

CRICKET MEAL AS AN ALTERNATIVE TO FISHMEAL IN  
DIETS FOR AFRICAN CATFISH (*Clarias gariepinus*)

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## ABSTRACT

In Malaysia, extensive farming of African catfish over the past decades has increased the utilization of fishmeal as the preferred aquafeeds owing to its highly nutritional properties. However, various pressures have been put on the fish farming industry to switch to more sustainable diet in order to reduce the dependency on fishmeal as the sole fish feed. Therefore, the aim of this thesis was to evaluate the potential of cricket in diets for African catfish in terms of growth performance, nutrient and amino acids digestibility, immunostimulant properties and susceptibility to oxidative stress. Four feeding trials were conducted to determine the use of cricket meal in African catfish diet. Cricket meal was capable of serving as an alternative protein replacement for fishmeal in the diet of farmed African catfish up to 100% without negatively affecting body composition and feed utilisation. Besides, fish fed cricket meal exhibited significantly higher nutrient and amino acids digestibility than fishmeal-fed fish. Mortalities at 12 days post-challenge with *Aeromonas hydrophila* was significantly decreased at dietary of 35% and 40% crude protein (CP) compared to control (fishmeal diet). From the pathogenic test, *Aeromonas hydrophila* isolation shows the highest count in the intestine of the control group while the lowest in the liver of the fish fed with 35% CP diet suggesting that dietary cricket meal enhanced the innate immune system and survivability of African catfish. Antioxidant activity of catalase was higher in 100% cricket meal substitution with 35% CP compared with fish fed other diets while glutathione S-transferase (GST) and superoxide dismutase (SOD) showed an increasing trend with higher incorporation of cricket meal although no significant difference was observed between all diets. These results signify that cricket meal could be a potential alternative for fishmeal as a protein source in African catfish diet without having any adverse health effect while at the same time improving growth and feed efficiency.

## ABSTRAK

Di Malaysia, penternakan ikan keli Afrika yang telah bermula sejak beberapa dekad yang lalu telah meningkatkan penggunaan tepung ikan sebagai makanan ikan keutamaan selaras dengan kandungan nutrisinya yang tinggi. Walaubagaimanapun, pelbagai tekanan telah dikenakan terhadap industri penternakan bagi menukar kepada sumber yang lebih lestari untuk mengurangkan kebergantungan terhadap tepung ikan sebagai makanan keutamaan dalam makanan ikan. Justeru, tesis ini bertujuan untuk menentukan keberkesanan tepung cengkerik bagi makanan ikan keli Afrika dari segi kadar tumbesaran, penghadaman nutrisi dan amino asid, stimulasi imun serta kesan terhadap oksidasi stres. Empat eksperimen telah dijalankan bagi menentukan keberkesanan tepung cengkerik sebagai makanan ikan keli. Tepung cengkerik mampu dijadikan alternatif dan menggantikan sehingga 100% tepung ikan tanpa memberi kesan terhadap komposisi badan dan penggunaan makanan. Selain itu, ikan yang diberi makan tepung cengkerik menunjukkan penghadaman nutrisi dan amino asid yang lebih tinggi berbanding ikan yang diberi makan tepung ikan (diet kawalan). Kemortalan pada hari ke 12 selepas disuntik bakteria *Aeromonas hydrophila* menunjukkan penurunan yang signifikan bagi ikan yang diberi diet 35% protein dan 40% protein berbanding tepung ikan. Berdasarkan analisis patogenasi, bakteria *Aeromonas hydrophila* menunjukkan jumlah yang lebih tinggi pada usus ikan yang diberi makan diet kawalan manakala jumlah yang paling rendah di bahagian hati ikan yang diberi diet 35% protein. Ini menunjukkan tepung cengkerik dapat meningkatkan sistem imuniti dasar dan kelangsungan hidup ikan keli. Aktiviti antioksidan catalase lebih tinggi bagi ikan yang diberi makan 100% tepung cengkerik dengan 35% protein berbanding ikan yang diberi diet lain. Tambahan pula, Glutathione S-transferase (GST) dan superoxide dismutase (SOD) menunjukkan trend yang meningkat bagi diet yang mempunyai peratusan tepung

cengkerik yang tinggi walaupun tiada perbezaan yang signifikan antara semua diet. Keputusan ini menunjukkan tepung cengkerik mampu menjadi alternatif kepada tepung ikan sebagai sumber protein untuk makanan ikan keli Afrika tanpa menjejaskan kesihatannya dan dalam masa yang sama meningkatkan kadar pembesaran serta keberkesanan penggunaan makanannya

