INVESTIGATION OF ALTERNATIVE INGREDIENTS FOR THE REPLACEMENT OF FISH MEAL IN FORMULATION OF FEED FOR MALAYSIAN MAHSEER FINGERLINGS, *TOR TAMBROIDES*

By

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A dissertation submitted to the Department of Mechatronics and BioMedical Engineering,
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DEDICATION

I would like to dedicate this thesis to my family, who has always been supportive without judgment of what I am doing or have done, and to both my supervisors who have steered me on the right path throughout these years. I would have been nothing without these people in my life.

ABSTRACT

INVESTIGATION OF ALTERNATIVE INGREDIENTS FOR THE REPLACEMENT OF FISH MEAL IN FORMULATION OF FEED FOR MALAYSIAN MAHSEER FINGERLINGS, *TOR TAMBROIDES*

Wan Mei Sze

Demand for fish meal (FM) has resulted in dwindling trash fish stocks, which caused the rising cost of feed. As awareness for the environment increases, much research has been performed on utilization of alternative protein sources. This research aims to substitute FM with agriculture by-products in an attempt to produce a sustainable diet for the growth and nutrition of *Tor tambroides*. The alternative protein sources used for diet formulation were krill, anchovy head and chicken offal (CO), from animal protein origins; soy waste and palm kernel cake (PKC), from plant protein origins. Nutrient analysis was conducted on all ingredients and diets; digestibility trials and a preference test were used to screen for the most preferred alternative protein sources, which were CO and PKC. Substitution level with CO was set at 33%, 66% and 100%, whereas PKC was at 16.5%, 33% and 50% of the total FM content in the experimental diets. The amino acid and fatty acid contents of the muscle and liver samples were determined at the end of the growth studies. A FM diet was used as the control to determine the performance and efficiency of the experimental diets in a 6-month growth study using *T. tambroides* fingerlings. Specific growth rate (SGR) did not differ between diets although

there were significant differences between the amino acids and fatty acids content in the muscle samples of fingerlings from the control diet and experimental diets. The best substitution levels of FM in the diets were found to be at 100% CO and 33% PKC. All fingerlings had a good relative condition factor after consuming the experimental diets, proving that fish meal could be substituted successfully in diets for *T. tambroides* fingerlings.

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A special shout out goes to my arms, for always being by my side, my legs for supporting me, my fingers and toes because I can always count on them.

APPROVAL SHEET

This dissertation entitled "**INVESTIGATION OF ALTERNATIVE INGREDIENTS FOR THE REPLACEMENT OF FISH MEAL IN FORMULATION OF FEED FOR MALAYSIAN MAHSEER FINGERLINGS, *TOR TAMBROIDES*"** was prepared by WAN MEI SZE and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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SUBMISSION OF THESIS

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I, WAN MEI SZE, hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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Date 19th January 2015

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LIST OF ABBREVATIONS

AA	Arachidonic acid
ADC	Apparent digestibility coefficient for protein
ADM	Apparent digestibility coefficient for dry matter
ALA	α-linolenic acid
ANOVA	Analysis of variance
ASEAN/US CRMP	Association of Southeast Asian Nations/United States Coastal Resources Management Project
DHA	Docosahexanoic acid
EPA	Eicosapentanoic acid
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed conversion ratio
FMOC-Cl	Fluorenylmethyloxycarbonyl chloride
GC-FID	Gas chromatograph with a flame ionized detector
GLA	γ-linolenic acid
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HMSO	Her Majesty's Stationery Office
HNO_3	Nitric acid
HPLC	High performance liquid chromatography

IENICA	Interactive European Network for Industrial Crops and their Applications

LIST OF ABBREVIATIONS (CONT.)

LA	Linoleic acid
MUFA	Monounsaturated fatty acid
n-3 fatty acid	Omega-3 fatty acid
n-6 fatty acid	Omega-6 fatty acid
NaH_2PO_4	Sodium phosphate
NaOH	Sodium hydroxide
OECD	Organisation for Economic Co-operation and Development
OPA	o-phthaldialdehyde
PER	Protein efficiency ratio
PUFA	Polyunsaturated fatty acid
SEM	Standard error of mean
SFA	Saturated fatty acid
SGR	Specific growth rate
UNEP	United Nations Environment Programme
UPM	Universiti Putra Malaysia

CHAPTER 1

INTRODUCTION

Human and marine life stock both depend on wild stock fish as food. Lately, investment of modern fishing fleets and processing factories are used to take advantage of the force behind this division of the food industry, in a natural reaction to cater for the increasing global requirements for fish and fishery products. The doubling in increased fish consumption in the developing countries since the 1970s (Delgado *et al*., 2003) is contributing to the collapse of natural fish stocks where it is caused by factors such as illegal fishing, unfair fishing and over-fishing. Thus, to combat these problems faced by the fishing industry, many are turning to aquaculture for a more reliable and steady supply of fish as food.

Aquaculture comprises of a very wide range of farming practices, many which involve seaweeds, mollusks, crustaceans and fish, where different environments, systems and resource patterns are being involved and utilized, thus which offer many farmers an option to be able to diversify and to enhance food production and generate incomes from different revenues (Hewitt, Campbell and Gollasch, 2006). One of the fastest growing food production systems globally is aquaculture, at an average increase of 8.8% per year since 1950 (FAO, 2006a), where world fish food supply steadily increased 3.2% per year (FAO, 2012) with China being the major contributor at 12.4% (Merino *et al.*, 2012).

FAO (1997) hoped that aquaculture will continue to increase the contributions to food security and alleviate poverty in many third world and developing countries. Yields from many capture fisheries have been stagnant at a total of 85 million tons, yet demand for fish and fishery products still increases, thus there is much eagerness for aquaculture to increase its involvement to the global production of aquatic foods (Finegold, 2009). Capture fisheries and aquaculture, combined, supplied the world with about 154 million tons of food fish in 2011, and out this of total, aquaculture accounted for 41%. In this 41%, 56.4% of cultured fish consists of freshwater fish (FAO Fisheries and Aquaculture Department, 2012).

Aquaculture in Malaysia began in the 1980s, starting at a production of 14, 863 tons (Department of Fisheries, 2009). Current schemes in the country like the Zone Industry Aquaculture High Impact Project as well as the e-Kasih Program have been launched to increase commercial aquaculture production by the public. Loans have been offered by established financial institutions with low interest rates to attract those looking for a boost in the industry (Idris *et al.*, 2013). Figure 1.1 shows the statistics for aquaculture production in Malaysia since 1950 (FAO Fishery Statistic, 2008).

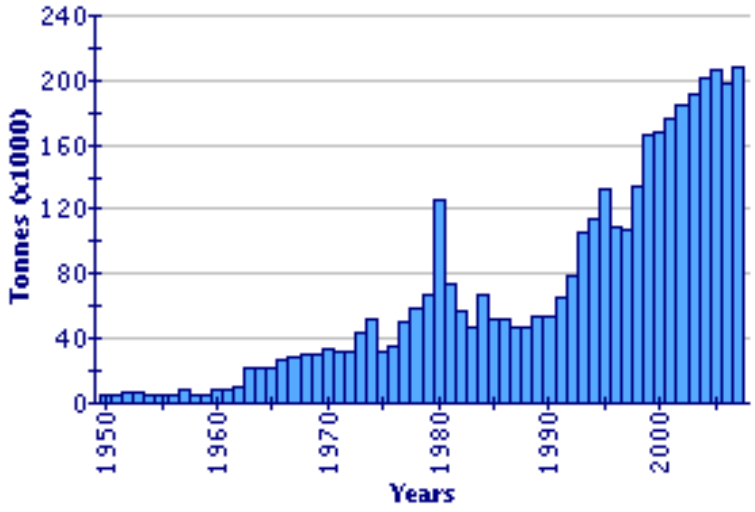

Figure 1.1: The reported aquaculture production in Malaysia from 1950

(FAO Fishery Statistic, 2008)

Compared to traditional aquaculture systems, intensive and semi-intensive aquaculture systems use up to five times more wild fish products which are fish meal and fish oil based to supply the aquaculture industry, than is produced in the form of farmed fish (Naylor *et al.*, 2001). Competition is also raging between aquaculture and the livestock sector for fish meal for feeds (FAO, 2006a). All these actually add to the burden on the wild stocks, and even without any new types of feed ingredients, aquaculturists' demand for wild fish will still grow as aquaculture continues to boom (Goldburg and Naylor, 2005). In order to sustain aquaculture, wild trash fish are farmed for fish meal. It is a cycle where aquaculture is used to reduce stress on wild fish, yet wild fish are used to feed the growing aquaculture market, where it is estimated three kilograms of wild caught fish are needed in order to produce one kilogram of farmed fish for consumption (Food & Water Watch, 2011).

Global fish meal usage reached a high of 4.2 million tons in 2005, but dropped to 3.8 million tons in 2007, and was stable at 3.6 million tons in 2011, and it is predicted that the usage will further reduce in 2015 due to stricter regulations and increased prices (Tacon, Hasan and Metian, 2011). Malaysia produces her own fish meal, estimated at 48, 200 tons in 2007, but only 40% of this fish meal is generated from fish byproducts, meaning a whopping 60% are still made up from whole fish (Jackson, 2009). In spite of the reducing global fish meal demand, the price of fish meal in 2012 hit USD 1, 500/MT (RM 4, 900), and is expected to remain so until at least 2022 (OECD/FAO, 2013).

The cost of feeds and feeding in aquaculture usually accounts from between 30% to 60% of the total production cost (Chong, 1992), of which 45% of the total cost is allocated to protein sources, namely fish meal (Asgard *et al.*, 2007). In 1995, global fish feed demand was only a total of 7.6 million tons. However, in 2010, the demand for fish feed hit 35.3 million tons, where a five-fold increase had taken place, and 30% of the feed was used mainly in carp culturing. This demand is estimated to double in 2020 to an astounding demand of 70 million tons (Tacon, Hasan and Metian, 2011). The fish feed production and demand in Malaysia in 2009 was calculated to be at 226, 000 tons, and is expected to rise further in the next decade (Aquaculture Asia Pacific, 2010).

Fish meal is usually incorporated in fish feed at different levels for carnivorous fishes. It currently the most essential dietary protein source in

most of the industrially produced aquafeeds (Anwahi, Cherian and Al-Janahi, 2008). Fish meal of high quality could contain up to 72% crude protein by weight. From a nutritional viewpoint, it is the most preferred animal protein supplement in the diets of farmed animals, and is often the key source of protein in feeds for fish and shrimp. Typical diets for fish typically contain from about 32% to 45% total protein by weight (Miles and Chapman, 2006).

Tor tambroides, locally known as the *kelah*, *empurau* or Malaysian mahseer, is economically valuable as game (Lim, 2006), ornamental (Agro-Biotechnology Institute Malaysia, 2013), and as food. The kelah is also one of the relatively expensive freshwater river fish in Malaysia, costing up to USD 100 (RM 330) per kilogram as food fish, both locally and internationally (Devindran, 2011). The fish is highly prized by anglers, commercial fishermen and poachers, each whom through relentless hunting is causing the reduction in *T. tambroides* population in the wild.

Reduction of natural habitats through soil erosion and dry weather, coupled with increased fishing pressure is causing the declination of *T. tambroides* (Baird & Mean, 2005; Government of Sarawak, 2007). Deforestation, in the name of development, and consisting of land clearing as well as timber harvesting activities upriver, has endangered the habitat of these valuable species (Seet, 2010). Eutrophication from sewage, and siltation caused by deforestation, has also resulted in the decrease of production and biodiversity of *T. tambroides* (Yusoff, 2007). To counter the declining numbers, *T. tambroides* is fast gaining popularity as a cultured fish for

conservation and food, which leads to the desperate need of a local feed that is suitable for the fish.

Kaushik *et al.* (2004), Wang *et al.* (2006) and Ahmed *et al*. (2008), have conducted studies on fish feed for the Nile tilapia, cuneate drum and European sea bass respectively, to produce a feed from local foodstuff or waste products that have economical values. Such feed for the Malaysian mahseer has not yet been extensively studied in the previous years and as a slow growing fish, there is an urgency to search for the optimal feed to accelerate growth of the Malaysian mahseer, especially in the commercial sector, although it has been determined that the mahseer needs a high protein and carbohydrate diet (UPM, 2013). Malaysia, as the fourth biggest exported of palm oil, produces ample of waste or byproducts, such as palm kernel cake that could be utilized as ingredients for the aquaculture feed industry. Byproducts from the poultry processing plants namely chicken offal is also viable and available as alternative ingredient for feed in the form of chicken offal powder.

As awareness for sustainability increases, more demand is being placed on using natural and locally produced feed consisting of vegetable proteins, waste products or foods that are available in abundance; in place of the conventional animal proteins (fish meal) which are dwindling. Alternative proteins have long been used to supplement aquaculture feed, and much research is done to determine the best possible fish meal substitutions in an effort to further reduce the usage of fish meal. Regardless, research on

alternative proteins for *T. tambroides* is relatively scarce; therefore this project was carried out with several objectives in mind:

- To investigate the potential of different alternative protein from plant and animal sources, to replace conventional fish meal for the growth and nutrition of *T. tambroides.*
- To formulate a sustainable diet using the selected alternative protein sources as fish meal replacement for the growth and nutrition of *T. tambroides*
- To determine the quality and performance of the diets formulated from alternative protein sources with that from fish meal.
- To identify the optimum inclusion levels for each type of alternative proteins and comment on the possibility of usage of an alternative protein source in diets for *T. tambroides* in aquaculture.

CHAPTER 2

LITERATURE REVIEW

2.1 Aquaculture in Malaysia

Aquaculture in Malaysia is considered to be one of the more significant sectors in agriculture, although not yet being as established as our neighboring countries, such as Thailand, Philippines and China. It started off with the freshwater culturing of the carp species, and progressed with marine shrimp and blood cockles (*Anadara granosa*) culture in the peninsula (FAO Fishery Statistics, 2008). East Malaysia's aquaculture sector started later, in the 1990s (Hamdan *et al*., 2012). The three aquaculture types in Malaysia currently are the freshwater, brackish water and marine aquaculture, with the brackish water and marine aquaculture comprising of 74% of the total aquaculture sector, and freshwater aquaculture at 26% in 2003 (WorldFish Center, 2006). However, the distribution has changed drastically as in 2012, where freshwater aquaculture took up the most of the sector at 77%, while the brackish and marine aquaculture has reduced to 23% (Department of Fisheries Malaysia, 2012).

Marine aquaculture makes up 23% of seafood production in Malaysia (Chowdhury and Yahya, 2012), the most popularly cultured species including the grouper family and Asian sea bass, where the grouper is regularly exported to countries such as Hong Kong and China (Ransangan *et al*., 2013). Brackish water ponds are usually used for culturing of species such as the sea bass,

grouper, snapper, sea perch, tiger shrimp and white shrimp through cage farming, generating up to USD 1, 000, 000 revenue for farmers (ASEAN/US CRMP, 1991; Idris *et al.*, 2013; Ramzani, Ismail and Abdurofi, 2013). Freshwater aquaculture is normally conducted in mining pools, earthen ponds and reservoirs, where the most commonly cultured species include the tilapia, the carp family, various species of catfish, sultan fish, gourami and the large freshwater prawn (ASEAN/US CRMP, 1991; Kechik, 1995; FAO, 2007, Department of Fisheries Malaysia, 2012).

Malaysia has a relatively small aquaculture sector, compared to the other Southeast Asia countries, but consumption of fish in Malaysia per capita is estimated to be three times higher than the global average, at 55 kg/year (FAO, 2006b; FAO, 2008). Feed is widely available for aquaculture; however, the cost is the restraining factor. With feed costing up to 80% of total costs for tilapia, or 50% for sea bass and shrimp culture, aquaculture is an expensive sector to sustain the food demand, yet Malaysians rely heavily on aquaculture for animal protein (Hishamunda *et al.*, 2009). Efforts by the government to look for alternative ingredients started more than 10 years ago, to obtain local ingredients essential in producing quality feed products (Subasinghe *et al.*, 2002). These efforts have been embarked in The Third National Agricultural Policy that emphasized development of aquaculture through sustainable utilization of natural resources (Ng *et al.*, 2013).

Malaysia's Third National Agricultural Policy identified the tilapia (*Oreochromis niloticus*) as the major species for freshwater aquaculture

(WorldFish Center, 2006), gaining popularity besides others such as the different carp species (*Cyprinus carpio*, *Ctenopharyngodon idella*, *Aristichthys nobilis* and *Hypophtalmichthys molitrix*) and the barb (*Puntios gonionotus*) (Pathansali, 1976). The most popular species for marine aquaculture is the sea bass (*Lates calcarifer*) (Kechik, 1995), nevertheless, the grouper family (*Epinephelus fuscoguttatus, Cromileptes altivelis*, *Epinephelus malabaricus*, *Epinephelus coioides* and *Epinephelus sexfasciatus*) is gaining recognition as the demand from both local and international markets grow (Othman, 2008). Besides fish, marine shrimp such as the black tiger shrimp (*Penaeus monodon*) along with the white shrimp (*Litopenaeus vannamei*) are both commonly cultured due to their fast growth, despite *L. vannamei* being banned until up to 2005 (Othman, 2008).

