nutrition* 1, 85-94.

Ngo, D.T., Pirozzi, I., Glencross, B., 2015. Digestibility of canola meals in barramundi (Asian seabass; *Lates calcarifer*). *Aquaculture* 435 442-449.

Norambuena, F., Lewis, M., Hamid, N.K.A., Hermon, K., Donald, J.A., Turchini, G.M., 2013. Fish oil replacement in current aquaculture feed: is cholesterol a hidden treasure for fish nutrition. *PloS one* 8, 1-9.

Nose, T., 1960. On the digestion of food protein by gold-fish (*Carassius auratus* L.) and rainbow trout (*Salmo irideus* G). *Bulletin of Freshwater Fisheries Research Laboratory* 10, 11-22.

NRC (National Research Council), 1993. Nutrient Requirements of Fish. National Academy Press, Washington, DC, USA.

NRC (National Research Council), 2011. Nutrient Requirements of Fish and Shrimp. The National Academies Press, Washington DC, USA.

Nyina-wamwiza, L., Wathelet, B., Richir, J., Rollin, X., Kestemont, P., 2010. Partial or total replacement of fishmeal by local agricultural by-products in diets of juvenile African catfish (*Clarias gariepinus*): growth performance, feed efficiency and digestibility. *Aquaculture Nutrition* 16, 237-247.

Ogino, C., 1980. Requirements of carp and rainbow trout for essential amino acids. *Bulletin of the Japanese Society of Scientific Fisheries* 46, 171-174.

Ogino, C., Kakino, J., Chen, M.S., 1973. Protein nutrition in fish II. Determination of metablic faecal nitrogen and endogenous nitrogen excretions of carp. *Freshwater Fisheries Research Laboratory* 39, 519-523.

Olim, C.P.R., 2012. Apparent Digestibility Coefficient of Feed Ingredients for Juvenile Meagre (*Argyrosomus regius,* Asso 1801). (MSc Thesis) Faculdade de Ciências. Universidade doPorto 79.

Oliva-Teles, A., Enes, P., Peres, H., 2015. Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish. In: Davis, D.A. (Ed.), *Feed and Feeding Practices in Aquaculture*. Woodhead Publishing, Cambridge, 203-233.

Olsen, R.L., Hasan, M.R., 2012. A limited supply of fishmeal: Impact on future increases in global aquaculture production. *Trends Food Science and Technology* 27, 120-128.

Opstvedt, J., Miller, R., Hardy, R.W., Spinelli, J., 1984. Heat-induced changes in sulfhydryl groups and disulfide bonds in fish protein and their effect on protein and amino acid digestibility in rainbow trout (*Salmo gairdneri*). *Journal of Agricultural and Food Chemistry* 32, 929-935.

Owens, F.N., Hanson, C.F., 1992. External and internal markers for appraising site and extent of digestion in ruminants. *Journal of Dairy Science* 75, 2605-2617.

Percival, S., Lee, P., Carter, C., 2001. Validation of a technique for determining apparent digestibility in large (up to 5 kg) Atlantic salmon (*Salmo salar L.)* in seacages. *Aquaculture* 201, 315-327.

Péres, A., Cahu, C., Infante, J.Z., 1997. Dietary spermine supplementation induces intestinal maturation in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiology Biochemistry* 16, 479-485.

Peres, H., Oliva-Teles, A., 2008. Lysine requirement and efficiency of lysine utilization in turbot (*scophthalmus maximus*) juveniles. *Aquaculture* 275, 283-290.

Peres, H., Aires Oliva-Teles, 2009. The optimum dietary essential amino acid profile for gilthead seabream (*Sparus aurata*) juveniles. *Aquaculture* 296, 81-86.

Pirozzi, I., Booth, M.A., Allan, G.L., 2010. A factorial approach to deriving dietary specifications and daily feed intake for mulloway, *Argyrosomus japonicus,* based on the requirements for digestible protein and energy. *Aquaculture* 302, 235-242.

PIRSA, 2001. Mulloway Aquaculture in South Australia. In: Fact Sheet. Department of Primary Industries and Resources South Australia, 6 pages.