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## LIST OF ABBREVIATIONS

AAC	– Amino acid coefficient
ADC	– Apparent digestibility coefficient
ADF	– Acid detergent fiber
BW	– Body weight
CAT	– Catalase
CFU	– Colony forming unit
CM	– Cricket meal
DCP	– Di calcium phosphate
DE/P	– Digestible energy to crude protein ratio
df	– Dilution factor
EAA	– Essential amino acid
EFA	– Essential fatty acid
FCR	– Feed conversion ratio
FM	– Fishmeal
GE	– Gross energy
GST	– Glutathione S-transferase
Hb	– Hemoglobin
HPLC	– High performance liquid chromatography
HSI	– Hepatic somatic index
Ht	– Hematocrit
HUFA	– Highly unsaturated fatty acid
MAS	– Motile Aeromonas Species
MCH	– Mean corpuscular hemoglobin

MCHC – Mean corpuscular hemoglobin concentration

MCV – Mean corpuscular volume

NFE – Nitrogen free extract

PER – Protein efficiency ratio

PUFA – Polyunsaturated fatty acid

ROS – Reactive oxygen species

RPS – Relative percentage survival

SGR – Specific growth rate

SR – Survival rate

WG – Weight gain

## **CHAPTER 1: INTRODUCTION**

Human consumption of aquatic food products has been increasing throughout the years. Demands for aquatic food products including fish have escalated rapidly due to the annual increase in growth of human population (FAO, 2014). People from rural areas and developing countries have been recognized as having macronutrients (protein and energy) and micronutrients (vitamins and minerals) deficiencies (FAO, 2011). Lack of micronutrients, for example, iodine has been related to goiter and mental retardation (FAO, 2011). According to FAO (2011), the recording of more than 800,000 child deaths per year corresponded to zinc deficiency and almost 2 billion people worldwide are at risk for iron deficiency.

Based on the reports from FAO / WHO consultation expert (FAO & WHO, 2010), there exists convincing evidence to show that fish consumption can reduce the risk of death from coronary heart disease. The supply of high quality protein mainly from fish is vital due to the presence of “good” source of amino acid and fatty acid that could contribute to primary prevention against cardiovascular disease (Kris-Etherton et al., 2009). Improved neurodevelopment had been reported in infants and young children whose mother consumed fish during pregnancy (FAO, 2011).

Aquaculture paved the way in increasing the production of aquatic food products as a result of the declining number of captured wild fish. According to the statistics by FAO (2012) in the Global Aquaculture Production Volume and Value Statistics Database, the world aquaculture production in 2012 was estimated at 66.63 million tonnes for food fish, which was produced for human consumption. On average, aquaculture supplied approximately 9.41 kg of food fish per person for consumption in

2012 although the production distribution is extremely variable across the globe and on all continents owing to the uneven development.

In Malaysia, African catfish is nominated as the highest number of freshwater fish in aquaculture production due to its hardiness and palatability (DOF, 2014). However, feed supply and feed costs are the most expensive components in sustainable aquaculture farming including African catfish. This is due to the fact that fishmeal has been utilized as a major protein source in fish and poultry feed. Hence, alternative protein sources to replace the diminishing production of fishmeal have to be explored to overcome the increasing market price of fish feed and production of sustainable products.

Sustainable resources such as insect meals are believed to be a good candidate for fishmeal replacement given the presence of essential amino acid for the fish and high content of protein (Henry et al., 2015). To date, numerous researches on insect meal have been widely studied as an alternative resource for fishmeal. There is no reliable evidence on the use of cricket meal in formulated fish feed although previous studies have proven that these insects could give promising result in poultry feed (Wang et al., 2005).

When introducing new feed materials, it is important to ensure that the feed does not compromise fish growth and welfare. This is to ensure that the important aspects enabling healthy growth development especially digestibility, stress and immune response of the fish could function normally. For this reason, African catfish was chosen as the species to be researched upon in the current study.