2.2 *Tor tambroides*

T. tambroides is a well known sport fish in Southeast Asia that belongs to the family Cyprinidae, and this family dominates the rivers found in Southeast Asia and parts of Asia, including Thailand, Indonesia, India, Vietnam, Cambodia and *et cetera*. They prefer the rocky bottom streams that are clear and swift flowing during the dry season, while seeking shelter in slack water during the rainy season (Lee, Ling and Adha, 1999). Nevertheless, the mature mahseers prefer a deeper, slightly slower running and more turbid water than the juveniles. The distribution of the mahseer has been suggested to also be dependent on the temperature of the water (Desai, 2003). Spawning happens uphill of streams, and is generally around July and August.

Nonetheless, the females can be induced to spawn, given the appropriate environmental stimuli such as heavy rains (Kunlapapuk and Kulabtong, 2011).

As poaching is a constant threat to the survival of the mahseer, the Malaysian government has implemented bans in several states, preventing the removal of the fish, and harsher still, inhibiting fishing in order to preserve and conserve the fish. The earliest state to be involved in the fishing ban was Perak in 1994, followed by Johor, Pahang, Kelantan, Terengganu and Kedah. The government decided to lift the ban in Terengganu in 2009, although anglers are still prohibited from keeping the fish, and a catch-and-release system was enforced by the locals (Leong, 2003; The Star, 2006; Bernama Media, 2012). In 1992, the Malaysian government realized the appreciation of the mahseer by the locals in terms of food fish, and has consideration its potential in aquaculture (Ismail, 1992).

In the wild, *T. tambroides* is slow-growing and can take up to a year just to reach 500 to 600 g (Ng, C. K., 2004) and pilot trials in ponds using captive-bred fish showed that an average weight of 790 g (range 390 g to 1200 g) could be reached by three years of age (Ingram *et al.*, 2007), although some reported a growth of only 200 g in the first year. Since fish of one to two kg are usually the ideal serving sizes in restaurants, the *T. tambroides* may require a prolonged grow-out period. Reproduction maturity is based on the size and weight of the mahseer, with males and females weighing 2.5 kg and 3.9 kg, and measuring 60 cm and 70 cm respectively (Kunlapapuk and Kulabtong,

2011). Currently hormonal procedures are relied on to induce spawning, where the males and females are housed in tanks at a 1:1 ratio (Ingram *et al*., 2005).

The Malaysian mahseer is an omnivorous creature, feeding on natural jungle fruits and nuts, insects and their larvae, small fishes and crustaceans, tadpoles and sometimes even small frogs (Chan, 2009). Their favorite foods include the local forest fig (*ara* or *Ficus auriculata*), and *engkabang* (*Shorea macrophylla*) (Chan, 2009; UPM, 2013). Popular foods used in the fishing of *T. tambroides* are usually ripe palm kernels, worms, small prawns and sometimes slightly rotten and fermented tapioca roots. In captivity, adult mahseers are usually fed commercial feeds containing high levels of animal protein, while being supplemented with the local fig and *engkabang* (Bista *et al.*, 2002; The Borneo Post, 2011).

2.3 Conventional Protein Source for Fish Feed

The conventional protein source for fish feed is fish meal. Fish meal is obtained after cooking, pressing, drying and milling fresh raw or trash fish and fish trimmings. It can be made from practically any type of seafood, but is most commonly manufactured from wild-caught, but small, marine fishes which are usually considered not suitable for direct human consumption (Schipp, 2008). Sugiyama, Staples and Funge-Smith (2004) define trash fish as the portion of catch that has almost no value based on their small size or lack of consumer partiality. Juveniles of fish species of commercial interest, juveniles and adults of lesser known food fish species, species of minor

importance and spoilt fishes that are of high value species make up what is known as trash fish (Edwards, Le and Allan, 2004; Malvas, 2005).

In Malaysia, there is currently no particular sector in the fishing industry specific for catching trash fish. Currently the west coast of peninsular Malaysia is the dominant provider of trash fish, as compared to the east coast, and East Malaysia. Although the contribution of trash fish from the west coast declined from 85% in 1987 to 60% in 2003, the other coasts of Malaysia have contributed to increasing amounts of trash fish, the east coast producing an estimate of 30% from a previous of 5%, and East Malaysia with a rising trend of 15% from 5% (Che Musa and Nuruddin, 2005). Trash fish capture from trawlers in the west coast of Peninsular Malaysia consists of up to 65% of the total catch (Chowdhury and Yahya, 2012).

Fish meal in Malaysia is made up from several different kinds of local marine fish, such as the scad, mackerel, sardine and tuna, to name a few (Leadbitter, 2011). In 2012, there was a call to ban the usage of fish meal in aquaculture feeds, due to the pressure exerted on the marine ecosystem where trash fish was heavily harvested to feed the fish meal market (Small Scale Fisheries, 2012), where was calculated more than five kg of wild caught fish was be needed just to produce one kg of farmed fish (Schipp, 2008). However, the juvenile species such as the sea bass still require fish meal as part of their feed (Merican, 2012); therefore the fish meal ban might not be viable, until more research is conducted.

2.4 Alternative Protein Sources

About two decades ago, aquaculture was considered an unimportant user with an estimate annual 10% usage of fish meal. And as the industry is growing at an increase of three million tons per year (FAO Fisheries and Aquaculture Department, 2010), stress made on fish meal as the main ingredient in fish feed is also increasing. To continue to provide for this rate in the industry, alternative ingredients are needed to substitute and reduce pressure on fish meal, while still supplying the nutrients needed for fish development. Animal waste products, seafood processing waste, and plant waste products are viewed as ingredients with the most potential as alternative ingredients (Hardy, 2000).

Non-conventional feed resources, or alternative protein sources, are recognized for a few factors. Firstly they are non-competitive, whether as a resource for humans or animals, and since the demand is in a completely different sector, the cost is reduced, thus contributing to a final lower-costing feed. Secondly, because they are the recycled material in the form of byproducts or waste from different agriculture sectors, farms or processing plants, use of these alternative protein sources serve as a waste management. These sources are divided into three types, the animal and plant proteins, and even other waste such as dung. Any alternative protein source could be salvaged, and reprocessed, into viable feeds, as long as the process required is practical and economical (Sogbesan and Ugwumba, 2008).

There are a few factors that need to be taken into account when using alternative ingredients. First and foremost, the energy and nutrient content has to be reasonable to sustain the energy usage. The more fiber in an ingredient, the more the digestibility of energy and nutrients are lowered. Secondly, there are anti-nutritional factors that interfere with the digestibility and absorption of the nutrients. Anti-nutritional factors include trypsin inhibitors, tannins and lectins. The ingredients have to be processed suitably before usage to prevent loss of performance. Thirdly, the palability of the ingredients will control the feed intake. The fish are able to taste and will decide on which type of ingredients they prefer. All these factors influence the inclusion rate of the alternative ingredients in the feed. As long as all are in balance with each other, the alternative ingredient can be used successfully without compromising the quality of feed (National Pork Board, 2008).

2.4.1 Plant Protein Sources

2.4.1.1 Corn Gluten Meal

Corn gluten meal is a byproduct left over from corn processing, where it has been often used in animal husbandry, especially in feed for ruminants. It contains a high percentage of crude protein, a minimum of 60%, as well as a good digestibility coefficient, making it extremely nutritious in terms of protein content. The meal contains feasible amino acid content, although there is a lack of lysine and arginine. Where other plant protein sources usually contain anti-nutritional factors, corn gluten meal hardly contains any, and with its low fiber content, causing it to be an ideal alternative for fish meal replacement (Pereira and Oliva-Teles, 2003).

Kaushik *et al.* (2004) used corn gluten meal to replace up to 50% of fish meal in European sea bass diets. However, in studies by Dias (1999) and Gomes, Rema and Kaushik (1995), the results obtained were different from Kaushik *et al.* (2004) where there was no change in voluntary feed intake due to high levels of a single protein source. Sea bream juveniles could only accept up to 10% substitution of corn gluten meal (Yigit *et al*., 2012), where any additional substitution would not have a growth rate comparable to fish meal, as is the case with the striped catfish (Bicudo *et al.*, 2012).

In contrast, as corn gluten meal contains high levels of carotenoids, which in turn affects the color of the fish muscles, the usage of corn gluten meal might not be practical, as any pigmentation of the flesh in supposedly white fillets would reduce its value on the market (Lovell, 1984). On the other hand, fish fillets which are valued for its pinkish muscle tone, such as the salmonids, would benefit from this natural pigmentation, and consumers of such fish will not be exposed to any artificial coloring (Robaina *et al*., 1997). Therefore, to prevent coloration of the flesh, bleaching of the pigments are usually conducted at its source to reduce the amount of carotenoids in the meal (Saez, 2013).

2.4.1.2 Soybean Meal

Soybean meal appears to be an appropriate alternative protein source because of its abundant supply, price and balanced amino acid profile. The high nutrient content makes it very suitable as a feed ingredient in the agriculture and aquaculture sectors. The meal itself is naturally clean, free

from contaminants such as fungi and bacterium that are harmful to the animals (Swick *et al*., 1995). However, soybean meal contains several antinutritional factors such as trypsin inhibitors, lectins and oligosaccharides that may affect the digestion or absorption of nutrients (Dersjant-Li, 2002). These factors can be deactivated, as long as the soybean meal is properly processed prior to incorporation in the feeds (Deng et al., 2006).

Osborne and Mendel found out heat treatment on soybeans could improve the growth rate of the animals fed the soybean meal by destroying the anti-nutritional factors such as the protease inhibitors (Willis, 2003). The two most commonly used soybean meal available in the market are the normal soybean meal, and the dehulled soybean meal. Both contain fairly similar crude protein content; but dehulled soybean meal is lower in fiber, and has a higher energy content than the normal soybean meal (Cromwell, 1999).

Soybean meal has already been used as a substitute for fish meal in diets of several crustaceans, and Alvarez *et al.* (2007) has received good results when used with the white shrimp. Feedings of the African catfish (Fafioye *et al.*, 2005) and the rose snapper (Silva-Carrillo *et al.*, 2012) showed positive effects on growth when fish meal was partially replaced with soybean meal. Soy meal has also been used as an alternative ingredient in diets for humpback grouper (Laining *et al*., 2003), rainbow trout (Barrows, Stone and Hardy, 2007) and cobia (Zhou *et al*., 2004). Feeding trials for trout since the 1940s has shown no significance in growth rates when soy meal was used, while the cost of production was reduced significantly (Hardy, 2003). Locally

conducted research on feeding of the sutchi catfish and marble goby show up to 45% and 10% of dietary fish meal could be substituted with soybean meal (Phumee *et al*., 2011; Yong, Ooi and Shapawi, 2013). Except, in a country like Malaysia which does not produce soybeans, soy meal can be an expensive commodity (Ng, W. K., 2004).

2.4.1.3 Sunflower Meal

Sunflower meal is a high fiber feed ingredient, is widely used in feeds for ruminants. The presence of the hull plays a major part in the nutrient content, where non-dehulled and partially dehulled sunflower meal have a more inferior nutrient composition as compared to the dehulled sunflower meal (Harrington *et al.,* 2003). However sunflower seeds contain a large quantity of phenolic compounds which may complex with proteins, causing discoloration of the protein isolates and lowering the nutritional content due to their interaction with the amino acids (Gandhi, Jha and Gupta, 2008).

Despite the lower protein content in sunflower meal as compared to the other oil seed meals, sunflower meal substitution of the other oil seed meals have produced positive results. In ruminants such as heifers, steers, and cows, inclusion of sunflower meal yielded similar results as diets containing soybean meal, canola meal or other plant protein meals (Harrington *et al.,* 2003). In fact, broiler chicks and other poultry benefited from sunflower meal diets, reflected in their growth and flesh (Rad and Keshavarz, 1976; Slavica, Jovanka and Olivera, 2006). Diets for the Nile tilapia and rainbow trout could contain up to 67% sunflower meal, in replacement of fish and soybean meal,

although full substitution is not recommended due to the lack of methionine (Fagbenro and Davis, 2000; Hertrampf and Piedad-Pascual, 2000).

2.4.1.4 Palm Kernel Cake

A by-product from the palm oil industry, palm kernel cake contains 14.5% to 19.6% crude protein, 1.75% methionine and 2.68% lysine (Alimon, 2004). It is a high energy source and is a very cost effective ingredient to be incorporated into ration formulations for various livestock. The ample availability of palm kernel cake in Malaysia throughout the year is well suited for agriculture, and in a country which produces and exports up to 2.4 tons of palm kernel cake in 2010 (Choo, 2011), its high availability and relatively low price in Malaysia readily makes it one of the best plant protein sources in feeds.

Palm meal has yet to show potential in aquaculture, due to the limited research studies utilizing it as an alternative ingredient (Zahari and Alimon, 2004), due lack of essential amino acids and a relatively low protein content (Ng, 2003). Although palm kernel cake has been successfully used in animal feed formulations such as cattle (Alimon, 2004; Oluwafemi, 2009), swine (Adesehinwa, 2007; Boateng *et al.*, 2008) and poultry (Boateng, *et al.*, 2008; Perez, Gernat and Murillo, 2000), its usage within compound fish feeds is still uncommon, caused by the non-starch polysaccharides antinutritional factor, despite its availability of lower cost compared with fish meal.

Studies involving the usage of palm kernel cake as protein source include feeds for catfish (American Palm Oil, 2006) and tilapia (Ng and Chong, 2002, Iluyemi *et al.*, 2010) at low levels of inclusion. More often than not, the palm kernel cake is fermented either with bacterium or digested with feed enzymes before usage to increase the nutritional, especially crude protein content (Ng, 2003, Iluyemi *et al.*, 2010). Sulfur amino acids are commonly added to supplement the lack of amino acids in the feed (Ng, 2003). Bioconversion of palm kernel cake with maggots has also been studied to for extraction of protein and fat from the cake (Hem *et al.,* 2008).

2.4.1.5 Seaweed Meal

Malaysia is one of the world's renowned top ten producers of seaweed, where seaweed is actively cultured and farmed. Production of seaweed in Malaysia comes from Sabah, the only state involved in seaweed aquaculture, with *Kappaphycus alvarezii* and the sea birds' nest (*Eucheuma spinosum)* being the two main species culture, although both species are only part of 10% and 3% of the global production respectively. Three types of culturing methods were used, namely the conventional long line method, the improved long line method and the basket method, whereby the seaweed were suspended in the water using either lines or baskets (Kaur and Ang, 2009).

In previous years, seaweed usage for both human consumption and animal feeds were more supplementary, for the mineral contents, or the polysaccharide properties such as alginate as a binder. Moreover, more focus is now being placed in the protein content of different seaweeds, the common

ones being brown, green and red seaweeds, with the former containing the least amounts of crude protein (Arasaki and Arasaki, 1983).

The amino acid compositions of the seaweeds are comparable to several other different foods, such as eggs (ovalbumin) and legumes (soy) (Fleurence, 1999). Essential amino acids and fatty acids are present in most seaweeds, and high digestibilities of the proteins were recorded through enzymatic digestion studies (Fleurence, 1999; MacArtain *et al.*, 2007). Seaweeds of the green and red varieties have a higher protein content, and are currently of interest as potential alternative feeds with good nutritional content and its likeliness to be used in fish feed (Valente *et al.*, 2006). As most commercial foodstuff are pricey for small time fish farmers, seaweed meal would be a cost effective way of including the essential amino acids into the diets of the fish (Swain and Padhi, 2011).

A few types of seaweeds such as the brown seaweed *(Enteromorpha intestinalis, Sargassum muticum*), the green seaweed (*Ulva rigida*), along with the red seaweed (*Gracilaria bursa-pastoris*, *Gracilaria cornea*, *Gracilaria vermiculophylla*, *Gracilaria verrucosa*, *Grateloupia filicina*, *Polysiphonia sertularioides, Porphyra dioica*) are presently being utilized in feed for the European sea bass, rohu, mrigal, rainbow trout, Nile tilapia, and other finfish larvae (Valente *et al.*, 2006; Swain and Padhi, 2011; Azad and Teo, 2012; Pereira *et al.*, 2012). All the above studies have reported the digestibility of the seaweed in the diets was favorable, where the experimental diets had values similar to the reference diet made from fish meal.

2.4.1.6 Kenaf Meal

Kenaf (*Hibiscus cannabinus L*) is known as a warm season annual herbaceous plant and closely related to the cotton and okra plant. The fibrous stem and stalk are usually used to weave jute and produce sacks or cardboard products such as paper, biocomposites, fiber boards as well as bioplastics (IENICA, 2002; Chan *et al.*, 2013). Besides, humans have been consuming its leaves, which are low in calories but rich in protein, essential oils, calcium, and phosphorus and have considerable amounts of Vitamin C. On the contrary, the seeds are rich in essential fatty acids and calories (Adebayo, 2010).