Poli, B.M., Parisi, G., Mecatti, M., Lupi, P., Iurzan, F., Zampacavallo, G., Gilmozzi, M., 2001. "The meagre (*Argyrosomus regius*), a new species for Mediterranean

aquaculture" Morphological, merchantable and nutritional traits in a commercial wide size-range. *European Aquaculture Society Special Publication* 29, 209-210.

Portz, L., Cyrino, J.E.P., 2003. Comparison of the amino acid contents of roe, whole body and muscle tissue and their A/E ratios for largemouth bass *Micropterus salmoides* (Lacepéde, 1802). *Aquaculture Research* 34, 585-592.

Portz, L., Cyrino, J.E.P., Martino, R.C., 2001. Growth and body composition of juvenile largemouth bass Micropterus salmoides in response to dietary protein and energy levels. *Aquaculture Nutrition* 7, 247-254.

Pozza, P.C., Gomes, P.C., Pozza, M.S.S., Nunes, R.V., Ferreira, R.A., Rodrigueiro, R.J.B., 2013. Use of markers to determine the ileal digestibility of amino acids for swine. *Ciência e Agrotecnologia* 37, 259-265.

Rahnema, S., Borton, R., Shaw, E., 2005. Determination of the effects of fish vs plant vs meat protein-based diets on the growth and health of rainbow trout. *Journal of Applied Animal Research* 27, 77-80.

Ravi, J., Devaraj, K.V., 1991. Quantitative essential amino acid requirements for growth of catla, *catla catla* (hamilton). *Aquaculture* 96, 281-291.

Rawles, S., Thompson, K., Brady, Y., Metts, L., Gannam, A., Twibell, R., Webster, C., 2010. A comparison of two faecal collection methods for protein and amino acid digestibility coefficients of menhaden fishmeal and two grades of poultry by-product meals for market-size sunshine bass (*Morone chrysops*× *M. saxatilis), Aquaculture Nutrition* 16, 81-90.

Rawles, S.D., Green, B.W., Gaylord, T.G., Barrows, F.T., McEntire, M.E., Freeman, D.W., 2012. Response of sunshine bass (*Morone chrysops x M. saxatilis*) to digestible protein/dietary lipid density and ration size at summer culture temperatures in the Southern United States. *Aquaculture* 356, 80-90.

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

Reyes, M.L.E., Velázquez, E.A.R., Hernández, L.H.H., Araiza, M.A.F., 2015. Effect of the Substitution of Fish Oil with a Mixture of Plant-Based Oils in Diets of Rainbow Trout (*Oncorhynchus mykiss* Walbaum) Fingerlings on Growth, Phosphorus and Nitrogen Excretion. *The Israeli Journal of Aquaculture-Bamidgeh* 67. http://hdl.handle.net/10524/49216

Ribeiro, M.J.P., Vidotti, R.M., Ferreira, L.A., Gonçalves, G.S., 2016. Evaluation of Soy Protein Concentrate and Meat and Bone Meal as a Replacement for Fish Meal in the Diet of Nile Tilapia Fingerlings. *Journal of the World Aquaculture Society* 47, 369-375

Riche, M., White, M., Brown, P., 1995. Barium carbonate as an alternative indicator to chromic oxide for use in digestibility experiments with rainbow trout. *Nutrition Research* 15, 1323-1331.

Richter, H., Lückstädt, C., Focken, U., Becker, K., 2003. Evacuation of pelleted feed and the suitability of titanium (IV) oxide as a feed marker for gut kinetics in Nile tilapia. *Journal of Fish Biology* 63, 1080-1099.

Riley, W., Higgs, D., Dosanjh, B., Eales, J., 1996. Influence of dietary arginine and glycine content on thyroid function and growth of juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Nutrition* 2, 235-242.

Rodehutscord, M., Becker, A., Pack, M., Pfeffer, E., 1997. Response of rainbow trout (*Oncorhynchus mykiss*) to supplements of individual essential amino acids in a semi purified diet, including an estimate of the maintenance requirement for essential amino acids. *Journal of Nutrition* 127, 1166-1175.

Rollin, X., Mambrini, M., Abboudi, T., Larondelle, Y., Kaushik, S.J., 2003. The optimum dietary indispensable amino acid pattern for growing Atlantic salmon (*Salmo salar* L.) fry. *British Journal of Nutrition* 90, 865-876.