### Objectives of the study

1. To determine the effect of fishmeal replacement with cricket meal on the Growth Performance of African catfish, *Clarias gariepinus*
2. To observe the effect of Digestibility of cricket meal and fish meal diets when fed to African catfish, *Clarias gariepinus*
3. To assess the impact of dietary cricket meal on Immune Function in African catfish, *Clarias gariepinus*
4. To study the influence of cricket meal diet on the Anti-oxidative response and Haematological effect in African catfish, *Clarias gariepinus*

### Hypothesis of the study

1. Cricket meal can replace conventional fishmeal without any negative effect on African catfish Growth Performance
2. Cricket meal diet increases the nutrient and amino acid digestibility of African catfish
3. Cricket meal diet increases the immune response and antioxidant enzyme activity of African catfish

## **CHAPTER 2: LITERATURE REVIEW**

### **2.1 Status of world aquaculture**

#### **2.1.1 Global aquaculture**

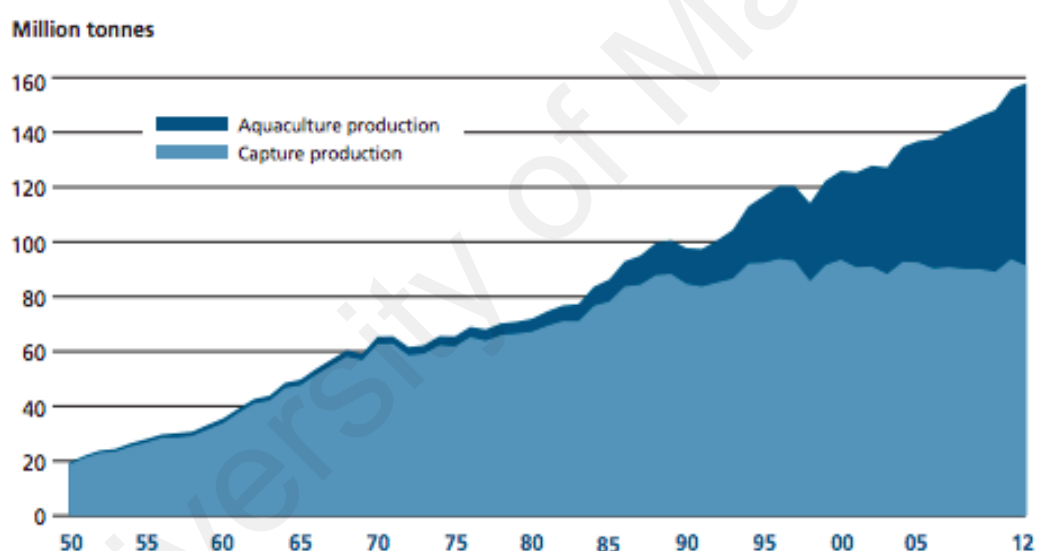
Aquaculture is related to the husbandry of aquatic organisms for profitable purposes. It can be distinguished from captured fisheries by two factors, which include intervention to enhance stock and ownership of the stock (FAO, 2006). In general, it is one of the most important industries that play a vital role in generating economic values in most developing countries including Malaysia. In fact, aquaculture has been recognized as the fastest growth sector in agribusiness globally with nearly 10% annual increase (FAO, 2012).

The contribution from aquaculture for global total fish production increased by 16.5 % from 2000 to 2012, which signifies a good growth development. Asia is the only continent producing more fish in aquaculture than captured fisheries. China has been dominating the chart of top food fish aquaculture production with 69.8% of global production followed by India (7.1%), Vietnam and Indonesia, both at 5.2% and Malaysia, which produced about 0.5% of total fish production worldwide (Table 2.2) (FAO, 2012).

Driven by high demand and environmental factors, aquaculture is and will be dominated by developing countries where a great number of employments, with an estimated of 16 million people especially in rural areas were created through aquaculture business (FAO, 2012). As highlighted in Table 2.1, inland aquaculture registered 2-fold higher production compared to mariculture in which finfish was the main source of production estimated at 66% total value in 2012.