The leaves of the plant have a crude protein content of 30% (Odetola and Eruvbetine, 2012), and are readily consumed by ruminants. After being grounded into kenaf meal, the meal exhibits a higher protein digestibility coefficient (Sethuraiman and Naidu, 2008), theoretically making it a satisfactory alternative protein source to substitute for fish meal. Yet, despite the good protein profile, kenaf meal might not be applicable in agriculture and aquaculture, due to the high crude fiber content.

Rabbits fed diets containing kenaf meal showed decreasing health conditions (Odetola, Ewuola and Adu, 2012), while broiler chicks could accept up to 10% supplementation of kenaf (Odetola, 2011). Substitution of soybean meal with kenaf meal in lactating cows would best be complemented with a supplement of amino acids (Chantiratikul, 2005). Currently there is no research on kenaf meal for fishes. Nonetheless, based on findings for the

terrestrial animals, it is possible minor supplementation with kenaf meal in aquaculture feed might be viable, especially with herbivorous fishes.

2.4.1.7 Guar Seed Meal

Guar is a leguminous plant grown in semiarid places in the world, such as Saudi Arabia and India. This protein-rich meal is an inexpensive byproduct of the guar gum manufacturing, where the protein content ranges from 30% to 45% and is comparable to the nutrient content of soybean meal (Salehpour and Qazvinian, 2011). However, guar gum is still detected in the meal, whereby it is an anti-nutritional factor (Nagpal, Agrawal and Bahtia, 1971), along with a trypsin inhibitor, although the trypsin inhibitors present were relatively lower than those in soybean meal (Conner, 2002). Both these factors have an adverse effect on the growth of broiler chicks, hence enzyme supplementation was used to combat the effect (Vohra and Kratzer, 1965).

Guar seed meal is often used as ruminant feed due to the high protein content, and is being suggested as an alternative feed for fishes, such as the Nile tilapia (Al-Hafedh and Siddiqui, 1998). The Nile tilapia, could accept feeds containing up to 50% of guar seed meal, without sacrificing the growth of the fish (Al-Hafedh and Siddiqui, 1998). Despite the positive properties of guar seed meal, only low levels of the meal could be incorporated into diets for broiler chicks due to the guar gum, without compromising the growth of the chicks (Gheisari *et al*., 2011). On the contrary, 50% incorporation of guar seed meal in feed for lactating cows improved the milk quality significantly,

indicating guar seed meal would be more suited for ruminants, rather than the other animals (Salehpour and Qazvinian, 2011).

2.4.2 Animal Protein Sources

2.4.2.1 Poultry Byproduct Meal

Poultry byproduct meal is a practical protein source for carnivorous fishes because of the high protein content and many essential amino acids. However, poultry byproducts vary between batches and producers, in terms of quality, and some are usually deficient in one or more essential amino acids. Marine fish oil is usually used to ensure sufficient amounts of essential amino acids are included in the diets, and vegetable oil such as canola used to increase the amounts of *n*-fatty acids (Subhadra *et al.*, 2006). Chicken offal is the waste product from slaughtered chickens, and it is made up of the kidneys, liver, intestines, esophagus and proventricus (Omole *et al.*, 2008).

Several studies have found chicken offal meal to be a viable replacement of fish meal in poultry (El Boushy *et al*., 1990; Ravindran, 2010), swine (Iheukwumere, Ndubisi and Etusiim, 2008; Tibbetts, Seerley and McCampbell, 1987), ruminants (Lallo and Garcia, 1994; Gonzalez *et al*, 2007) and the common carp (Machin, 1999; Zabihi *et al*., 2011), with different inclusion levels in their diets. While chicken offal has already been used widely in chicken feeds, incorporations in fish feeds are still relatively new. Care must be taken when using chicken offal, proper cleaning and processing procedures are essential, as bacteria such as the *Salmonella* sp from chicken

can be transmitted from different organisms up the food chain (Budiati *et al*., 2013).

2.4.2.2 Krill Meal

Wild fish, usually marine, often consume amphipods, krill and small crustaceans as part of their natural diet. The name krill is commonly used to describe crustaceans from the Euphausiacea order, with the Antarctic krill (*Euphausia superba*) being the most widely known species. Krill is a high nutrient low calorie food, rich in *n*-3 fatty acids and minerals (Tou, Jaczynski and Chen, 2007). The fishes seem to be able to tolerate the presence of high levels of fluorine in the exoskeleton, which is detrimental to terrestrial animals. Unwanted metals such as cadmium and copper could be eliminated by proper processing of the krill (Moren *et al.*, 2006).

Malaysia has access to the sea, but major krill fisheries are located in mostly in Japan and Antarctica. Once an unused resource from the ocean, it is now commercially harvested for bait, supplement oil or as aquaculture feed (Yoshitomi, Aoki and Oshima, 2007). The popularity of krill meal was based on the ease of capture and the abundance of each harvest. Overfishing of krill, for either human or animal usage, has caused the disruption of the ecosystem. The massive increase of krill usage in aquaculture feed and as bait has caused krill fishing to be banned all along Alaska and the West Coast of U. S. A. (Alexander, 2009).

Almost all the krill which has been harvested from the sea is used in the aquaculture sector. One third of the Japanese capture is frozen and used either directly as feed, or processed into fish meal for further usage, while krill from the Canadian catch is utilized as an ingredient in the aquaculture feeds. Krill as additives are used to enhance taste and nutrient content, where it acts as a stimulus to increase feeding in fish such as the rainbow trout and sea bream, thus contributing to the growth rate of the fish. Disease resistance was also found to be increased in salmon consuming krill as part of their diet (Nicol, Forster and Spence, 2000).

The effect of krill meal has been studied on several fish species like the rainbow trout, salmon (*Salmo salar*) and the yellowtail (*Seriola quinqueradiata*). Partial replacement of fish meal with krill in diets for the rainbow trout show promise at low levels of inclusion. However, once the inclusion level reached 30%, the rainbow trout showed a marked decrease in growth rate (Yoshitomi *et al*. 2006). Yoshitomi and Nagano (2011) tried full substitution of krill meal with the yellowtail with negative effects although the yellowtail fed 15% inclusion level of krill meal had no significant difference in growth compared to the reference diet. Hansen *et al*. (2010) found deshelled krill could influence the growth rate, and could successfully be incorporated as a single protein source in diets for the salmon.

2.4.2.3 Squid Meal

Squid meal is a high protein content feed ingredient, with crude protein levels comparable to those found in fish meal and other fish products, and is

commonly used in shrimp culturing due to its growth enhancing properties and as a feed attractant (Cruz-Suarez, Ricque and AQUACOP, 1992; Li *et al.*, 2009; Mohanta, Subramanian and Korikanthimath, 2013). Its ideal amino acid profile is similar to fish meal, with the exception of arginine, which is more frequently found in squid meal (Valverde *et al.*, 2013). Another common factor between squid meal and fish meal is the price. Squid meal is expensive, and prices often fluctuate between years (Conklin, 2009).

Crude protein digestibility of squid meal is as good as what has been obtained for fish meal. In fact, in some instances, squid meal has proven to have a better crude protein digestibility than fish meal in feedstuff for the mud crab and spiny lobster (Catacutan, Eusebeo and Teshima, 2003; Rathinam *et al.*, 2009). Growth of prawns consuming a diet supplemented with squid meal showed growth improvements of up to 35% of the reference diet (Cruz-Suarez, Ricque and AQUACOP, 1992). The Japanese flounder could accept up to 36% fish meal replacement with soybean-squid meal (Kader *et al.*, 2012) while the blue gourami had optimal growth at 100% substitution (Mohanta, Subramanian and Korikanthimath, 2013).

2.4.2.4 Blood Meal

Blood meal is a lysine-rich feed ingredient, containing up to 8% lysine (El-Haroun and Bureau, 2007). Different techniques are used to produce blood meal, which include disc-dried, spray-dried, rotoplate-dried, steam-tube-dried, ring-dried and flash-dried (Bureau, Harris and Cho, 1999), and each processing method results in significantly different apparent digestibility

coefficients for crude protein. The studies on the rainbow trout and rohu fingerlings recorded at least 80% and 70% digestibility for crude protein respectively (El-Haroun and Bureau, 2007; Hussain *et al*., 2011).

Despite the good nutritional content of blood meal, inclusions in fish feeds would best be limited to a supplementation. A maximum of 50% blood meal inclusion in the diet was recorded for fish such as the Chinook salmon, rainbow trout, Nile tilapia (Hertrampf and Piedad-Pascual, 2003), juvenile Palmetto bass (Gallagher and LaDouceur, 1995), mudfish fingerlings (Eyo and Olatunde, 1999), gilthead sea bream (Nogueira *et al.*, 2012) and largemouth bass (Tidwell *et al*., 2005), whereas, in feeds for medium-sized shrimps, the fish meal portion in the grow-out diets could be fully substituted with blood meal (Hertrampf and Piedad-Pascual, 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of Ingredients

Two different categories of alternative protein sources were used in this study, i.e. plant protein source, and animal protein source. Plant protein sources used were soy waste and palm kernel cake, whereas animal protein sources used were anchovy waste, krill and chicken offal.

Fish meal made from fresh local torpedo scad (*Megalaspis cordyla*) was used to formulate a diet as the positive control. Soy waste which was the soy residue after soy milk extraction was supplied by a local soy milk vendor, whereas palm kernel cake was provided by a palm oil processing plant. Anchovy waste was supplied by the local fishermen after the daily processing of each catch, while krill was purchased from a local market and chicken offal was obtained from a poultry product processing plant. All ingredients were sorted and dried before being ground or blended into powder form (FAO, 2004).

3.2 Nutrient Analysis

Nutrient analysis was carried out on all the potential protein sources, and the experimental diets formulated for the digestibility trial as well as the growth study.

3.2.1 Crude protein

Crude protein was determined using the Kjeldahl method according to Rhee, in the Handbook of Food Analytical Chemistry (2005). The digestion block (Kjeldahltherm, Gerhardt, Germany) was pre-heated to 375 °C. A dried sample weighing 250 mg was placed into a 250 mL Kjeldahl digestion tube. A pre-mixed Kjeldahl catalyst mixture, made up of copper, was added together with 1.0 g anti-bumping granules. Digestion of sample was conducted in 10 mL of 16 M H_2SO_4. The samples were left to digest for two hours, after which they were cooled slightly before distillation.

Care was taken not to allow the acid solution to bubble and overflow out from the tube when 100 mL of distilled water was added slowly into the boiling tube to dilute the acid. A 12 M NaOH solution was added slowly to the boiling tube to make the solution strongly alkaline. Once the sodium hydroxide was added completely, the tube was immediately inserted into the distillation unit. The distillate was collected in a conical flask filled with 25 mL of 0.1 M H_2SO_4 and marked with a methyl red indicator.

The collected distillate was slowly titrated with 0.5 M NaOH while the flask was swirled to ensure complete neutralization. Titration was stopped when the methyl red indicator turned to yellow. The total volume of NaOH used was noted. Sample blank was prepared in the exact method as described for the sample. The nitrogen content in each sample was calculated using the formula below:

$$\%N = \{[M_{acid}\ (mL_{acid}) - mL_{bk}\ (M_{NaOH)} - mL_{NaOH}\ (M_{NaOH})] * 1400\} / mg_{sample}$$

mL_{bk} = volume of 0.5 M NaOH needed to titrate 1 mL 0.1 M H_2SO_4 – volume of 0.5 M NaOH needed to titrate reagent blank

mL_{acid} = milliliters of H_2SO_4 used to collect distillate

mL_{NaOH} = milliliters of NaOH used to titrate sample

The nitrogen content from each sample was then multiplied with a conversion factor, where 6.25 was used for the animal protein source, while 5.71 was used for the plant protein sources to obtain the crude protein content (Chang, 2003; Rhee, 2005).

3.2.2 Crude Lipid

Crude lipid analysis was conducted according to the method by Bligh and Dyer (1959). A sample weighing 15 g was mixed with 35 mL of water and homogenized with 100 mL of methanol in a Waring blender. Chloroform (50 mL) was then added to make a 2:1 methanol:chloroform ratio and the mixture was homogenized for two minutes. An extra 50 mL of chloroform was added and mixed for 30 seconds after which 50 mL water was added and thoroughly combined. The liquid was collected by filtering through a Whatman No. 1 filter paper using a Buchner funnel applied with slight suction.

The solid retentate was pressed with the bottom of a beaker for maximum solvent recovery and the liquids were retained. The liquids were then transferred into a separatory funnel and were left to separate into two

different layers. The bottom chloroform layer was allowed to flow through an anhydrous sodium sulfate layer (2.5 cm thick) using a Whatman No. 1 filter paper in a funnel. The solvent was removed using a rotary evaporator (Rotovapor R-200, Buchi, Switzerland) under vacuum, at 40 °C. The final weight of the lipids was obtained from the formula below:

Weight of lipids = (weight of container + extracted lipids) - (weight of container)

The weight of the lipids in the samples was determined by weight difference:

Lipid content (%) = [weight of lipids extracted (g) / weight of samples (g)] * 100

3.2.3 Crude Fiber

Samples were subjected to fiber analysis based on the procedure described by Aberoumand and Deokule (2009). Residual sample after crude lipid extraction weighing 10 g was added with 200 mL of boiling 1.25% H_2SO_4. The sample-acid mixture was boiled for 30 minutes, cooled, and then filtered through a filter paper. The mixture residue was washed trice with 50 mL aliquots of boiling water, after which the washed residue was placed in the original vessel and further digested, this time through more boiling in 200 mL of 1.25% NaOH for an additional 30 minutes. The digestate was filtered to obtain the residue and washed trice with 50 ml aliquots of boiling water and lastly with 25 mL ethanol. The digested and washed residue was allowed to

dry in an oven at 130 ºC and cooled in a desiccator. The residue was placed into a porcelain crucible, weighed, ashed at 550 ºC for two hours, cooled in a desiccator and reweighed. Crude fiber content was conveyed as percentage loss in weight on ignition in the formula below:

Crude fiber (%) = [Loss in weight on ignition (g) / weight of sample (g)] x 100

3.2.4 Ash

Dry ashing was conducted based on the protocol recommended in Food Analysis, 3rd Edition, by Harbers and Nielsen (2003). A sample of 10 g was weighed in a tarred crucible. The crucible was placed in a cooled muffle furnace (Lindberg/Blue M, Thermo Scientific, USA) and was ignited overnight at 550 °C. After the temperature was allowed to drop, the crucible was transferred quickly to a desiccator and allowed to cool to room temperature prior to weighing. Percentage of ash is calculated using the formula below:

Ash (%) = mass of ash (g)/mass of dry sample (g) * 100

3.2.5 Mineral Content

The calcium, phosphorus and potassium in the samples were extracted using the ultrasound assisted extraction method by Costas *et al.* (2010). A dried sample weighing 100 mg was placed in a 15 mL centrifuge tube. The extraction solution used was 5 mL 1% HNO_3. The suspension was sonicated (FB15061, Fisher Scientific, USA) at room temperature (27 °C) for five

minutes. In order to obtain the extracted minerals, the suspension was then centrifuged at 4000 rpm for ten minutes. A volume of 4 mL supernatant was filtered and transferred to a volumetric flask, made up to 10 mL, and stored in polypropylene centrifuge tubes prior to inductive coupled plasma with optical emission spectrometry (ICP-OES) analysis.

ICP (Optima 7000 DV, PerkinElmer, USA) instrumental parameters set based on recommendations by Besecker and Duffy (2000) were used to determine the mineral content of the samples. Five standards of different concentration (20, 40, 60, 80, 100 mg/L) were prepared from a commercial stock solution for calcium, phosphorus and potassium, while ultrapure water was used as the blank. A calibration curve of 99.9% was obtained before sample analysis was started. The wavelengths used (Besecker and Duffy, 2000; PerkinElmer Inc, 2010) for each element are as in Table 3.1:

Table 3.1: Wavelengths (nm) used for detection of the minerals

Element	Wavelength
Calcium	317.933
Phosphorus	178.221
Potassium	766.49

3.2.6 Amino Acid Analysis

The amino acid composition in the experimental diets and muscle samples of the fingerlings after being fed the experimental diets was determined using a high performance liquid chromatography (HPLC) method.

Frozen filleted muscle samples were freeze dried under vacuum (FreeZone 2.5, Labconco, USA) for 24 hours. Freeze-dried samples weighing

30 mg were placed in microwave assisted hydrolysis digestion vessels (Kroll, Rawel and Krock, 1998). Four mL of 6 N HCl containing 1% of phenol was added to each vessel and the vessels were capped and positioned according to the microwave digestion system (Speedwave four, Berghof, Germany). After digestion was completed, the digestate was allowed to cool and made up to 50 mL volume with MiliQ water. The solution was then filtered and stored at -20 °C until analysis.