Rossi, W., Newcomb, M., Gatlin, D.M., 2016. Assessing the nutritional value of an enzymatically processed soybean meal in early juvenile red drum, *Sciaenops ocellatus* *Aquaculture* 467, 94-101.

Rowan, A.M., Moughan, P.J., Wilson, M.N., 1991. Acid-insoluble ash as a marker compound for use in digestibility studies with humans. *Journal of the Science of Food and Agriculture* 54, 269-274.

Sabahelkhier, M., Adam, S.I., Rayan, M., Amana, I., Maab, M., Nusiba, A., 2014. Research Article Assessment of Protein Content and Study Effect of Anti-Nutrition Factor on in-vitro protein digestibility. *Nova* 3, 1-5.

Sadiku, S., Jauncey, K., 1995. Digestibility, apparent amino acid availability and waste generation potential of soybean flour: poultry meat meal blend based diets for tilapia, *Oreochromis niloticus* (L.) fingerlings. *Aquaculture Research* 26, 651-657.

Sakomura, N.K., Rostagno, H.S., 2007. Métodos De Pesquisa Em Nutrição De Monogástricos. Funep 283, 1-8.

Sales, J., 2009. The error associated with the prediction of digestible protein contents of fish diets from tabulated values. *Czech Journal of Animal Science* 54, 498-509.

Sales, J., Britz, P., 2002. Evaluation of the reference diet substitution method for determination of apparent nutrient digestibility coefficients of feed ingredients for South African abalone (*Haliotis midae L.*). *Aquaculture* 207, 113-123.

Sales, J., Janssens, G., 2003. The use of markers to determine energy metabolizability and nutrient digestibility in avian species. *World's Poultry Science Journal* 59, 314-327.

Salze, G., Craig, S., Schwarz, M., McLean, E., 2008. Novel live feed enrichments beneficially impact digestive ongogeny in larval cobia *Rachycentron candadum. Abstract of Aquaculture America* 8, 337.

Saravanan, S., Schrama, J.W., Figueiredo-Silva, A.C., Kaushik, S.J., Verreth, J.A., Geurden, I., 2012. Constraints on energy intake in fish: the link between diet composition, energy metabolism, and energy intake in rainbow trout. *Plos One 7*, 34743.

Serrano, J.A., Nematipour, G.R., Gatlin, D.M., 1992. Dietary protein requirement of the red drum (*Sciaenops ocellatus*) and relative use of dietary carbohydrate and lipid. *Aquaculture* 101, 283-291.

Shamushaki, V.A.J., Kasumyan, A.O., Abedian, A., Abtahi, B., 2007. Behavioural responses of the Persian sturgeon (*Acipenser persicus*) juveniles to free amino acid solutions. *Marine Freshwater Behavior and Physiology*. 40, 219-224.

Shiau, S., Liang, H., 1995. Carbohydrate utilization and digestibility by tilapia, Oreochromis niloticus crossed with *O. aureus*, are affected by chromic oxide inclusion in the diet. *The Journal of Nutrition* 125, 976-982.

Shiau, S., Lin, S., 1993. Effect of supplemental dietary chromium and vanadium on the utilization of different carbohydrates in tilapia, *Oreochromis niloticus*× *O. aureus. Aquaculture* 110, 321-330.

Shipton, T., Britz, P., 2001. An assessment of the use of chromic oxide as a marker in protein digestibility studies with *Haliotis midae L. Aquaculture* 203, 69-83.

Shipton, T., Britz, P.J., 2007. A Study on the Status of Aquaculture Production and Trade in South Africa. Volume 1: Industry Status and Diagnostic Report. A report for the Department of Trade and Industry produced by Enviro-Fish Africa, Grahamstown.

Short, F., Gorton, P., Wiseman, J., Boorman, K., 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. *Animal Feed Science and Technology* 59, 215-221.

Silberschneider, V., Gray, C., 2008. Synopsis of biological, fisheries and aquaculture-related information on mulloway *Argyrosomus japonicus* (*Pisces: Sciaenidae*), with particular reference to Australia. *Journal of Applied Ichthyology* 24, 7-17.

Small, B.C., Soares, J.H., 2000. Quantitative dietary lysine requirement of juvenile striped bass *Morone saxatilis*. *Aquaculture Nutrition* 6, 207-212.