**Table 2.1: World aquaculture production of finfish, crustacean, molluscs and other aquatic species in 2012 from inland aquaculture and mariculture (in live weight equivalent) (FAO, 2012)**

	Inland aquaculture	Mariculture	Sub-total	
Aquatic animals	(Thousand tonnes)	(Thousand tonnes)	(Thousand tonnes)	(Percentage)
Finfish	38,599	5,552	44,151	66.3
Crustacean	2,530	3,917	6,447	9.7
Molluscs	287	14,884	15,171	22.8
Other species	530	335	865	1.3
Total	41,946	24,687	66,635	100



**Figure 2.1: Total world aquaculture productions and capture fisheries from 1950 to 2012. (FAO, 2014)**

**Table 2.2: Top food aquaculture producers in Asia 2012. (FAO, 2012)**

<b>Asia</b>	<b>Tonnes</b>	<b>Percent</b>
China	41,108,306	69.8
India	4,209,415	7.1
Vietnam	3,085,500	5.2
Indonesia	3,067,660	5.2
Bangladesh	1,726,066	2.9
Thailand	1,233,877	2.1
Myanmar	885,169	1.5
Philippines	790,894	1.3
Japan	633,047	1.1
Korea	484,404	0.8
Taiwan	344,404	0.6
Iran	296,575	0.5
Malaysia	283,780	0.5
Turkey	212,805	0.4
Pakistan	142,832	0.2
Rest of Asia	395,334	0.7
Total	58,900,068	100

### **2.1.2 Aquaculture in Malaysia**

As in other countries of the world, aquaculture industry in Malaysia has shown considerable growth in fish production since the 1920s. Nowadays, several culture practices are used to rear aquatic organisms. However, at present, brackish water farming is gaining more prominence than freshwater farming, producing approximately 414,000 tonnes and 107,000 tonnes respectively as a result of substantial demand for land and freshwater for the local population (Abdel-Warith, 2002; DOF, 2014).

Higher demand for animal protein will eventually occur throughout Malaysia over the coming decades owing to the growing population. Many jobs have been created to enhance the production level as well as increasing the economic sectors for the areas involved. According to Anon (2003), an estimate of 20,976 employments were generated in 2003, in which more than 70 percent were recruited in freshwater pond and concrete tank culture systems.

Aquaculture in Malaysia has significantly contributed to the high quality of protein sources for world population as well as food security since the Seventh Malaysia Plan (1996 – 2000) (FAO, 2008). Thereafter, it has been recognized as one of the potential commodity that could enhance export revenues besides rubber and oil palm. For this reason, aquaculture appears to be crucial for the improvement of livelihoods in a community and contributing to poverty reduction.

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**Table 2.3: Aquaculture productions from freshwater culture system by species, 2014 in Malaysia**

Species	Total (Tonnes)
Milkfish	68.00
River catfish	1,648.29
Snakehead	22.28
Jade Perch	16.80
River carp	1,151.57
Big head carp	1,299.57
Grass carp	426.62
River carp	18.05
Freshwater catfish	46,122.01
Carp / Barb	151.83
Goby	13.09
Javanese carp	1,584.66
River carp	334.80
Common carp	1,759.49
Mrigal carp / Indian carp	134.00
Pacu	59.28
River catfish	11,625.54
Climbing perch	207.69
Rohu	2,171.02
Snakeskin gourami	2.83
Freshwater seabass	5.69
Giant catfish	0.19
Carp / Barp	0.02
Black tilapia	4,145.11
Red tilapia	31,203.09
Giant snakehead	1,121.79
Giant freshwater rrawn	398.10
Red claw shrimp	76.48
Miscellaneous	867.40
Total	106,731.41

## 2.2 Biology of African catfish

The genus of *Clarias* has been widespread throughout African continents with several species (*C. mossambicus*, *C. lazera*) and synonymized as *C. gariepinus*. It is typically air-breathing fish species with a scaleless, bony elongated body as well as long dorsal and anal fin. The head shaped like a helmet and colour varies from dark to light brown with olive and greyish shades while the underside of the dorsal range from pale cream to white (Skelton, 2001).