Amino acid analysis was carried out using a 4.6 mm x 150 mm x 3.5 µm reverse phase Zorbax Eclipse Plus C18 column (Agilent Technologies, USA) with an Agilent 1100 (USA) HPLC system. Solution buffers and solvents were prepared according to Henderson *et al.* (2000). Mobile phase A used was 40 mM sodium phosphate buffer (NaH_2PO_4) (pH 7.8). Mobile phase B used was a mixture of acetonitrile:methanol:water at 45:45:10 v/v ratio.

The samples were derived with reagents from Agilent Technologies (USA) at the following volumes: 50 µL borate buffer (0.4 M, pH 10.2), 10 µL sample, 10 µL o-phthaldialdehyde (OPA) reagent, 10 µL fluorenylmethyloxycarbonyl chloride (FMOC-Cl) reagent and 640 µL deionized water. The mixture was vortexed and allowed to stand for 2 minutes for complete derivatization of the amino acids. Injection was facilitated by an Agilent 1100 (USA) autosampler, and eluted at 2.0 mL/min through the column using a gradient program by manipulating the percentage of mobile phase B (Henderson *et al.*, 2000) (Table 3.2). Detection was at 338 nm using a diode array detector (Agilent 1100, USA) and amino acid composition was

calculated using a standard curve and represented as % total amino acid (National Institute of Health Science, 2002).

Table 3.2: Gradient program for amino acid analysis

Time (minutes)	Mobile phase B (%)
0	0
1.9	0
18.1	57
18.6	100
22.3	100
23.2	0
26	0

3.2.7 Fatty Acid Analysis

Fatty acid analysis was performed to determine the fatty acid composition in the experimental diets, muscle and liver samples of the fingerlings after being fed the experimental diets. A gas chromatograph (GC) with a flame ionized detector (FID) was used for the analysis.

Lipids were extracted using the method in 3.2.2. Fatty acid methyl esters (FAMEs) were converted using the protocol from Sigma-Aldrich (1997). Lipid samples weighing 20 mg were placed in a reaction vessel and 2 mL of 10% methanolic H_2SO_4 (Sigma-Aldrich) was added. The mixture was heated at 60 °C for 30 minutes, vortexing every 15 minutes to ensure proper heating. The mixture was cooled to room temperature and 1mL saturated sodium bicarbonate solution was added to neutralize the reagent. In order to extract the esters, 1 mL hexane was added to the mixture, vortexed, and centrifuged to allow the phases to separate. The upper hexane layer was carefully transferred to an airtight vessel for storage prior to analysis.

Fatty acid analysis was conducted using a 30 m x 0.25 mm x 0.25 µm BP20 (SGE, Australia) column with a GC (Clarus 500, PerkinElmer, USA) system. Chromatographic conditions for the GC used were modified from Canadian Life Science (2012), as shown in Table 3.3.

Table 3.3: GC conditions for fatty acid analysis

Component	Condition
Injector	Temperature: 250 °C
Carrier gas	Helium gas
	Flow rate: 0.5 mL/min
Detector	Temperature: 250 °C
Oven	Initial temperature: 50 °C, hold for 2 minutes
	Ramp: 4 °C/minute
	Final temperature: 250 °C, hold for 15 minutes
Split ratio	30:1
Flame ignition	Hydrogen gas
	Flow rate: 45 mL/min
	Purified air
	Flow rate: 450 mL/min

FAMEs were analyzed using the FID of the GC system. A 37 FAME mix standard (Supelco, USA) was used for calibration and peak identification purposes. Tricosanoic acid (C23:0 fatty acid) was used as the internal standard. Results were calculated using the areas of the internal standard and expressed as % total fatty acids (Hribar *et al.*, 2013).

3.3 Digestibility Trials

3.3.1 Formulation of Experimental Diets

A prototype experimental diet was formulated based on various research studies reported by Fontainhas-Fernandes *et al.* (1999), Maina *et al.* (2002), Tibbetts, Milley and Lall (2006), Ng, Abdullah and de Silva (2008) and Misieng, Kamarudin and Musa (2011).

The basal diet was made up of spirulina, casein, flour mix, yeast, lecithin, multivitamins and minerals and palm carotene oil, while the protein portion was manipulated to contain the different alternative protein sources. Fish meal was substituted at 100% inclusion level by the alternative animal protein sources, whereas the alternative plant protein sources substituted fish meal at 50% inclusion level. Lecithin at 1% inclusion level was used as an emulsifier and binder in the diet. This was based on the results obtained from the nutrient analysis of the individual ingredients, where the plant protein sources had relatively low crude protein content, coupled with other factors such as high crude fiber content as well as antinutritional factors. Chromium oxide was incorporated at 1% dietary inclusion as an inert internal marker (Table 3.4).

Table 3.4: Composition of experimental diets for digestibility trials

	Diet					
	FMD	SWD	PKCD	COD	AWD	KRD
Fish meal	50.0	25.0	25.0	-	-	-
Soy waste	-	25.0	-	-	-	-
Pam kernel cake	-	-	25.0	-	-	-
Chicken offal	-	-	-	50.0	-	-
Anchovy waste	-	-	-	-	50.0	-
Krill	-	-	-	-	-	50.0
Spirulina	6.0	6.0	6.0	6.0	6.0	6.0
Casein	10.0	10.0	10.0	10.0	10.0	10.0
Flour mix	25.0	25.0	25.0	25.0	25.0	25.0
Yeast	2.0	2.0	2.0	2.0	2.0	2.0
Lecithin	1.0	1.0	1.0	1.0	1.0	1.0
Multivitamins and minerals*	2.0	2.0	2.0	2.0	2.0	2.0
Chromic oxide	1.0	1.0	1.0	1.0	1.0	1.0
Palm carotene oil	3.0	3.0	3.0	3.0	3.0	3.0

*Multivitamins and minerals: Vitamin A, 5000 iu; Vitamin D, 400 iu; Thiamine HCI (B1), 3.5 mg; Riboflavin (B2), 2.5 mg; Pyidoxine HCI (B6), 2.5 mg; Cyanocobalamine (B12), 0.005 mg; Ascorbic acid (C), 50 mg; Nicotinamide, 25 mg; Calcium Pantothenate, 4 mg; Calcium, 25 mg; Copper, 0.75 mg; Iron, 5 mg; Iodine, 0.05 mg; Magnesium, 0.5 mg; Manganese, 0.5 mg; Phosphorus, 20 mg; Zinc, 2 mg; Folic acid, 0.5 mg.

The basal ingredients were placed in a mixer and small amounts of water were added to form a smooth paste. The protein samples (fish meal, anchovy waste, krill, chicken offal, palm kernel cake or soy meal) were added slowly to the paste and the paste was stirred continuously until a soft dough was formed. The dough was kneaded to ensure homogenous mixing and put through an extruder (Ch-888, Taiwan) where the pellets extruded were of 2*2* 2 mm. The pellets were put in a convection oven and the temperature was set to 105 °C. The pellets were dried for 3 hours before being stored until used.

3.3.2 Feeding of Fingerlings

A total of six experimental diets were formulated, with the fish meal diet as the control. Thirty *T. tambroides* fingerlings obtained from a local hatchery, weighing 5.5 ± 0.3 g and measuring 4.7 ± 0.3 cm were used for each diet formula, totaling to 180 fingerlings. The fingerlings were allowed to adapt to their surroundings for two weeks followed by four days to acclimatize towards the new diets and fed twice a day to apparent satiation.

Fecal collection was modified slightly based on a review of the Guelph system (Bureau and Cho, 1999). The tanks were cleaned after the second feeding to ensure that there was no food contamination. The faeces were allowed to settle at the bottle of the tank until the following day where they were siphoned out into bottles before centrifuging. The supernatant was discarded and the faeces were freeze-dried, ground and stored for digestibility analysis. Both diets and faeces were analyzed for crude protein content according to the methods described in sections 3.2.1. Chromium content in the diets and faeces were analyzed using the protocol in section 3.2.5, with wavelength of 267.716 nm.

The digestibility index was expressed as apparent digestibility coefficient for dry matter (ADM) and protein (ADC), and was calculated based on the following formulae (Martins, Valente and Lall, 2009):

$$ADM = 100 - (100 * (\%\ Cr_2O_3 \text{ in diet} / \%\ Cr_2O_3 \text{ in faeces}))$$

ADC = 100 – (100 * (% Cr_2O_3 in diet / % Cr_2O_3 in faeces) * (% protein in faeces / % protein in diet))

A preference test was conducted simultaneously as the digestibility trial. The fingerlings were presented with a no-choice possible test (Stallings, 2010) where they were fed designated diets and the preference of each diet was based on the amount of food consumed over a period of time. The fingerlings were fed a measured amount of the experimental diets twice daily, and the remaining uneaten food collected and weighed to determine how much feed was consumed. Ratings were reported as +, ++, +++ and ++++ for 4.1-5.0 g, 5.1-6.0 g, 6.1-7.0 g and 7.1-8.0 g of the experimental diet consumed respectively.

3.4 Growth Studies

3.4.1 Formulation of Experimental Diets

After the digestibility trials, chicken offal from the animal protein category and palm kernel cake from the plant protein category were selected as the alternative protein sources as fish meal replacements in growth studies. Seven isoproteic diets containing 42% crude protein were formulated (Table 3.5), with the reference diet containing 100% fish meal (FMG) as the main protein source which was used as the positive control and six alternative protein based diets. Three diets containing chicken offal at increasing replacement levels of fish meal at 33.3% (1CO), 66.7% (2CO) and 100% (3CO), and three diets from palm kernel cake as fish meal replacement at

16.5% (1PKC), 33.0% (2PKC) and 50% (3PKC) were evaluated in the growth studies.

Table 3.5: Composition of experimental diets for growth studies

	Diet						
	FMG	1CO	2CO	3CO	1PKC	2PKC	3PKC
Fish meal	50.0	33.5	17.0	-	41.8	33.5	25.0
Pam kernel cake	-	-	-	-	8.2	16.5	25.0
Chicken offal	-	16.5	33.0	50.0	-	-	-
Casein	8.0	9.0	13.0	17.0	11.0	16.0	21.0
Flour mix	30.0	28.0	23.0	17.0	27.0	20.0	13.0
Spirulina	2.0	3.0	4.0	6.0	2.0	4.0	6.0
Yeast	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Lecithin	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Multivitamins and minerals*	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Palm carotene oil	3.0	3.0	3.0	3.0	3.0	3.0	3.0

*Multivitamins and minerals: Vitamin A, 5000 [Type a quote from the document or the summary of an interesting point. You can position the text box anywhere in the document. Use the Drawing Tools tab to change the formatting of the pull quote text box.]
iu; Vitamin D, 400 iu; Thiamine HCI (B1), 3.5 mg; Riboflavin (B2), 2.5 mg; Pyidoxine HCI (B6), 2.5 mg; Cyanocobalamine (B12), 0.005 mg; Ascorbic acid (C), 50 mg; Nicotinamide, 25 mg; Calcium Pantothenate, 4 mg; Calcium, 25 mg; Copper, 0.75 mg; Iron, 5 mg; Iodine, 0.05 mg; Magnesium, 0.5 mg; Manganese, 0.5 mg; Phosphorus, 20 mg; Zinc, 2 mg; Folic acid, 0.5 mg.

3.4.2 Feeding of Fingerlings

Feeding trials were performed to evaluate the potential for growth and the nutrient utilization of the *T. tambroides* fingerlings fed the diets containing increasing levels of chicken offal and palm kernel cake. A total of seven experimental diets were studied whereby 210 fingerlings were used. For each diet, 30 homogenous fingerlings were randomly assigned to a cement tank with dimensions of 60*90*180 cm. The initial body weight of the fingerlings was 17.2 ± 0.6 g, the standard length measuring 8.6 ± 0.5 cm and the total length measuring 10.8 ± 0.7 cm. The growth studies were conducted over a period of six months, and the fingerlings were fed the diets twice a day to apparent satiation. Prior to the experiment, the fingerlings were allowed 14 days to adapt to their new rearing conditions followed by four days to acclimatize towards the new diets

3.4.3 Sampling Procedure

Fingerlings were randomly selected and measured at the beginning of every week for the first two months, and fortnightly for the following four months. The fingerlings were sedated with the use of NIKA Transmore® Fish Stabilizer that contains α-methylquinoline as the active ingredient. The standard length, total length and weight gain were used as indicators for growth. Figure 3.1 shows the diagram which the measurement of length was based on.

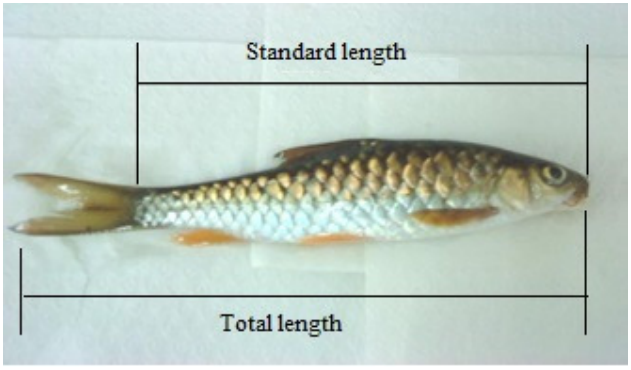

Figure 3.1: Measurement of standard length and total length

Diet performance was reviewed based on the data obtained through the specific growth rate (SGR), the feed conversion ratio (FCR) and the protein efficiency ratio (PER) (Shapawi, Ng and Mustafa, 2007; Wang *et al.*, 2006).

The formulae used were:

SGR (%) = 100 * ((ln FBW – ln IBW) / x days)

FCR = diet consumed/weight gain

PER = weight gain/protein intake

Where IBW = initial body weight and FBW = final body weight

Length-Weight (L-W) relationship and the relative condition factor (K) were determined based on the cube law and using the formula by Froese (2006):

$W = a L^b$

$K = W/a L^b$

Where W = body weight, L = total length in cm, coefficient a = intercept of the regression and b = the regression coefficient.

At the end of the growth studies, fish from each tank were sacrificed. Each fish sample was filleted, with bones removed before labeling and stored at -80 °C for further body composition analysis. Similarly, the liver was removed, labeled and stored under the same conditions. For control and experimental diets, muscle and liver samples were prepared for fatty acid analysis, whereas only the diets and muscle samples were used in the amino acid analysis. Both analyses were conducted to determine the effect of the experimental diets on the body composition of the fingerlings.

3.5 Statistical Analysis

All data collected from nutrient analysis, digestibility trials and growth studies were expressed as mean and standard error mean (SEM) and used to determine the efficacy of the alternative protein source on the growth of the fingerlings. SEM was used as a guide to determine the precision of the estimated mean as well as the standard deviation. Standard length, total length and weight of the fingerlings were analyzed using Levene's test ($P>0.05$) to ensure homogeneity of variance between the fingerlings at the start of the growth study. Data were analyzed using one way analysis of variance (ANOVA) and treatment means were evaluated using Duncan's multiple range tests (to provide additional information on the differences between means) through SPSS Statistics 11.5 package (IBM, USA) where the significance was tested at 95% confidence level and significant differences were considered when P was less than 0.05.

CHAPTER 4

RESULTS

4.1 Nutrient Analysis

4.1.1 Proximate Analysis for Individual Ingredients

Nutrient proximate analysis was conducted on each ingredient prior to diet formulation to determine the nutrient composition. The animal proteins sources contained the highest levels of crude protein, while the plant protein sources had crude protein levels which were less than half the amount of the animal proteins. Crude fat levels varied among ingredients due the different lipid content in each protein source. Crude fiber levels were the highest in plant protein sources which is attributed to the plant cell wall structures, whereas these structures were absent in the animal protein sources. Mineral levels (ash, calcium, phosphorus and potassium) obtained from the animal protein sources were higher when compared with the plant protein sources, as the animal sources were prepared from whole carcasses, bones and offal which contributed to the mineral content. Table 4.1 compiles the nutrient contents of each ingredient.

Table 4.1: Nutrient composition of the individual ingredients* (g/100 g)

Proximate analysis[§]	FM	SW	PKC	CO	AW	KR	SEM[¥]
Crude protein	69.82^{f}	21.57^{b}	16.95^{a}	49.46^{c}	56.53^{d}	57.34^{e}	4.71
Crude fat	6.23^{b}	1.47^{a}	6.78^{bc}	10.35^{e}	8.46^{d}	7.50^{c}	0.67
Crude fiber	0.13^{a}	9.27^{c}	10.14^{c}	0.33^{a}	0.69^{a}	1.89^{b}	1.02
Ash	11.36^{c}	3.24^{a}	5.56^{b}	25.25^{d}	32.27^{f}	31.71^{e}	2.90
NFE[#]	12.56^{b}	64.60^{e}	61.74^{d}	14.89^{c}	2.31^{a}	1.63^{a}	6.45
Calcium	22.09^{e}	4.05^{a}	6.17^{b}	48.99^{f}	21.94^{d}	21.50^{c}	3.56
Phosphorus	14.83^{e}	2.20^{a}	5.14^{b}	24.50^{f}	13.63^{d}	11.10^{c}	1.74
Potassium	8.98^{e}	9.36^{f}	6.49^{c}	4.63^{a}	8.56^{d}	6.33^{b}	0.41

*FM (fish meal), SW (soy waste), PKC (palm kernel cake), CO (chicken offal), AW (anchovy waste), KR (krill)

[§]Values are means for triplicate analysis. Mean values within any single row with different superscripts are noted to be significantly different ($P \leq 0.05$)

[¥]Standard error of mean

[#] Nitrogen free extract

4.1.2 Proximate Analysis for Digestibility Trial Diets

Crude protein content of all the digestibility trial experimental diets ranged from 32% to 43%, where only the protein portion of the diets was substituted, without any modification to the basal diet composition. Crude protein levels differed significantly (P=0.00) among all the diets, with the fish meal-based diet contained the most crude protein. Although soy waste and palm kernel cake individually have low crude protein content, only 50% of the protein portion in the experimental diets was replaced to compensate for differences in protein composition of the animal and plant protein sources.