Smith, B.W., Lovell, R.T., 1971. Digestibility of nutrients in semi-purified rations by channel catfish in stainless steel troughs. In: Proceedings Annual Conference Southeast *Association Game Fish Community* 25, 425-459.

Smith, R.R., Peterson, M.C., Allred, A.C., 1980. Effect of leaching on apparent digestion coefficients of feedstuffs for salmonids. *The Progressive Fish Culturist* 42, 195–199.

Smith, R.R., Winfree, R.A., Rumsey, G.W., Allred, A., Peterson, M., 1995. Apparent digestion coefficients and metabolizable energy of feed ingredients for rainbow trout *Oncorhynchus mykiss*. *Journal of the World Aquaculture Society* 26, 432-437.

Song, L., Mao, S., Wang, B., Xu, P., Wang, C., 2014. Effect of Dietary Lipids on Growth, Feed Utilization, and Protein Sparing in Black Rockfish, *Sebastes schlegeli*. *The Israeli Journal of Aquaculture-Bamidgeh* 66, http://hdl.handle.net/10524/49107.

Souffrant, W.B., 1991. Endogenous nitrogen losses during digestion in pigs. In: Verstegen, M., Huisman, J., den Hartog, L.A. (Eds.), Proceedings of the 5th International Symposium on Digestive Physiology in Pigs. Wageningen, 147–166.

Spyridakis, P., Metailler, R., Gabaudan, J., 1989. Studies on nutrient digestibility in European sea bass (*Dicentrarchus labrax*): Effect of sodium alginate on protein and lipid digestibility. *Aquaculture* 77, 71-73.

Suenaga, R., Tomonaga, S., Yamane, H., Kurauchi, I., Tsuneyoshi, Y., Sato, H., Denbow, D., Furuse, M., 2008. Intracerebroventricular injection of L-arginine induces sedative and hypnotic effects under an acute stress in neonatal chicks. *Amino Acids* 35, 139-146.

Sugiura, S.H., Dong, F.M., Rathbone, C.K., Hardy, R.W., 1998. Apparent protein digestibility and mineral availabilities in various feed ingredients for salmonid feeds. *Aquaculture* 159, 177-202.

Sullivan, J.A., Reigh, R.C., 1995. Apparent digestibility of selected feedstuffs in diets for hybrid striped bass (Morone saxatilis♀ X Morone chrysops♂). *Aquaculture* 138, 313-322.

Tacon, A., Rodrigues, A., 1984. Comparison of chromic oxide, crude fibre, polyethylene and acid-insoluble ash as dietary markers for the estimation of apparent digestibility coefficients in rainbow trout. *Aquaculture* 43, 391-399.

Tacon, A.G., 1989. Nutrición y alimentación de peces y camarones cultivados. Manual de capacitación. Programa cooperativo Gubernamental. Documento de campo 4. FAO, Brasil: FAO-Italia, 572.

Tacon, A., Cowey, C., 1985. Protein and amino acid requirements. Tyler, P., Calow, P. (Eds.), In: Fisg Energetics: New Perspectives, 155-183.

Tacon, A.G., Metian, M., 2008. Global overview on the use of fishmeal and fish oil in industrially compounded aqua feeds: Trends and future prospects. *Aquaculture* 285, 146-158.

Tacon, A.G., Metian, M., 2009. Fishing for aquaculture: non-food use of small pelagic forage fish - a global perspective. *Reviews in Fisheries Science* 17, 305-317.

Tacon, A.G., Metian, M.R., Tacon, M.A.G., Hasan, M.R., Metian, M., 2011. Demand and Supply of Feed Ingredients for Farmed Fish and Crustaceans: Trends and Prospects. FAO Fisheries and Aquaculture Technical Paper, No. 54. FAO, Rome, 87.

Temminck, C.J., Schlegel. H., 1842-1850. Pisces. In: von Siebold, P.F. (Ed.), Fauna Japonica. Lugduni Batavorum, Batavia. 323.

Tibaldi, E., Tulli, F., Lanari, D., 1994. Arginine requirement and effect of different dietary arginine and lysine levels for fingerling sea bass (*dicentrarchus labrax*). *Aquaculture* 127, 207-218.