African catfish can tolerate very low oxygen concentrations and survived a considerable amount of time out of water by using specialized suprabranchial organ (Safriel & Bruton, 1984) which is a large paired chamber of branches above the gill arches specifically adapted for air breathing. The ability of the catfish in tolerating extreme condition allows it to survive in moist sand or burrows with an air-water interface (Van der Waal, 1998).

In terms of growth, African catfish has been considered to have rapid growth rate (in length and weight) with ambient condition and habitat (Britz & Pienaar, 1992) as well as density dependent (Hecht & Appelbaum, 1987).

## 2.3 Catfish production in Malaysia

African catfish, *Clarias gariepinus* is a freshwater aquaculture fish, native to the African continents and has been successfully cultured commercially around the globe within the tropical and subtropical environments (Adewolu et al., 2008; Çek & Yilmaz, 2009). This species is well known for its hardiness, high fecundity and ease of production in captivity, besides their capabilities to grow in high densities, which made them an excellent candidate for commercial freshwater fish (Haylor, 1991; Toko et al., 2007). As African catfish provides a good resource for human protein intake, studies on

feed nutrition on this particular species is essential to establish quality feed resources, both practical and economical for the fish farmers.

The global production of catfish within the past decade has been rising exponentially as a result of market demand. However, in 2014, the total production of catfish in Malaysia dropped to approximately around 46,000 tonnes (DOF, 2014), a 0.08% reduction from 2013 (Table 2.3). Commercial production of African catfish in Malaysia has begun since the year 2000 with the development of artificial mass seed production (Tuan et al., 2003). More researches and technical advances in the mass production of African catfish were explored to intensify the fish yield not only for the live market but also for the production of other side products such as catfish fillet. Indeed, the development of African catfish and other aquaculture commodities have created an economic solution for agriculture-based countries such as Malaysia (DOF, 2014).

### **2.3.1 Feed and feeding practices in African catfish**

The primary cost in aquaculture production at present is fish feed, which accounts for 50 to 70 percent of operating cost whereby protein is the limiting factor that influences market price for fish feed (FAO, 2002). Generally, catfish feed comprises of two different types; pelleted feed and farm-made feeds, which differ in terms of formulation and quality (Nguyen & Oanh, 2009; Phan et al., 2009). Farm-made feed generally contains trash fish and other animals, which constituted roughly 50 to 70 percent of overall feed formulation. On the other hand, pelleted feed normally consists of fishmeal, as the main protein ingredients and formulated according to fish nutritional requirements. As these sources are costly especially for the small-scale farmers hence, further studies are required to find cheaper alternatives to replace trash fish and fishmeal as protein sources.

African catfish nutrition has been studied extensively in recent decades. As an omnivorous species, they can utilize plants in their diet efficiently but are more oriented towards animal-based diet. In addition, they can digest dietary carbohydrate effectively compared to other fish (Jantrarotai et al., 1994; Phonekhampheng, 2008). Besides fishmeal, a wide range of under-utilized agricultural products such as rice bran, cotton seed cake, blood meal and groundnut cake have shown positive results in their growth development (Abdel-Warith, 2002). Among other non-conventional sources that have been successfully tested in African catfish feed are hydrolyzed feather meal (Madu & Ufodike, 2004), toad meal (Ayinla, 2007), rumen epithelial meal (Sotolu & Adejumoh, 2008) and pigeon pea meal (Ogunji et al., 2008). Generally, catfish required 8 -10 percent of fishmeal in their diet requirement. However, Phonekhampheng et al. (2009) reported total replacement of fishmeal with golden apple snail meal (*Pomacea canaliculata*) increased growth performance and did not affect feed efficiency of African catfish.

## **2.4 Nutrition requirement for catfish**

### **2.4.1 Protein requirement**

Generally, protein is the costliest component in formulated aquafeed. Fish consume higher percentage of protein compared to other terrestrial animals due to their lower energy requirements (NRC, 1983). However, as African catfish is an omnivorous species, lower dietary protein is sufficient to facilitate their growth compared to carnivorous fish. High dietary protein content will lead to leakage and wastage of nitrogen into the environment. In fact, diets that contain sufficient protein for growth and metabolic requirements together with carbohydrate and lipids to satisfy the energy need will be environmentally friendly and cost effective (Lucas & Southgate, 2012).