The shell and bones from the krill and anchovy led to high levels of ash in both the krill and anchovy waste diets, while the soy waste diet contained the lowest ash content. The plant-based diets had the highest amounts of crude fiber, due to the plant materials used. There was no significant differences (P=0.05) between the nitrogen free extract for anchovy

waste, krill and fish meal diets while crude fat amounts in all the diets varied among each other. Table 4.2 shows the nutritional composition of the experimental diets used in the digestibility trials.

Table 4.2: Nutrient composition of the experimental diets in digestibility trial (g/100 g)

Diet*	Proximate analysis[§]				
	Crude protein	Crude fat	Crude fiber	Ash	NFE
FMD	42.31[e]	10.35[a]	0.35[a]	11.93[c]	35.06[a]
SWD	32.73[a]	10.85[a]	3.10[c]	10.45[b]	42.87[c]
PKCD	32.12[a]	10.67[a]	3.13[c]	9.34[a]	44.74[c]
COD	35.80[b]	16.70[c]	0.52[a]	13.80[d]	33.18[b]
AWD	37.90[c]	16.34[c]	1.54[b]	15.61[e]	28.74[a]
KRD	39.13[d]	12.70[b]	1.35[b]	18.13[f]	28.77[a]
SEM[¥]	0.87	0.64	0.27	0.73	1.53

*Diets formulated from fish meal (FMD), soy waste (SWD), palm kernel cake (PKCD), chicken offal (COD), anchovy waste (AWD), krill (KRD)
[§]Values are means for triplicate analysis. Mean values within any single column with different superscripts are noted to be significantly different ($P \leq 0.05$)
[¥]Standard error of mean

4.1.3 Proximate Analysis for Growth Study Diets

All the experimental diets were formulated to contain an estimation of 42% crude protein, as demonstrated by the relatively similar crude protein level (P=0.12). Crude fat content of the all formulated diets were high, due to the addition of palm carotene oil in the diet. When the substitution level of fish meal with chicken offal increased, the crude fat content in the chicken offal diets also increased, since chicken offal contained more crude fat than fish meal. A similar trend was observed in the diets containing palm kernel cake, where the levels of crude fat show a proportional increase with the incorporation of fish meal.

Table 4.3 shows that crude fiber content increased as the inclusion level of the alternative protein increased due to both chicken offal and palm kernel cake contained more crude fiber than fish meal. As 3PKC contained the most plant protein, it was expected the crude fiber levels were the highest of all the experimental diets. Ash content in all experimental diets was inversely proportional to the substitution level in the chicken offal diet, while the opposite trend was observed in the palm kernel cake diet.

Table 4.3: Nutrient composition of the experimental diets in growth study (g/100 g)

Diet*	Proximate analysis[§]				
	Crude protein	Crude fat	Crude fiber	Ash	NFE
FMG	42.13^{a}	10.01^{a}	0.35^{ab}	6.38^{b}	37.87^{d}
1CO	41.90^{a}	13.20^{d}	0.12^{a}	5.80^{a}	37.78^{d}
2CO	42.67^{a}	15.52^{e}	0.40^{ab}	8.63^{e}	31.60^{b}
3CO	41.83^{a}	16.30^{f}	0.52^{bc}	12.80^{f}	27.14^{a}
1PKC	41.93^{a}	10.33^{ab}	0.70^{c}	6.44^{b}	38.70^{d}
2PKC	42.67^{a}	10.64^{bc}	2.07^{d}	7.21^{c}	34.41^{c}
3PKC	42.50^{a}	10.96^{c}	3.10^{e}	7.90^{d}	32.53^{b}
SEM[¥]	0.12	0.57	0.23	0.57	0.88

*Replacement levels of fish meal at 0% (FMG), 33.3% (1CO), 66.7% (2CO) and 100% (3CO), 16.5% (1PKC), 33.0% (2PKC) and 50% (3PKC)
[§]Values are means for triplicate analysis. Mean values within any single column with different superscripts are noted to be significantly different ($P \leq 0.05$)
[¥]Standard error of mean

The mineral content of potassium ranged from 2 to 5 g/kg for the experimental diets. The calcium and phosphorus contents in chicken offal-based diets increased proportionally with the inclusion of chicken offal, whereas the opposing trend was noted in the potassium content. As for the palm kernel cake-based diets, calcium levels decreased as the inclusion increased, though potassium levels increased and phosphorus levels remained constant (Table 4.4).

Table 4.4: Mineral content of the experimental diets in growth study (g/100 g)

Diet	Proximate analysis*		
	Calcium	Phosphorus	Potassium
FMG	7.83[d]	5.17[b]	4.64[g]
1CO	10.45[e]	6.08[c]	4.35[f]
2CO	12.44[f]	6.61[d]	3.72[d]
3CO	12.72[g]	6.84[e]	3.10[b]
1PKC	5.67[c]	3.69[a]	2.98[a]
2PKC	5.48[b]	3.65[a]	3.45[c]
3PKC	5.34[a]	3.64[a]	4.08[e]
SEM[§]	0.68	0.30	0.13

*Values are means for triplicate analysis. Mean values within any single column with different superscripts are noted to be significantly different ($P \leq 0.05$)

[§]Standard error of mean

4.2 Diet Digestibility

The apparent digestibility values and the preference for the experimental diets are summarized in Table 4.5. There were significant differences (P=0.00) between the diets based on the estimations of apparent digestibility coefficient for dry matter (ADM) and protein (ADC). The highest values of ADM and ADC of protein were observed for control (fish meal) diet. As the apparent digestibility values were affected by the nature of the diet, it was noted the substitute animal protein sources had slightly reduced ADM when compared to the fish meal diet, but it was significantly reduced (P=0.00) for plant-based diets. The palm kernel cake diet had a higher ADC level and was the most preferred diet in the plant protein category; although chicken offal was not the most digestible animal protein source, the fingerlings preferred the chicken offal diet over the other animal protein sources, with the exception of the fish meal diet.

Table 4.5: Apparent digestibility coefficient values in percentage and preference for the experimental diets

Diet	Apparent digestibility coefficient*		Preference
	ADM	ADC	
FMD	92.28[e]	98.78[f]	++++
SWD	66.90[b]	86.23[a]	+
PKCD	65.11[a]	87.42[b]	+++
COD	82.12[c]	89.77[c]	+++
AWD	85.09[d]	93.39[d]	++
KRD	83.70[c]	96.12[e]	+
SEM[§]	2.40	1.11	-

*Values are means for triplicate analysis. Mean values within any single column with different superscripts are noted to be significantly different ($P \leq 0.05$)

[§]Standard error of mean

4.3 Growth Performance

Based on the results of the preference test, chicken offal and palm kernel cake were selected as the best alternative animal and plant protein to substitute fish meal at different inclusion levels. Levene's test was used to verify the assumptions of equality of variance, whereby all the fingerlings at the start of the study were homogenous in weight (P=0.884), total length (P=0.270) and standard length (P=0.216).

There was no mortality of the fingerlings reported throughout the whole six-month period for growth studies. As shown in Table 4.6, there was no significant difference in weight gain (P=0.10) and standard length gain (P=0.173) percentage respectively between fingerlings fed the fish meal diet or the experimental alternative protein diets, except for fingerlings consuming the 3CO diet. Significant differences (P=0.00) were observed for total length gain. There was also no significant difference (P=0.84) in the specific growth rate (SGR) between the groups, although 3CO achieved the highest SGR when compared with the others, including the control group. Feed conversion ratio

(FCR) was similar among diets, where there were no significant differences (P=0.28). Protein efficiency ratio (PER) of the experimental diets indicated that the protein utilization of the experimental diets were comparable to the fish meal diet, with 1CO as the exception.

Table 4.6: Growth and diet utilization efficiency of the fingerlings

Diet	Parameters*					
	Weight gain (%)	Standard length gain (%)	Total length gain (%)	SGR (%)	FCR	PER
FMG	221.88^{a}	133.91^{b}	114.19^{g}	0.44^{a}	2.58^{ab}	0.92^{b}
1CO	219.28^{a}	131.61^{ab}	112.16^{f}	0.43^{a}	2.90^{b}	0.82^{a}
2CO	224.34^{a}	129.13^{a}	110.32^{b}	0.44^{a}	2.63^{ab}	0.89^{b}
3CO	230.46^{b}	129.46^{ab}	110.51^{c}	0.46^{a}	2.52^{a}	0.95^{b}
1PKC	220.96^{a}	131.21^{ab}	111.94^{e}	0.43^{a}	2.61^{ab}	0.91^{b}
2PKC	222.87^{a}	129.75^{ab}	110.74^{d}	0.44^{a}	2.54^{ab}	0.92^{b}
3PKC	220.49^{a}	128.59^{a}	109.75^{a}	0.43^{a}	2.59^{ab}	0.91^{b}
SEM§	0.96	0.58	0.31	0.01	0.04	0.01

*Values are means for triplicate analysis. Mean values within any single column with different superscripts are noted to be significantly different ($P \leq 0.05$)

§Standard error of mean

Based on the formulae $W = a L^{b}$ and $K = W/a L^{b}$, a length-weight relationship as well as the relative condition factor was determined (Table 4.7). No significant differences (P=0.233) were found for the relative condition factor between fingerlings from different diets, although it ranged from 1.07 to 1.14. Equations for the length-weight relationship were calculated by applying the Log function to the formula $W = a L^{b}$.

Table 4.7: Length-weight relationship and the relative conditioning factor of the fingerlings

Diet	Equation	Parameters* Regression			r
		a	b	K	
FMG	Log W = -0.28 +1.45 log L	0.52^{d}	1.45^{c}	1.12^{c}	0.96
1CO	Log W = -0.20 + 1.39 Log L	0.63^{f}	1.39^{a}	1.13^{d}	0.97
2CO	Log W = -0.30 + 1.49 Log L	0.50^{c}	1.49^{d}	1.07^{a}	0.97
3CO	Log W = -0.37 + 1.53 Log L	0.43^{b}	1.53^{f}	1.12^{c}	0.96
1PKC	Log W = -0.51 + 1.51 Log L	0.31^{a}	1.51^{e}	1.10^{b}	0.97
2PKC	Log W = -0.36 + 1.63 Log L	0.44^{b}	1.63^{g}	1.13^{d}	0.95
3PKC	Log W = -0.25 + 1.43 Log L	0.56^{e}	1.43^{b}	1.14^{e}	0.94
SEM[§]	-	0.02	0.02	0.01	-

*Values are means for triplicate analysis. Mean values within any single column with different superscripts are noted to be significantly different ($P \leq 0.05$)

[§]Standard error of mean

4.4 Amino Acid Composition

Tryptophan was not measured in this study, as it was degraded by the acid during hydrolysis. Asparagine and glutamine formed complexes with aspartic acid and glutamic acid respectively and were detected as such after being hydrolyzed.

The 3PKC diet contained the least amounts of the essential amino acids, and the most non-essential amino acids. Essential amino acid content in all experimental diets was constant to the incorporation of the substitute proteins and each inclusion level. Aspartic acid and glutamic acid were highest in all the experimental diets, with the 3CO diets containing the highest for both. Lysine content in the diets ranged from 3.36-3.93% of the total amino acids (Table 4.8).

Essential amino acid in muscle samples (Table 4.9) increased with the inclusion of chicken offal and palm kernel cake in the experimental diets,

while the non-essential amino acid content decreased. There was no direct relation of the amino acids in the diet samples and the muscle samples. Nevertheless, the amino acid trend line in the muscle samples was similar to the trend line from the diet samples. Total essential amino acids in the muscle samples were not found to be directly affected by the total essential amino acids in dietary samples. Table 4.9 shows the amino acid content obtained from the fingerling muscle samples.

Table 4.8: Amino acid analysis of diet samples (% total amino acids)

Amino acid*	Diet sample							
	FMG	1CO	2CO	3CO	1PKC	2PKC	3PKC	SEM§
Threonine	5.63^{c}	5.31^{bc}	5.15^{ab}	4.86^{a}	5.60^{c}	5.31^{bc}	5.40^{bc}	0.07
Valine	6.50^{ab}	6.83^{bc}	7.16^{cd}	7.28^{d}	6.39^{a}	6.61^{ab}	6.83^{bc}	0.07
Isoleucine	5.20^{c}	5.28^{c}	4.64^{b}	4.25^{a}	4.90^{bc}	5.03^{c}	4.97^{bc}	0.08
Leucine	8.67^{c}	8.95^{cd}	9.45^{d}	9.50^{d}	6.60^{b}	6.43^{b}	5.63^{a}	0.34
Phenylalanine	3.57^{d}	4.59^{e}	5.78^{e}	5.86^{e}	2.57^{c}	2.32^{b}	2.04^{a}	0.34
Lysine	3.47^{a}	3.42^{a}	3.44^{a}	3.43^{a}	3.36^{a}	3.88^{b}	3.93^{b}	0.05
Histidine	2.40^{a}	2.31^{a}	2.25^{a}	2.21^{a}	2.18^{a}	2.33^{a}	2.31^{a}	0.03
Arginine	5.18^{b}	5.26^{b}	5.27^{b}	5.33^{b}	5.14^{b}	4.78^{a}	4.77^{a}	0.06
Methionine	3.45^{cd}	3.10^{bc}	2.76^{ab}	2.73^{a}	3.62^{d}	3.59^{d}	3.79^{d}	0.10
Aspartic acid	10.40^{ab}	11.26^{ab}	11.67^{ab}	11.85^{b}	10.20^{a}	10.36^{ab}	10.46^{ab}	0.20
Glutamic acid	15.02^{a}	17.17^{b}	18.01^{b}	18.79^{b}	14.71^{a}	14.86^{a}	15.31^{a}	0.39
Serine	3.35^{ab}	3.28^{a}	3.55^{bc}	3.61^{c}	4.49^{d}	4.85^{e}	4.75^{e}	0.14
Glycine	8.69^{b}	8.33^{b}	7.33^{a}	7.35^{a}	8.79^{b}	8.51^{b}	8.37^{b}	0.16
Alanine	10.33^{c}	8.05^{ab}	7.54^{a}	7.02^{a}	9.72^{c}	9.44^{bc}	9.54^{c}	0.29
Tyrosine	2.44^{b}	2.37^{b}	2.02^{a}	1.86^{a}	3.95^{c}	3.84^{c}	3.97^{c}	0.19
Cysteine	1.86^{a}	1.94^{ab}	2.16^{bc}	2.38^{c}	1.82^{a}	1.74^{a}	1.72^{a}	0.06
Proline	3.84^{c}	2.55^{b}	1.82a	1.69^{a}	5.96^{d}	6.`1de	6.21^{e}	0.42

*Values are means for triplicate analysis. Mean values within any single row with different superscripts are noted to be significantly different ($P \leq 0.05$)
§Standard error of means

Table 4.9: Amino acid analysis of muscle samples (% total amino acids)