Tibaldi, E., Zittelli, G.C., Parisi, G., Bruno, M., Giorgi, G., Tulli, F., Venturini, S., Tredici, M., Poli, B., 2015. Growth performance and quality traits of European sea bass (*D. labrax*) fed diets including increasing levels of freeze-dried Isochrysis sp.(T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives. *Aquaculture* 440, 60-68.

Tibbetts, S.M., Milley, J.E., Lall, S.P., 2006. Apparent protein and energy digestibility of common and alternative feed ingredients by Atlantic cod, *Gadus morhua* (Linnaeus, 1758). *Aquaculture* 261 1314-1327.

Trosvik, K.A., Webster, C.D., Thompson, K.R., Metts, L.A., Gannam, A., Twibell, R., 2013. Effects on growth performance and body composition in Nile tilapia, *Oreochromis niloticus,* fry fed organic diets containing yeast extract and soybean meal as a total replacement of fish meal without amino acid supplementation. *Biological Agriculture and Horticulture* 29, 173-185.

Turchini, G., Hermon, K., Cleveland, B., Emery, J., Rankin, T., Francis, D., 2013. Seven fish oil substitutes over a rainbow trout grow-out cycle: I) Effects on performance and fatty acid metabolism. *Aquaculture Nutrition* 19, 82-94.

Van Keulen, J., Young, B., 1977. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *Journal of Animal Science* 44, 282-287.

Vandenberg, G., De la Noüe, J., 2001. Apparent digestibility comparison in rainbow trout (*Oncorhynchus mykiss*) assessed using three methods of faeces collection and three digestibility markers. *Aquaculture Nutrition* 7, 237-245.

Vázquez, G.R., Guerrero, G., 2007. Characterization of blood cells and haematological parameters in *Cichlasoma dimerus* (Teleostei, Perciformes). *Tissue and Cell* 39, 151-160.

Vidal, L.V.O., Xavier, T.O., Michelato, M., Martins, E.N., Pezzato, L.E., Furuya, W.M., 2015. Apparent Protein and Energy Digestibility and Amino Acid Availability of Corn and Co-products in Extruded Diets for Nile Tilapia, *Oreochromis niloticus.* *Journal of the World Aquaculture Society Society* 46, 183-190.

Vogtmann, H., Pfirter, H., Prabucki, A., 1975. A new method of determining metabolisability of energy and digestibility of fatty acids in broiler diets. *British Poultry Science* 16, 531-534.

Wang, S., Liu, Y.J., Tian, L.X., Xie, M.Q., Yang, H.J., Wang, Y., Liang, G.Y., 2005. Quantitative dietary lysine requirement of juvenile grass carp, *ctenopharyngodon idella*. *Aquaculture* 249, 419-429.

Wang, T., Fuller, M., 1989. The optimum dietary amino acid pattern for growing pigs. *British Journal of Nutrition* 62, 77-89.

Wang, Y., Kong, L., Li, C., Bureau, D., 2010. The potential of land animal protein ingredients to replace fishmeal in diets for cuneate drum, *Nibea miichthioides,* is affected by dietary protein level. *Aquaculture Nutrition* 16, 37-43.

Wang, Y., Wang, F., Ji, W., Han, H., Li, P., 2015. Optimizing dietary protein sources for Japanese sea bass (*Lateolabrax japonicus*) with an emphasis on using poultry by-product meal to substitute fishmeal. *Aquaculture Research* 46, 874-883.

Watanabe, T., Takeuchi, T., Satoh, S., Kiron, V., 1996. Digestible Crude Protein Contents in Various Feedstuffs Determined with Four Freshwater Fish Species. *Fisheries Science* 62, 278-282.

Weatherup, R., McCracken, K., 1998. Comparison of estimates of digestibility of two diets for rainbow trout, *Oncorhynchus mykiss* (Walbiaum) using two markers and two methods of faeces collection. *Aquaculture Research* 29, 527-533.

Webster, C., Rawles, S., Koch, J., Thompson, K., Kobayashi, Y., Gannam, A., Twibell, R., Hyde, N., 2015. Bio-Ag reutilization of distiller's dried grains with solubles (DDGS) as a substrate for black soldier fly larvae, *Hermetia illucens,* along with poultry by-product meal and soybean meal, as total replacement of fish meal in diets for Nile tilapia, Oreochromis niloticus. *Aquaculture Nutrition* 22, 976-988.

Wei, Y., He, G., Mai, K., Xu, W., Zhou, H., Mei, L., 2015. Apparent Digestibility of Selected Feed Ingredients in Juvenile Turbot (*Scophthalmus maxima L.*). *The Israeli Journal of Aquaculture-Bamidgeh*. DOI: http://hdl.handle.net/10524/49191

Wetherbee, B.M., Gruber, S.H., 1993. Use of acid-insoluble ash as a marker in absorption efficiency studies with the lemon shark. *The Progressive Fish-Culturist* 55, 270-274.

Williams, K.C., Barlow, C.G., Rodgers, L., Hockings, I., Agcopra, C., Ruscoe, I., 2003. Asian seabass *Lates calcarifer* perform well when fed pelleted diets high in protein and lipid. *Aquaculture* 225, 191-206.

Wilson, R.P., 2003. *Amino acid requirements of finfish and crustaceans.* In: Amino Acids in Animal Nutrition (D'Mello, J.P.F.ed.), 427–447. CAB International, Wallingford, Oxon, UK.

Wilson, R.P., 2002. Amino acid and proteins. In: Halver, J.E., Hardy, R.W. (Eds), Fish Nutrition. 162-164. Academic Press, New York, USA.

Wilson, R.P., Halver, J.E., 1986. Protein and amino acid requirements of fishes. *Annual Review of Nutrition* 6, 225-244.

Wilson, R.P., Poe, W.E., 1985. Relationship of whole body and egg essential amino patterns to amino acid requirement patterns in channel catfish, *Ictalurus punctatus*. *Comparative Biochemistry Physiology* 80B, 385-388.

Wilson, R.P., Robinson, E.H., Poe, W.E., 1981. Apparent and true availability of amino acids from common feed ingredients for channel catfish. *Journal of Nutrition* 111, 923-929.

Windell, J.T., Foltz, J.W., Sarokon, J.A., 1978. Methods of fecal collection and nutrient leaching in digestibility studies. *The Progressive fish-culturist* 40, 51-55.

Woolley, L., Jones, C.L., Britz, P., 2010. Effect of dietary protein to energy ratio on growth and nitrogenous waste production of cultured dusky kob *Argyrosomus japonicus*. *African Journal of Marine Science* 32, 625-631.

Wright, P.A., Fyhn, H.J., 2001. In: [Anonymous] *Ontogeny of Nitrogen Metabolism and Excretion*. In: Fish Physiology, 20: Nitrogen Excretion. Wright, P.A., Anderson, P.M. (Eds.), 149-200. New York, London, Academic Press.

Wu, G., Bazer, F.W., Dai, Z., Li, D., Wang, J., Wu, Z., 2014. Amino acid nutrition in animals: protein synthesis and beyond. *Annual Review of Animal Biosciences* 2, 387-417.

Wu, G., Wu, Z., Dai, Z., Yang, Y., Wang, W., Liu, C., Wang, B., Wang, J., Yin, Y., 2013. Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. *Amino Acids* 44, 1107-1113.

Wu, X., Liu, Y., Tian, L., Mai, K., Yang, H., 2006. Apparent digestibility coefficients of selected feed ingredients for yellowfin seabream, *Sparus latus*. *Journal of the World Aquaculture Society* 37, 237-245.

Xia, S., Yang, H., Li, Y., Liu, S., Xu, Q., Rajkumar, M., 2013. Effects of food processing method on digestibility and energy budget of *Apostichopus japonicus*. *Aquaculture* 384, 128-133.

Xie, F., Zeng, W., Zhou, Q., Wang, H., Wang, T., Zheng, C., Wang, Y., 2012. Dieatry lysine requirement of juvenile pacific white shrimp (*litopenaeus vannamei*). *Aquaculture* 358, 116-121.

Yamada, S., Simpson, K.L., Tanaka, Y., Katayama, T., 1981. Plasma amino acid changes in rainbow trout (*salmo gairdneri*) force fed casein and a corresponding amino acid mixture. Bulletin of the *Japanese Society of Scientific Fisheries* 47, 1035-1040.

Yang, S., Liu, F., Liou, C., 2011. Assessment of dietary lysine requirement for silver perch (*bidyanus bidyanus*) juveniles. *Aquaculture* 312, 102-108.