Amino acid*	Muscle sample[§]							
	FMF	1COF	2COF	3COF	1PKCF	2PKCF	3PKCF	SEM[¥]
Threonine	4.17^{a}	3.93^{a}	3.94^{a}	3.92^{a}	3.73^{a}	3.94^{a}	3.86^{a}	0.15
Valine	4.07^{a}	3.55^{a}	3.21^{a}	3.14^{a}	3.22^{a}	3.03^{a}	3.15^{a}	0.16
Isoleucine	3.96^{ab}	3.72^{ab}	3.73^{ab}	3.80^{ab}	2.57^{a}	3.30^{ab}	4.23^{b}	0.18
Leucine	8.48^{a}	10.54^{b}	10.57^{b}	10.65^{b}	8.37^{a}	8.22^{a}	8.13^{a}	0.28
Phenylalanine	3.79^{b}	3.54^{ab}	3.59^{ab}	3.62^{ab}	3.48^{ab}	3.40^{a}	3.27^{a}	0.05
Lysine	3.99^{a}	3.90^{a}	4.20^{ab}	4.46^{b}	4.16^{ab}	4.11^{ab}	3.97^{a}	0.05
Histidine	1.93^{a}	2.10^{ab}	2.22^{bc}	2.35^{c}	2.83^{e}	2.72^{de}	2.60^{d}	0.07
Arginine	5.32^{c}	4.96^{a}	4.96^{a}	4.94^{a}	5.18^{bc}	5.05^{ab}	5.03^{ab}	0.04
Methionine	2.66^{c}	2.49^{bc}	2.56^{bc}	2.60^{c}	2.56^{bc}	2.39^{b}	2.21^{a}	0.04
Aspartic acid	11.76^{ab}	11.59^{a}	11.67^{a}	11.83^{ab}	12.75^{abc}	13.44^{bc}	13.83^{c}	0.25
Glutamic acid	15.63^{a}	15.31^{a}	13.46^{a}	15.55^{a}	17.71^{b}	16.98^{ab}	16.73^{ab}	0.25
Serine	7.89^{b}	7.98^{b}	7.91^{b}	7.86^{b}	7.56^{ab}	7.27^{a}	7.12^{a}	0.09
Glycine	9.32^{bc}	9.51^{c}	9.36^{bc}	9.28^{bc}	8.54^{abc}	8.28^{ab}	8.05^{a}	0.17
Alanine	11.33^{a}	11.42^{a}	11.37^{a}	11.25^{a}	12.05^{a}	12.80^{a}	12.74^{a}	0.22
Tyrosine	3.23^{a}	3.29^{ab}	3.32^{ab}	3.34^{ab}	3.54^{ab}	3.60^{ab}	3.63^{b}	0.05
Cysteine	1.46^{b}	0.53^{a}	0.49^{a}	0.46^{a}	0.97^{b}	0.84^{b}	0.81^{b}	0.08
Proline	1.01^{c}	1.64^{e}	1.44^{d}	0.95^{bc}	0.78^{ab}	0.63^{a}	0.64^{a}	0.08

*Values are means for triplicate analysis. Mean values within any single row with different superscripts are noted to be significantly different ($P \leq 0.05$)

[§]Muscle samples of fingerlings consuming replacement levels of fish meal at 0% (FMF), 33.3% (1COF), 66.7% (2COF) and 100% (3COF), 16.5% (1PKCF), 33.0% (2PKCF) and 50% (3PKCF)

[¥]Standard error of means

4.5 Fatty Acid Composition

The 3PKC diet contained the highest amounts of total saturated fatty acids (SFA). SFAs in the experimental diets increased as more palm kernel cake was used in each diet, as well as in relation to the inclusion of chicken offal. Despite the monounsaturated fatty acid (MUFA) in the palm kernel cake diets increasing with the incorporation of the alternative proteins, polyunsaturated fatty acid (PUFA) decreased proportionally with the substitution of fish meal in all the experimental diets. Both *n*-3 and *n*-6 fatty acids decreased too with the addition of the experimental proteins.

Total unsaturated fatty acids decreased with the inclusion of fish meal in both the chicken offal and palm kernel cake experimental diets. The 1CO diet had the highest unsaturated fatty acids content. Palmitic acid and stearic acid were the two most abundant SFA while *n*-9 oleic acid was the major contributor in MUFA. Essential fatty acids (*n*-3 and *n*-6) precursors i.e. α-linolenic acid (ALA) and linoleic acid (LA) were detected in varying amounts in all diets. Eicosapentanoic acid (EPA), docosahexanoic acid (DHA), γ-linolenic acid (GLA) and arachidonic acid (AA) were present in every diet (Table 4.10).

Similar to those observed in the diet samples, SFA content in fingerling muscle samples was predominantly palmitic acid and stearic acid, and oleic acid was the main MUFA, and both palmitic and oleic acid were fairly constant throughout the groups. MUFA levels in the muscle ranged from 43.6-46.8% of total fatty acids, irrespective of the experimental diet groups, as

with SFA levels at 29.6-33.5% of total fatty acids (Table 4.11). Both muscle MUFA and PUFA seemed to be affected by the dietary unsaturated fatty acids, where they were both influenced by the increase or decrease of MUFA and PUFA in the diet samples. Although the levels of *n*-6 in the diets were low, results of muscle and liver samples show much higher *n*-6 content as compared to the diets.

In the liver samples, MUFA levels increased proportionally to the inclusions of the substitute proteins and were composed of mainly oleic acid at an average of 93%. Increasing amounts of oleic acid in the liver were observed as the incorporation of chicken offal and palm kernel cake in the diet increased. PUFA levels, in contrast, decreased proportionally to the substitution of fish meal in the experimental diets. AA was high in the liver of fingerlings consuming the animal protein diets, while there were lower amounts present in those from the experimental diets containing palm kernel cake. Liver LA decreased as substitution levels of chicken offal increased, though, the reverse was recorded for the palm kernel cake diet (Table 4.12).

Inclusion of chicken offal in the experimental diets increased the SFA and MUFA content of the dietary samples, whereas the levels for PUFA, *n*-3 and *n*-6 decreased with the incorporation of chicken offal. Likewise, SFA, MUFA and PUFA in the muscle samples from fingerlings consuming the chicken offal diet increased. SFA content in the liver samples, in contrast, were not affected by the dietary fatty acids, while the rest of the fatty acids seemed to be influenced.

Meanwhile, the palm kernel cake-based diets showed increase in SFA and MUFA, while PUFA decreased with the level of substitution of fish meal. *n*-3 and *n*-6 content decreased and were fairly constant in the fingerling muscle samples respectively, despite no obvious reflections in the diet samples. As the dietary MUFA and PUFA respectively increased and decreased, the MUFA content in the liver samples showed a similar trend, although PUFA levels were similar through out the experimental diets utilizing palm kernel cake as the substitute protein.

Table 4.10: Fatty acid analysis of diet samples (% total fatty acids)

Fatty acid*	Diet							
	FMG	1CO	2CO	3CO	1PKC	2PKC	3PKC	SEM[§]
C12	0.03[a]	0.04[a]	0.07[a]	0.11[a]	3.44[b]	4.63[c]	6.24[d]	0.55
C14	1.11[b]	1.27[c]	0.78[a]	1.50[d]	2.36[e]	2.79[f]	2.99[g]	0.18
C16	26.56[c]	21.14[a]	23.92[b]	24.76[b]	28.91[d]	29.15[d]	30.05[d]	0.69
C18	6.29[g]	2.45[b]	2.15[a]	3.38[c]	5.88[f]	4.87[ez]	4.26[d]	0.33
C20	0.20[b]	0.21[b]	0.20[b]	0.19[b]	0.15[a]	0.18[ab]	0.25[c]	0.01
C24	0.18[d]	0.08[a]	0.12[b]	0.22[e]	0.19[d]	0.16[c]	0.11[b]	0.01
C16:1	2.75[c]	2.80[c]	2.64[c]	2.96[d]	2.04[b]	1.94[b]	1.52[a]	0.11
C18:1*n*9	36.51[ab]	47.92[c]	48.10[c]	48.12[c]	35.46[a]	36.64[ab]	37.49[b]	1.30
C18:2*n*6	4.01[b]	3.86[ab]	3.80[ab]	3.57[a]	3.52[a]	3.83[ab]	4.07[b]	0.06
C18:3*n*3	0.24[b]	0.38[c]	0.47[e]	0.58[f]	0.22[a]	0.38[c]	0.45[d]	0.03
C18:3*n*6	0.44[d]	0.40[cd]	0.24[a]	0.33[bc]	0.35[bc]	0.32[b]	0.28[ab]	0.02
C20:2n6	2.02[d]	0.26[a]	0.26[a]	0.33[a]	2.41[e]	1.85[c]	1.17[b]	0.19
C20:3*n*6	2.13[e]	0.79[c]	0.50b	0.12[a]	1.84[d]	1.69[d]	0.93[c]	0.16
C20:4*n*6	0.33[e]	0.21[d]	0.19[c]	0.17[b]	0.19[c]	0.14[a]	0.13[a]	0.01
C20:5*n*3	4.14[c]	2.40[b]	2.56[b]	2.48[b]	3.91[c]	2.56[b]	1.80[a]	0.18
C22:1*n6*	0.55[d]	0.29[a]	0.31[ab]	0.42[c]	0.47[cd]	0.42[c]	0.39[bc]	0.02
C22:5*n*3	5.22[f]	5.88[g]	4.44[e]	1.87[a]	2.34[b]	3.16[c]	3.79[d]	0.31
C22:6*n*3	6.68[d]	8.96[f]	8.50[e]	8.10[e]	5.93[c]	5.22[b]	3.79[a]	0.39
C24:1	0.61[c]	0.66[c]	0.74[d]	0.81[d]	0.39[b]	0.31[ab]	0.29[a]	0.05
SFA	34.37[c]	25.19[a]	27.24[a]	30.16[b]	40.93[d]	41.78[d]	43.90[e]	1.57
MUFA	39.87[a]	51.67[b]	51.80[b]	52.31[b]	37.89[a]	38.65[a]	39.30[a]	1.46
PUFA	25.76[e]	23.14[d]	20.96[cd]	17.53[ab]	21.18[cd]	19.57[bc]	16.80[a]	0.68
n-3	16.28[c]	17.62[c]	15.97[c]	13.01[b]	12.40[b]	11.32[ab]	9.83[a]	0.62
n-6	9.48[d]	5.81[ab]	5.30[a]	4.94[a]	8.78[d]	8.25[cs]	6.97[bc]	0.39

*Values are means for triplicate analysis. Mean values within any single row with different superscripts are noted to be significantly different ($P \leq 0.05$)

[§]Standard error of mean

Table 4.11: Fatty acid analysis of muscle samples (% total fatty acids)

Fatty acid*	Muscle sample§							
	FMF	1COF	2COF	3COF	1PKCF	2PKCF	3PKCF	SEM¥
C12	1.17^{bc}	1.13^{b}	1.30^{d}	1.25^{cd}	0.51^{a}	1,55^{e}	2.11^{f}	0.10
C14	1.71^{b}	1.61^{a}	2.14^{d}	2.01^{c}	2.84^{e}	3.50^{f}	2.76^{e}	0.14
C16	21.08^{b}	19.17^{a}	21.22^{b}	22.57^{cd}	23.70^{d}	21.42^{bc}	22.09^{bc}	0.32
C18	7.99^{f}	7.38^{e}	5.96^{bc}	4.79^{a}	6.25^{d}	6.08^{cd}	5.85^{b}	0.22
C20	0.18^{bc}	0.15^{ab}	0.21^{c}	0.27^{d}	0.18^{bc}	0.13^{a}	0.15^{ab}	0.01
C24	0.14^{d}	0.15^{d}	0.08^{b}	0.06^{a}	0.10^{c}	0.07^{ab}	0.08^{b}	0.01
C16:1	2.90^{d}	2.09^{b}	3.22^{e}	3.57^{f}	1.49^{a}	2.11^{b}	2.32^{c}	0.15
C18:1*n*9	41.14^{a}	42.94^{a}	42.79^{a}	42.82^{a}	41.65^{a}	42.26^{a}	42.13^{a}	0.23
C18:2*n*6	3.53^{c}	2.96^{b}	2.45^{a}	2.24^{a}	3.41^{c}	3.60^{c}	3.60^{c}	0.12
C18:3*n*3	0.69^{b}	0.62^{a}	0.89^{d}	1.17^{e}	0.77^{c}	0.90^{d}	0.96^{e}	0.04
C18:3*n*6	0.88^{e}	1.06^{e}	0.74^{cd}	0.66^{b}	0.33^{a}	0.79^{d}	0.71^{bc}	0.05
C20:1	0.22^{b}	0.15^{a}	0.28^{c}	0.35^{d}	0.16^{a}	0.15^{a}	0.21^{b}	0.02
C20:2n6	1.22^{a}	1.62^{c}	1.71^{d}	1.85^{e}	1.47^{b}	1.44^{b}	1.28^{a}	0.05
C20:3*n*3	0.91^{c}	0.34^{b}	0.30^{ab}	0.23^{a}	0.28^{ab}	0.26^{ab}	0.23^{a}	0.05
C20:3*n*6	0.68^{d}	0.63^{cd}	0.57^{b}	0.46^{a}	0.57^{b}	0.68^{d}	0.58^{bc}	0.02
C20:4*n*6	2.61^{a}	3.56^{c}	3.13^{b}	2.79^{a}	3.33^{bc}	2.84^{a}	2.78^{a}	0.08
C20:5*n*3	3.76^{b}	8.39^{e}	6.20^{d}	5.91^{c}	3.15^{a}	3.75^{b}	3.26^{a}	0.41
C22:1*n6*	4.56^{e}	1.42^{a}	2.07^{b}	2.93^{c}	4.56^{e}	4.11^{d}	4.66^{e}	0.28
C22:5*n*3	1.19^{a}	1.25^{ab}	1.84^{d}	1.63^{cd}	2.42^{e}	1.57^{cd}	11.49^{bc}	0.09
C22:6*n*3	2.89^{b}	3.23^{c}	2.81^{b}	2.39^{a}	2.51^{a}	2.45^{a}	2.41^{a}	0.07
C24:1	0.56^{d}	0.15^{b}	0.08^{s}	0.05^{a}	0.32^{c}	0.34^{c}	0.34^{c}	0.04
SFA	32.27^{ab}	29.59^{a}	30.92^{ab}	30.95^{ab}	33.58^{b}	32.75^{b}	33.04^{b}	0.40
MUFA	44.82^{ab}	45.33^{ab}	46.37^{ab}	46.79^{b}	43.62^{a}	44.86^{ab}	45.00^{ab}	0.35
PUFA	18.35^{ab}	23.66^{c}	20.64^{b}	19.33^{ab}	18.24^{ab}	18.28^{ab}	17.30^{a}	0.52

Table 4.11 (continued)

Fatty acid	Muscle sample							
	FMF	1COF	2COF	3COF	1PKCF	2PKCF	3PKCF	SEM
n-3	9.43[a]	13.83[c]	12.04[b]	11.33[b]	9.13[a]	8.93[a]	8.35[a]	0.44
n-6	13.48[b]	11.25[a]	10.67[a]	10.93[a]	13.67[b]	13.46[b]	13.61[b]	0.34

*Values are means for triplicate analysis. Mean values within any single row with different superscripts are noted to be significantly different ($P \leq 0.05$)

[§]Muscle samples of fingerlings consuming replacement levels of fish meal at 0% (FMF), 33.3% (1COF), 66.7% (2COF) and 100% (3COF), 16.5% (1PKCF), 33.0% (2PKCF) and 50% (3PKCF)

[¥]Standard error of means

Table 4.11: Fatty acid analysis of liver samples (% total fatty acids)

Fatty acid*	Liver sample§							
	FMF	1COF	2COF	3COF	1PKCF	2PKCF	3PKCF	SEM¥
C12	0.07^{a}	1.06^{d}	1.07^{d}	1.17^{e}	0.49^{b}	0.86^{c}	1.63^{f}	0.10
C14	2.02^{e}	1.93^{d}	1.61^{b}	1.78^{c}	1.64^{b}	1.49^{a}	1.41^{a}	0.05
C16	30.69^{d}	24.50^{b}	22.27^{a}	21.69^{a}	21.68^{a}	26.14^{c}	25.88^{c}	0.69
C18	10.29^{e}	8.88^{c}	7.01^{a}	6.84^{a}	9.30^{d}	7.39^{b}	9.16^{d}	0.28
C20	0.28^{a}	0.33^{c}	0.29^{ab}	0.28^{a}	0.33^{c}	0.32^{bc}	0.34^{c}	0.01
C24	0.22^{c}	0.16^{a}	0.19^{b}	0.15^{a}	0.18^{b}	0.21^{c}	0.32^{d}	0.01
C16:1	1.78^{c}	2.04^{d}	2.11^{de}	2.24^{e}	1.34^{a}	1.57^{b}	1.35^{a}	0.08
C18:1*n*9	30.45^{a}	33.96^{b}	41.00^{c}	43.8^{d}	39.31^{c}	41.00^{c}	40.78^{c}	0.98
C18:2*n*6	1.18^{bc}	1.20^{bc}	1.04^{ab}	0.76^{a}	1.00^{ab}	1.39^{cd}	1.60^{d}	0.07
C18:3*n*3	0.89^{b}	1.25^{e}	0.97^{c}	0.66^{a}	0.97^{c}	1.09^{d}	1155^{e}	0.06
C18:3*n*6	0.21^{b}	0.69^{c}	0.66^{c}	0.65^{c}	0.10^{a}	0.07^{a}	0.08^{a}	0.06
C20:1	0.39^{b}	0.75^{f}	0.63^{d}	0.95^{g}	0.35^{a}	0.54^{c}	0.70^{e}	0.04
C20:2n6	3.57^{e}	2.50^{d}	2.68^{e}	3.57^{e}	1.81^{a}	2.36^{c}	2.24^{b}	0.14
C20:3*n*3	0.01^{a}	0.93^{c}	0.94^{c}	0,92^{c}	0.07^{b}	0.02^{ab}	0.04^{ab}	0.10
C20:3*n*6	1.52^{g}	0.79^{d}	0.61^{b}	0.37^{a}	1.02^{f}	0.86^{e}	0.71^{c}	0.08
C20:4*n*6	0.01^{a}	2.72^{b}	2.68^{b}	2.64^{b}	0.03^{a}	0.02^{a}	0.02^{a}	0.30
C20:5*n*3	5.18^{c}	6.16^{e}	5.47^{d}	3.53^{a}	4.94^{c}	4.38^{b}	4.53^{b}	0.18
C22:1*n6*	6.16^{d}	4.75^{bc}	4.63^{b}	4.95^{c}	10.45^{f}	7.07^{e}	4.30^{a}	0.45
C22:5*n*3	2.83^{e}	2.69^{e}	1.65^{bc}	1.24^{a}	2.33^{d}	1.51^{b}	1.90^{c}	0.13
C22:6*n*3	1.79^{c}	2.56^{f}	2.34^{ef}	2.03^{cd}	2.08^{de}	1.21^{b}	0.89^{a}	0.13
C24:1	0.48^{b}	0.15^{a}	0.16^{a}	0.19^{a}	0.58^{c}	0.50^{b}	0.57^{c}	0.05
SFA	43.57^{c}	36.86^{b}	32.44^{a}	31.91^{a}	33.62^{a}	36.41^{b}	38.74^{b}	0.89
MUFA	33.10^{a}	36.90^{b}	43.90^{c}	46.76^{d}	41.58^{c}	43.61^{c}	43.40^{c}	1.01
PUFA	17.17^{c}	21.49^{d}	19.03^{cd}	16.38^{bc}	14.35^{ab}	12.91^{a}	13.56^{a}	0.69

Table 4.12 (continued)

Fatty acid	Liver sample							
	FMF	1COF	2COF	3COF	1PKCF	2PKCF	3PKCF	SEM
n-3	10.69^{b}	13.59^{c}	11.37^{b}	8.38^{a}	10.39^{b}	8.21^{a}	8.91^{a}	0.42
n-6	12.64^{bc}	12.65^{bc}	12.29^{b}	12.95^{bc}	14.41^{c}	11.77^{b}	8.95^{a}	0.39

*Values are means for triplicate analysis. Mean values within any single row with different superscripts are noted to be significantly different ($P \leq 0.05$)

[§]Liver samples of fingerlings consuming replacement levels of fish meal at 0% (FMF), 33.3% (1COF), 66.7% (2COF) and 100% (3COF), 16.5% (1PKCF), 33.0% (2PKCF) and 50% (3PKCF)

[¥]Standard error of means

CHAPTER 5

DISCUSSION

5.1 Digestibility Trials

Digestibility testing plays a role in determining whether the food source is suitable for a certain subject. It helps in the analysis of feed quality, which facilitates in deciding which feed to be used for maximum nutrient absorption, and ultimately the growth rate of the subject (Reuss, 2001). Nutrient digestibility is usually reported as the apparent digestibility. The difference of a specific nutrient in the faeces and feed is used to calculate the percentage of intake of the nutrient. This method is a simpler way of assessing the digestibility, as it only takes into account the amount of fecal nutrients (Pagan, 1997).

To quantify digestibility of nutrients in a certain ingredient, the digestibility coefficient is used. The particular nutrient is often included in a basal diet at a certain percentage where calculations of the digestibility are then conducted through nutrient measurements in the diet and faeces (Guillaume and Choubert, 2001). The apparent digestibility coefficients for dry matter (ADM) and protein (ADC) are influenced by the type of raw ingredients used, as suggested by Gul, Salim and Rabbani (2007). Substitution of fish meal in the diets lowered both the ADM and ADC for all experimental diets. Plant proteins generally contain higher crude fiber and nitrogen free extract, and are expected to yield lower ADM when compared to alternative

protein sources from animal origins (Sugiura *et al.*, 1998). As the digestibility coefficient is an indicator of how much nutrients are absorbed by the subject, a higher digestibility coefficient is preferable for any type of feed. Due to this reason, the plant protein-based alternative ingredients were partially substituted whereas the animal protein sources were fully substituted.

The control fish meal diet in this study showed a higher ADM level at 92%, compared to that of 87% in juvenile cobia when Peruvian fish meal was used in feeds (Zhou *et al.,* 2004), and 71% when used in feeds for the humpback grouper (Shapawi, Ng and Mustafa, 2007). Soy ADM levels reported for the barramundi was at 56%, while the silver perch could digest dry matter better at 73%, according to Smith *et al.* (2001). Little information is available on the ADM of palm kernel cake for fish; a maximum of 50% digestibility was obtained for the red tilapia when *Trichoderma longibrachiatum* fermented palm kernel cake was used (Iluyemi *et al*., 2010). The ADM levels for soy waste and palm kernel cake fed to *T. tambroides* fingerlings were recorded at 67% and 65% respectively. If based on the reported values for other species, these levels were proven to be acceptable.

ADM results for anchovy-based diets ranged between 89% to 91% for the Senegalese sole and Nile tilapia, (Koprucu and Ozdemir, 2005; Dias *et al.*, 2010), whereas the Atlantic salmon and halibut could digest krill dry matter at a high of 96% (Suontama *et al.*, 2007). Both results were higher than what was obtained for the *T. tambroides* fingerlings, and could be due to the source of the proteins where anchovy and krill are both found in marine environments.

As stated by Tibbetts, Milley and Lall (2006) as well as Koprucu and Ozdemir (2005), variance in ADM is often dependent on the source of ingredients, whereby sources used in the diets have different digestibility values on different species. As nutrient composition of individual ingredients such as crude fiber and ash will affect the ADM (Koprucu and Ozdemir, 2005; National Pork Board, 2008), lower ADM for anchovy and krill could be due to the higher crude fiber and ash content as compared to fish meal. Anchovy waste which was supplied by the local fishermen (Chapter 3.1) contained the bones, head and offal while krill is naturally high in minerals. Dry matter digestibility for poultry meal when fed to the juvenile silver perch was 86% (Allan *et al.*, 2000), and the experimental CO diet did not differ much at 82% ADM levels for the *T. tambroides* fingerlings.

Current results of the soy waste ADC levels were within the range of soy meal ADC acquired by researchers for the Nile tilapia, Atlantic cod, rainbow trout and rohu ranged from 84% to 92% (Koprucu and Ozdemir, 2005; Tibbetts, Milley and Lall, 2006; Barrows, Stone and Hardy, 2007; Noreen and Salim, 2008), even though the soy waste which was used in the present study was the by-product of soy bean processing, containing less protein than soy meal, which is the leftover after oil extraction. Although without any additional information on the ADC for palm kernel cake in fish, it can be deduced that the protein digestibility of the palm kernel cake is still acceptable, as the 87% ADC obtained was within the range for soy meal ADC, and there are values lower in other well-known plant proteins for other species such as wheat meal for Senegalese sole (Dias *et al.*, 2010), cottonseed meal for

rainbow trout (Cheng and Hardy, 2002), corn meal in Nile tilapia (Ribeiro *et al*., 2011) and seaweed meal in trout and Nile tilapia (Pereira *et al.,* 2012).

The chicken offal, anchovy waste and krill diets showed protein ADC levels which were similar to other researches at 89%, 93% and 96% respectively. Maina *et al.* (2002) and Koprucu and Ozdemir (2005) reported protein ADC levels of 90% for anchovy in diets. Shapawi, Ng and Mustafa (2007) obtained protein ADC levels between 86% and 90% while Gaylord, Barrows and Rawles (2008) reported values of 88% for the digestibility of protein from chicken by-products. Tibbetts, Milley and Lall (2006) demonstrated results of 96% protein ADC levels for krill. The high ADC value obtained for fish meal is usually attributed to the more balanced amino acid profile (Asad *et al.*, 2005)

Decrease in preference for the experimental diets when compared with the fish meal diet could be attributed to the palability of the diet that affects feed intake, where the fish meal diet is the most palatable due to the fish meal content (Miles and Chapman, 2006). In the wild, diets of *T. tambroides* also include fruits like palm, fig, and *engkabang*. Since palm kernels make up part of the natural diet for *T. tambroides* (Geoffrey, 2006), the preference for palm kernel cake substituted diet over the soy waste diet was obvious. On the other hand, despite the higher protein digestibility of anchovy waste and krill, the *T. tambroides* fingerlings preferred the chicken offal diet over the former two. As fishes are able to differentiate physicochemical and nutritional properties of a diet (de la Higuera, 2008), it was assumed the chicken offal diet contains

nutrients which are more suited to the diets of *T. tambroides*, and was therefore chosen for the growth study.

Formulation of fish diets require careful nutrient compositions using ingredients at a specific inclusion rate, based on the availability of nutrients and energy in each. Diets are usually formulated based on nutrient figures obtained from literature. During occasions where literature cannot be found for a certain species, information from closely related species is utilized (Maina *et al*., 2002). As *T. tambroides* is still a relatively new species in aquaculture, results from other freshwater fish such as the carp and tilapia are often used as guidelines.

5.2 Growth Studies

The growth of an animal is described as a change in the animal, be it through size (weight and length), tissues, internal chemical compositions or even reproductive abilities. Many factors are involved to achieve maximum growth potential. Dietary input with sufficient, high quality digestible nutrients and environmental input especially through oxygen and water are vital to drive the growth rate (Bureau *et al.*, 2000). The growth rate of any animal is receptive to environmental changes and measurements of the growth rate are often used to gauge the growth performance (Elliott and Hurley, 1995).

In this study, isoproteic diets were employed at 42% crude protein level. Ng, Abdullah, de Silva (2008) conducted a pioneer research on *T. tambroides* fingerlings weighing 21 g, where the dietary crude protein content

needed for optimal growth was at 48%, which is higher than the current research. However, in another study by Misieng, Kamarudin and Musa (2011), *T. tambroides* fingerlings of estimated 5 g in weight could achieve a favorable growth rate after consuming feed with only 40% crude protein content. Therefore based on those results, 42% crude protein used in this study would be sufficient for the growth of the fingerlings.

T. putitora fry of an initial weight of 0.4 g had optimal growth when consuming diets with a minimum crude protein content of 45% (Sawhney and Gandotra, 2010), while bigger fingerlings at 1 g could survive on diets containing slightly less protein at 40% (Hossain *et al*., 2002). Islam (2002) recorded excellent growth results in *T. putitora* fingerlings weighing at least of 10 g being fed supplementary feeds with 30% crude protein, while optimal crude protein content for the pond-reared fingerlings of the same species was noted at 45% (Islam and Tanaka, 2004).

Even as Ng and Andin (2011) reported crude lipid levels for the *T. tambroides* were optimal at 5%, and any higher levels would not affect the growth performance in any way, Ramezani-Fard *et al.* (2011) proved that diets containing up to 20% crude dietary lipid made up mainly of oleic acid would be beneficial for *T. tambroides* over a certain period. Bazaz and Keshavanath (1993), on the other hand, stated optimal lipid content of 11% in feeds for *T. khudree* had a noteworthy effect on the fish and optimal growth could be achieved. Moreover, there are claims that induction of protein sparing could be reached when there is an increase of dietary lipid in the feed. For example,

Bazaz and Keshavanath (1993) observed protein sparring in *T. khudree* with the inclusion of sardine oil in their diets, where the growth was derived from the dietary lipids. In a study by Ng, Abdullah and de Silva (2008), the growth rate of *T. tambroides* was not affected; nevertheless, the increased lipid content provided extra energy which was stored in the form of body fat, particularly in the visceral cavity of the fish. But, this is not always applicable, as at least one study reported that such fish suffered from an adverse effect, such as a decreased growth performance (Chatzifotis *et al.*, 2010).

In the present study, all the experimental diets had significantly different (P=0.000) crude lipid content. Despite this, based on observations of the growth rate for all the diets, fingerlings fed the control diet had a similar growth rate as the other fingerlings on the experimental diets. Thus, it could be speculated that the crude lipid content in the diets did not influence the growth of the *T. tambroides* fingerlings, and the growth would most probably be credited to the crude protein content in the feeds.

Calcium, potassium and phosphorus play important roles in the development of the fish. Limiting calcium and phosphorus would affect the skeletal growth while potassium is essential physiologically (Davis and Gatlin, 1996). The more readily available minerals are calcium and potassium, which are often present in water, and easily absorbed, thus reducing the need for supplementation, however other minerals such as phosphorus, magnesium, iron and selenium should be found as part of the dietary requirements in the feed.

From the results in Table 4.4, all the experimental diets, including the control fish meal diet, contained 5.34-12.72 g/100 g and 2.98-4.64 g/100 g total mineral content of dietary calcium and potassium respectively, of which the minimum requirement for each was 0.5 g and 0.1-0.3 g/100 g dry feed as documented by Chow and Schell (1978) for warm water fishes. Ye *et al.* (2006) reported dietary calcium content of more than 0.33 g/100 g has no significant effect on the growth performance of the fish, which can also be seen in this study from the growth of the *T. tambroides* fingerlings. Phosphorus content in plant material is often lower than from animal sources (Koumi *et al.*, 2011), which explains the lower phosphorus level in the palm kernel cake-based diets when compared to the fish meal and chicken offal diets.

Growth is usually measured over a period of time, and the growth rate is represented in a sigmoid curve. Aquaculturists are often striving to achieve the maximum growth rate of fish, and this has led to the formulation of measurements such as the absolute growth rate, thermal unit growth coefficient and the specific growth rate (SGR). The absolute growth rate is used as a day-by-day measurement of the growth, whereas the SGR expresses the growth over a certain period of time, and is the more popularly used formula (Kjorsvik, Pittman and Pavlov, 2004; Strand, Magnhagen and Alanara, 2011). Protein efficiency ratio (PER) is an evaluation of the protein quality in the diet which contributes to growth of the fish rather than the overall diet. Assumptions formed when using this ratio were that weight gain of the fish

was contributed by the muscle formation, and all protein consumed from the diet was used in muscle tissue synthesis (Hepher, 1988).

The gibel carp has an SGR of at least 2.3% when the alternative protein sources obtained from animals such as poultry by-product meal as well as meat and bone meal were used in the diet (Hu *et al.,* 2008). Studies by Wang *et al*. (2006) on the cuneate drum feed substitution yielded SGR of 1.7%. Significant levels of substitution of animal-rendered proteins could replace fish meal in the diets for the rainbow trout, although the growth rate was not stated and thus it could not be compared with other reported studies (El-Haroun, Azevedo and Bureau, 2009). Despite the numerous positive reviews on animal protein substitution, fish meal diets replaced by alternative proteins were found to be unsuccessful for the growth of mirror carp fingerlings (Emre, Sevgili and Diler, 2003).

There is not much current data on studies of the SGR for palm kernel cake substitution for fish. Nevertheless, the control diet and the experimental diets showed no significant difference between groups for the SGR of the fingerlings and PER of the diets. This indicated that SGR was not affected by the level of inclusion of chicken offal and palm kernal cake (Table 4.6). However, the highest SGR and PER were demonstrated by fingerlings fed 3CO and 2PKC diets. In fact, it was found that experimental diets with alternative proteins could perform as well as the control diet, suggesting a possible replacement of the fish meal diet with the experimental diets.

Despite having comparable crude protein content in their diets for the fingerlings, *T. tambroides* fingerlings in this experiment had a slightly higher SGR than those reported by Misieng, Kamarudin and Musa (2011) when using fingerlings of similar size. Several factors which could influence these results include the feeding time, temperature, nutritional utilization and method (Sanchez-Muros *et al.*, 2003), types of protein sources use (Stankovic, Dulic and Markovic, 2011) and even possibly, the lunar cycle (Haroon and Pittman, 1997). The method of feeding was affected by the feeding time, where, in the wild, the fishes could feed at will, according to their natural feeding rhythm. As the fingerlings in this study were fed at specific periods of time, the most possible explanation for this SGR variance between the current study and the results reported by Misieng, Kamarudin and Musa (2011) could be due the type of protein used in the studies.

The mahseer family is known for its slow growth by anglers, and is further established by the results of several studies on the different *Tor* species. The *T. douronensis* studied by Ingram *et al.* (2005) had an SGR of 0.71%. The chocolate mahseer yearlings recorded a maximum SGR of 0.82%, while the adults were slightly slower at 0.6% (Laskar, Das and Tyagi, 2009). Golden mahseer hatchlings fed diets without any added supplements have a growth rate of 0.41% (Mohan, Bhanja and Basade, 2009). Fingerlings of a different age have a different growth rate (Ingram *et al.*, 2005), where *T. tor* has a reduced growth as it matures (Desai, 2003) agreeing with the reduction of SGR for *T. tambroides* from a high of 6.44% to a low of 0.09% (Ingram *et al.*, 2005). Fingerlings in this research had an SGR of between 0.43% and 0.46%,

and were considered to be acceptable based on comparisons with the other *Tor* species.