Yigit, M., Erdem, M., Koshio, S., Ergün, S., Türker, A., Karaali, B., 2006. Substituting fishmeal with poultry by-product meal in diets for black Sea turbot *Psetta maeotica*. *Aquaculture Nutrition* 12, 340-347.

Yoo, J.H., Takeuchi, T., Tagawa, M., Seikai, T., 2000. Effect of thyroid hormones on the stage-specific pigmentation of the Japanese flounder *Paralichthys olivaceus*. *Zoological Science* 17, 1101-1106.

Yun, H., Park, G., Ok, I., Katya, K., Hung, S., Bai, S.C., 2016. Determination of the dietary lysine requirement by measuring plasma free lysine concentrations in rainbow trout *Oncorhynchus mykiss* after dorsal aorta cannulation. *Fisheries and Aquatic Sciences* 19, 1-7.

Yuan, Y., Gong, S., Yang, H., Lin, Y., Yu, D., Luo, Z., 2010. Apparent digestibility of selected feed ingredients for Chinese sucker, *Myxocyprinus asiaticus*. *Aquaculture* 306, 238-243.

Zhou, F., Shao, J., Xu, R., Ma, J., Xu, Z., 2010. Quantitative l-lysine requirement of juvenile black sea bream (*sparus macrocephalus*). *Aquaculture Nutrition* 16, 194-204.

Zhou, Q.C., Wu, Z.H., Chi, S.Y., Yang, Q.H., 2007. Dietary lysine requirement of juvenile cobia, *rachycentron canadum*. *Aquaculture* 273, 634-640.

APPENDICES

Appendix 1

Sources and compositions of all the feed ingredients used in the formulation of experimental diets in this thesis.

	Feed ingredient composition		
Feed ingredient	Protein (%)	Lipid (%)	Gross energy (MJ/kg)
FMprime[1]	64.78	7.65	18.64
FMstandard[1]	57.19	7.51	17.13
PLM[2]	65.61	0.58	20.10
PKM[2]	49.16	1.46	16.76
CNM[1]	33.42	3.5	19.4
CGM[1]	47.74	8.49	21.03
SBM[1]	45.60	1.21	17.20
SFM[1]	34.90	11.22	17.51
BLM, spray dried[2]	89.5	0.17	22.78

FM, fishmeal, PLM, poultry meal, PKM, pork meal, CNM, canola meal; CGM, corn gluten meal, SBM, soybean meal; SFM, sunflower meal and BLM, blood meal, spray dried.
[1] Marifeed Pty Ltd, Hermanus, South Africa.
[2] Ingredients sourced and provided by Montego Pty Ltd, Eastern Cape, South Africa.

Appendix 2

Amino acid digestibility (%) in the reference diet (mean ± standard deviation, n=3).

Reference diet	Amino acid digestibility
EAA	
Arginine	83.80±5.7
Histidine	62.67±25.2
Isoleucine	78.57±8.7
Leucine	81.67±7.6
Lysine	85.00±7.3
Methionine	80.20±10.7
Phenylalanine	76.96±0.0
Threonine	78.32±9.0
Valine	78.15±8.9
NEAA	
Aspartic acid	48.38±20.2
Glutamic acid	71.83±11.6
Alanine	75.61±10.8
Glycine	64.72±16.9
Serine	77.08±9.34
Proline	70.67±14.3
Tyrosine	67.03±14.6

Appendix 3

Acid insoluble-ash content of dusky kob feed and faeces (mean value of three sample replicate expressed in % dry matter, ±standard deviation).

	Feed	Faeces
Single protein source		
Fishmeal prime	0.55	0.66±0.1
Fishmeal standard	0.52	0.87±0.2
Pork meal	0.80	0.85±0.2
Poultry meal	0.88	0.99±0.0
Substitution method		
Pork meal	1.03	1.23±0.3
Poultry meal	0.97	1.05±0.2
Canola meal	0.50	1.35±0.1
Corn gluten meal	0.40	1.19±0.4
Sunflower meal	0.63	1.19±0.2
Soybean meal	0.51	1.71±0.3
Blood meal	0.62	1.44±0.1
Reference diet	1.49	1.31±0.3