The length-weight relationship and the relative condition factor indicate the well-being of the fingerlings. Fulton (1902) stated the condition factor is an indicator of the welfare of a population whereby a factor of more than one would show how healthy a population is in relation to the growth. The condition factor obtained from the growth study was similar to other studies on the carp species such as *Scardinius erythrophtalmus, Carassius auratus gibelio, Tinca tinc,* and *Hemiculter leucisculus* wherein Moradinasab *et al*. (2012) obtained a value 1.0. The *Labeobarbus batesii* had a factor range of 1.0 to 1.3 due to the slightly different seasons in the country (Tomedi *et al*., 2014). A relative condition factor range of 0.98 to 1.09 was noted in *Tor tor* by Saini, Ojha, and Gupta (2014).

The growth coefficient 'b' in this study was less than three, which indicated a negative allometric growth, in which the fingerlings were growing in length, but not yet putting on enough weight (Nehemia, Maganira and Rumisha, 2012), despite the positive relative condition factor obtained. Pioneer studies in Pakistan on *Tor macrolepis* showed a result of 3.14 for the 'b' value for *T. macrolepis*, however the researchers admit it is not indicative of the whole *Tor* species, especially those in different ecosystems (Chatta and Ayub, 2010). In fact, the use the cube law itself has faced opposition in the past due to the lack of attention to the form of the fishes, in which different

stages of life would produce variations that would possibly affect the body (Ricker, 1958).

Chicken offal has been studied as part of the animal rendered proteins substituting for fish meal in a range of fish species, where different inclusion levels yielded different results in different species. Usman *et al.* (2006) realized the humpback grouper juveniles could accept feed containing up to 47% chicken offal, whereas the Nile tilapia and common carp would only consume feed containing a maximum of 20% chicken offal without any detrimental effect on the growth or full body composition (Belal, Al-Owaifeir and Al-Dosari, 1995; Ufodike, Adijetu and Ofor, 2003). Zhou *et al* (2011) substituted up to 60% of fish meal in diets for the juvenile cobia while Rawles *et al.* (2006) was more successful with the hybrid striped bass at 70% fish meal substitution with poultry by-products. This current research shows the *T. tambroides* fingerlings were able to accept full substitution of fish meal with chicken offal (3CO diet) without compromising the growth performance.

In Malaysia, where palm kernel cake is a commodity, extensive usage of palm kernel cake in ruminant feed, especially cattle, can be found, with up to 50% of daily feed comprising of palm kernel cake (Osman, 1986; Osman and Hisamuddin, 1999). Palm kernel cake contains high levels of crude fiber, and while ruminants can easily accept the fiber, palm kernel cake is usually included in diets for non-ruminants such as pigs (Adesihinwa, 2007) and hens (Adrizal *et al*., 2011) to contribute to the bulk of the feed, and at the same time

supply certain amounts of crude protein, energy, vitamins and minerals (Alimon, 2004).

Even though the usage of palm kernel cake is widespread in land animal diets, in the aquaculture sector, palm kernel cake has yet to gain popularity, as there are several limitations which contribute to the low acceptance of palm kernel cake. Still, up to 20% could successfully be substituted into feeds for the hybrid catfish (Ng and Chen, 2002) and in red tilapia diets, up to only 10% inclusion level was used (Iluyemi *et al*., 2010) although Zahari and Alimon (2004) suggested dietary palm kernel cake inclusion could reach up to 20% for freshwater fish. There was no significant difference in fingerling SGR between the palm kernel based diets and the control diet, implying fish meal could be partially substituted with palm kernel cake at 33% substitution level (2PKC diet), yet contribute to the same growth rate as the control diet.

5.3 Amino Acid Analysis

Glutamic acid and aspartic acid were the two most commonly found amino acids, and according to Zuraini *et al.* (2006) glutamic acid is more abundant than aspartic acid. Animal protein sources generally contain low cysteine levels and due to partial substitution of fish meal in the palm kernel cake-based experimental diets, the palm kernel cake diets did not contain as much cycteine as expected. Thus, resulting in lesser amounts in both diets and muscle samples of all the experimental diets (Adeyeye, 2009). Although there was no specific association of amino acids in the experimental diets and

muscle samples, the trend of the amino acids in the diets was similar to that in the muscle samples.

Lysine is an essential amino acid in diets for the growth of freshwater fish. According to Halver and Hardy (2002), the most favorable lysine levels for most fish species lie between 4%-5% of total protein content, which is estimated to be 1.6-2.0 g/100 g feed, based on a 40% crude protein diet. As a positive control, the fish meal-based diet is assumed to contain the optimal protein source. However, the chicken offal-based and palm kernel cake-based diets were not found to be lacking in lysine. Nevertheless, despite the slight variations in lysine content of all the experimental diets, muscle lysine contents were comparable to those obtained from the control diet group. The same observation was demonstrated by Yuan *et al* (2011) on the Chinese sucker (*Myxocyprinus asiaticus*) and Rawles *et al*. (2013) in his study of the hybrid striped bass.

Though herbivorous and omnivorous fish are believed to require less dietary lysine in contrast to carnivorous fishes (Zhou *et al.*, 2007), the different carp species have increased requirements at 2.1 g/100 g for the grass carp (Wang *et al.*, 2005), 1.9 g/100 g for the juvenile Jian carp, and 2.3 g/100 g for the Indian major carp (Ahmed and Khan, 2004). Although the dietary lysine content differed between the experimental diets, growth rate of the fingerlings consuming all the diets had no significant differences among the groups. Lower weight gain resulting in diminished growth would be an indicator of deficiency in lysine. Nevertheless, the chicken offal-based diet

performed just as well as the control diet in the growth of the fingerlings, even though the control diet contained more lysine than the experimental chicken offal diets. Also, it was noted the total essential amino acid content decreased with increased of palm kernel cake inclusion level (1PKC>2PKC>3PKC). Despite this, an excess in dietary lysine might not be as favorable as it might seem, since excess lysine may produce an opposite effect on the growth or health of the fish, as in the case of the Indian major carp (*Cirrhinus mrigala*) (Ahmed and Khan, 2004), the Nile tilapia (Furuya *et al.*, 2012) and the Atlantic salmon (Berge, Sveier and Lied, 1998).

Despite the similar crude protein contents of the control and experimental diets in the study, amino acid compositions in dried muscle samples varied from diets to diet. As in the case of the rainbow trout and the common carp, crude protein studies conducted on these two fishes have shown varied muscle amino acid compositions even though they consumed equal dietary crude protein contents (Harlioglu, 2011; Yuangsoi and Masumoto, 2012). A study on the jundia catfish has also proven the lack of relationship between dietary crude protein content and the muscle amino acids content (Meyer and Fracalossi, 2005). Results from this study agreed with the data obtained for these freshwater fishes, especially the carp, as the *T. tambroides* is part of the Cyprinid family.

In contrast, essential amino acid content in the muscles is affected by the essential amino acid content in the diet. The results obtained from this study correspond with those from the study on the Nile tilapia (El-Saidy and

Gaber, 1998) as well as salmonids (Lall and Anderson, 2005). Despite the fact that there was no obvious interaction between crude protein content in feed and the amino acid profile of the muscles, the quality of the protein source seem to play a part (Lall and Anderson, 2005) where fingerlings that consumed the palm kernel cake-based diets had lower essential amino acid contents even though the diets were partially substituted with fish meal.

5.4 Fatty Acid Analysis

Presence of both palmitic acid, as the dominant SFA, and oleic acid, as the main MUFA, obtained from the GC analysis were consistent with studies by others on the Nile tilapia (Teoh, Turchini and Ng, 2011), sea bass (Bhouri *et al.*, 2010), beluga (Ovissipour and Rasco, 2011) and common carp (Stancheva and Merdzhanova, 2011), although values recorded in this study were slightly higher than the rest. Oleic acid is the most abundant fatty acid in the muscles of *T. tambroides* fingerlings (Ng and Andin, 2011), and despite the variation of oleic acid content in the experimental diets, results showed somewhat little differences between groups, indicating the fingerlings being able to retain oleic acid selectively (Ramezani-Fard *et al.*, 2011).

Regost *et al.* (2003) reported lipid content in fish muscle tissue is affected by the dietary lipids and fatty acids. Based on the results obtained from this study, the dietary MUFA levels had an impact on the MUFA levels in the muscles of fingerlings. Freshwater fishes from warmer climates generally contain more SFA and MUFA (Dey *et al.*, 1993) while marine fishes are richer in PUFA (Vlieg and Body, 1988). This is based on the fact that both

utilizes different food sources, where the marine food source is made up of PUFA-rich plankton while freshwater fishes like the *T. tambroides* consume the vegetation around their habitats, which include fallen jungle fruits, such as wild figs, as well as small insects and worms (Jabeen and Chaudhry, 2011).

High crude lipid content in the diets has long been suspected to cause fatty liver disease in fish (Ramezani-Fard and Kamarudin, 2013). Several studies have been conducted on the juveniles of grass carp, tilapia, as well as the red drum where it was discovered that more than 5% dietary crude lipid levels would cause lipid deposition in the liver. It is this deposition which is detrimental to the health and increased the mortality rate of the juvenile red drum, although for the juvenile grass carp and juvenile tilapia, only the health was affected (Du *et al*., 2005; Gan *et al*., 2009; Jiang *et al*., 2010), whereby the mesenteric fat index and lipid deposition in the liver increased in the juvenile grass carp (Du *et al*., 2005) and the juvenile tilapia had a thin body but tumefaction of the abdomen (Gan *et al*., 2009). Both were regarded as symptoms of mild fatty liver disease. However, there were no significant differences the hepatosomatic index for *T. tambroides* fingerlings fed diets containing up to 19% dietary lipid (Ng and Andin, 2011).

Kamarudin *et al* (2012) proved the usage of palm oil was beneficial to the growth of *T. tambroides* fingerlings, although the long term effect on the development of the fingerlings have not yet been determined (Ramezani-Fard and Kamarudin, 2013). Ng and Wang (2011) have also established increased deposition of fatty acids from palm oil in the gonads and eggs of the female

Nile tilapia. As the fingerlings used in this study were not matured sexually, analysis was not conducted on the gonads. Histopathological studies were not performed on the liver of the fingerlings, and although the diets had increased crude lipid content due to the addition of palm carotene oil, it cannot be verified whether the *T. tambroides* fingerlings were suffering from fatty liver disease, as there were no changes in health or mortality rates throughout the six-month growth study. Still, detection of superoxide dismutase activity in the liver cells indicated potential oxidative stress of the experimental diets on the liver (Ong, unpublished data).

Although a minimum PUFA/SFA ratio was set at 0.45 by Her Majesty's Stationery Office (HMSO) (1997), and an optimal ratio of between 1.0 and 1.5 suggested by Kang *et al.* (2005), too high PUFA/SFA ratios contributed to increased liver cholesterol in human test subjects (Chang and Huang, 1998). As the PUFA/SFA ratio of the muscle samples in this study ranged from 0.52 to 0.79, the flesh of the fish would be able to fulfill the PUFA/SFA ratio requirements when consumed.

EPA levels detected in fingerling muscle samples were higher than other works, whereas DHA levels were lower when compared to similar studies with freshwater fishes (Ramezani-Fard *et al*., 2011; Teoh, Turchini and Ng, 2011). There is speculation of *n*-3 *de novo* synthesis in *T. tambroides* muscles, by enzymes such as elongase and desaturase (Ramezani-Fard *et al.*, 2011), rather than being obtained from dietary sources. Therefore, the fingerlings could synthesize EPA from the dietary ALA, where EPA was the

main contributor for *n*-3 fatty acids, and deposited in the muscles of fingerlings from all diets. Fish having difficulty synthesizing DHA could be due to the low activity of the said enzymes (Navaro *et al.*, 2012) which is partially results from higher than optimal water temperatures (Farkas, 1984).

n-6:*n*-3 ratios in fish muscles have been studied to improve on the benefits of fish to the human consumer. A range of optimal *n*-6:*n*-3 ratios have been recommended by Stancheva *et al.* (2012) to be between 0.2 and 1.5. As with the PUFA/SFA ratio, too high *n*-6:*n*-3 ratios have been proven to be detrimental for health, where it could lead to increased inflammatory response in the tissues, and increased incidents of blood thrombosis (Lenas and Nathanailides, 2011). All muscle *n*-6:*n*-3 ratios of fingerlings in this study varied between 0.81 and 1.62, thus making it suitable for human consumption.

5.5 Further Studies

The pellets in this study were produced using a meat extruder, causing the pellets to be slightly crumbly and not as compact as those produced using a pelletizer. As a good feed conversion ratio and uniform nutrient distribution within every fish is obtained through properly pelleted feeds, a poor quality pellet often results in fine waste production, which dirties the water, in turn affecting the oxygen quality and the filtering processes. Although pelleting technology may help to a certain extent, it is what the pellet contains, such as various binders and emulsifiers, certain protein or fat content, and functional addictives that will optimize the pellet quality (Gehring, Jaczynski and Moritz, 2009).

During the pelleting process, different attractants such as glycine betaine, trimethylglycine, pheromones and free amino acids (Moore, 2008; Ajiboye, Yakubu and Adams, 2012) could be added in order to facilitate the feeding of *T. tambroides* by increasing consumption volume and reducing wastage. Usage of attractants would enable the inclusion of alternative protein sources, and lessen the reliance on fish meal. Since *T. tambroides* has a keen sense of smell (Ogale, 2002) and often feeds on jungle fruits, it would be useful to examine the fruit extracts in attractants for the culturing of the *T. tambroides*.

The growth rate in the aquaculture sector is something which is always focused on. To induce a better growth rate, supplementations are often utilized. Supplementations are always being researched, to increase the nutrient content of feed, to increase the health benefits and thus improve on the growth rate of the fish. Additives commonly used include probiotics (Fuller, 1992). While feed additives provide benefits in terms of growth, certain additives affect the different species differently, and it should be noted the additives, particularly antibiotics, must not harm the environment, especially when the unconsumed feed is left to deteriorate and the additives dispersed into the water body (Ajiboye, Yakubu and Adams, 2012).

Supplementation in terms of enhancing the amino acid content in the *T. tambroides* muscles should be studied if *T. tambroides* were to be cultured as a food fish. Even so, care should be taken to ensure proper amino acid supplementation is included in the diets, as free amino acids are not as

effective as protein-bound amino acids in terms of absorption and availability (Higuera *et al*., 1998; Lemme, 2010). Yet, if amino acid supplementation in feed is conducted correctly, the cost of feed production could be reduced by cutting down on the crude protein content, thus reducing the need for fish meal in feeds too (Gaylord and Barrows, 2009).

As the idea of alternative protein sources in feeds for *T. tambroides* are still relatively novel, more in-depth research could be conducted on how the alternative ingredients affect the microflora of the fish, especially within the digestive tract. Since the microflora generally indicates the overall health of an organism, it would be helpful to monitor any changes in the microflora as it would be practical in determining the physical conditions of the fish (Uddin and Al-Harbi, 2012). It is important to remember some fish can be asymptomatic, yet continue to be a carrier for certain pathogens (Austin, 2006).

CHAPTER 6

CONCLUSIONS

During the digestibility trials, all six experimental diets (FM, SW, PKC, CO, AW and KR) were accepted by the *T. tambroides* fingerlings. The fish meal-based had the highest ADM and ADC values, since fish meal is the most favorable protein source for fish. This was followed by the animal protein substitutes chicken offal, anchovy waste and krill, and finally by the plant protein substitutes palm kernel cake and soy waste.

Based on the preference test, chicken offal and palm kernel cake were selected for subsequent experimental diets for the growth study, although chicken offal had the lowest ADC level within the animal protein category, while palm kernel cake gave a higher ADC level compared to soy waste. The preference test based on the amount of feed consumed during the feeding time was considered significant as the fingerlings would willingly consume the diet.

In the growth studies, three experimental diets were formulated for chicken offal and palm kernel cake respectively, making a total of six experimental diets, and one control diet which was the fish meal diet. Both chicken offal and palm kernel cake could be possible substitutes for the fish meal diet, as these groups had no significant difference in the specific growth rate.

Length-weight relationship and the relative condition factor of the fingerlings indicate a good correlation between the weight and the length of the fingerlings consuming the experimental diets, as well as the relative condition factor shows an overall well-being of the fingerlings. This verifies the experimental diets were good substitutes for the fish meal diet, and potentially be developed in a more commercial scale.

The optimal inclusion level for chicken offal was at 100% substitution of fish meal in the diet (3CO). At this degree of substitution, crude protein levels were maintained at a sufficient level, and at the same time still obtaining a similar growth rate while utilizing the least amounts of fish meal. However, in order to maintain suffice crude protein levels in the diet, palm kernel cake can only be substituted at 33% of dietary protein, and the remaining 67% made up with fish meal (2PKC).

Benefits from the experimental diets include a reduced dependency on fish meal, wherein it has been proven the experimental diets have a comparable nutrient content to the fish meal diet. Fingerlings were healthy and there was no mortality observed in any diet. The ingredients for the experimental diets were easily obtained locally, and are more affordable as compared to fish meal.

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