Li et al. (2006) has demonstrated that dietary level as low as 24% crude protein (CP) could produce the same growth effect and feed efficiency to the growing fish as traditionally high protein diets fed to channel catfish, *Ictalurus punctatus* (32% - 35%) despite its tendencies to increase body fat content. Therefore, they recommended a dietary crude protein level of 28% with a stocking density of less than 10,000 individuals per acre; daily fed with more than 80 pounds per acre of feed per day. These could generate good growth response and decrease fat content with improved yield quality. On the other hand, in striped catfish, Cho et al. (1985) reported that diet exceeding 45% crude protein (CP) resulted in depressed growth than a lower percentage of CP. Diets with 25%, 30% and 35% CP produced optimal growth rates while 20% and 40% CP supported similar growth response.

Currently, the commercial feed contains highly variable CP contents, which are supplied according to the fish life stages (NRC, 1993). The feed is given in relation to four phases; fry, juvenile, grow out and broodstock. However, some aquaculture producers feed their catfish the same amount of crude protein level throughout the growing season. Highest amount of protein is required in the fry and small fingerlings phases (40% to 50% CP) to facilitate their growth and subsequently, as they grow bigger to a juvenile stage, protein requirement reduces to 36 - 40% CP and finally 25 - 36% CP for grow out phase (Li et al., 2006). As for the broodstock, Quintero et al. (2011) reported that channel catfish fed dietary protein of 32 – 42% did not show any significant effect on spawning, fecundity or fertilization compared to the lower level of protein although it did affect eggs size and biochemical composition.

#### **2.4.2 Essential amino acid requirement**

Apparently, all vertebrates including fish require ten essential amino acids, as they are incapable of synthesizing them and therefore need to be supplied from external sources. According to Lucas and Southgate (2012), animals do not have any specific requirements for protein but they need to fulfill the amino acid requirements. Hence, it is commonly accepted that 'protein requirement' indicates the lowest level of high quality protein needed to comply with amino acid requirements. Generally, the ten amino acids need to be supplied in the diets are: arginine (arg), histine (his), isoleucine (ile), leucine (leu), lysine (lys), methionine (met), phenylalanine (phe), threonine (thr), tryptophan (trp) and valine (val).

There are factors that need to be taken into consideration in regulating dietary requirements. These include; dietary component, physiological need, pathological status and environmental factors (Wu, 2013). Methionine and lysine are the most common limiting amino acids in the practical diet of fish. Some deficient essential amino acid can be met with additional non-essential amino acid. For example, cysteine could replace about 60% of the methionine while tyrosine can spare up to 50% of phenylalanine requirement whereas lysine is the only deficient amino acid that cannot be replaced. Nevertheless, the problems can be overcome by supplementing lysine in the diet to meet the requirements (Li et al., 2006).

**Table 2.4: Estimated essential amino acid requirements of different fish species (Jimoh et al., 2014; NRC, 2011).**

Fish	Essential amino acids (g 100g <sup>-1</sup> crude protein)									
	Arg	His	Iso	Leu	Lys	Met	Phe	Thr	Trp	Val
Channel catfish <sup>1</sup>	4.3	1.5	2.6	3.5	5.1	2.3	2.1	2.2	0.5	3.0
African catfish <sup>2</sup>	3.6	1.2	2.0	3.5	4.8	2.4	4.0	2.8	-	2.4
Nile tilapia <sup>1</sup>	4.2	1.7	3.1	3.4	5.1	2.7	3.8	3.8	1.0	2.8
Common carp <sup>1</sup>	4.3	2.1	2.5	3.3	5.7	2.0	6.5	3.9	0.8	3.6
Rainbow trout <sup>1</sup>	4.2	1.2	2.8	2.9	5.3	1.9	2.0	2.6	0.4	3.4
Japanese eel <sup>1</sup>	4.2	2.0	3.8	4.7	5.1	4.8	5.8	3.8	1.1	3.8

<sup>1</sup> Amino acid requirements according to NRC (2011)

<sup>2</sup> Amino acid requirements according to Jimoh et al. (2014)

Indispensable amino acids are vital in aiding metabolic reactions and protein synthesis as well as being precursors for neurotransmitters, hormones and cofactors (NRC, 2011; Rodehutsord et al., 1997). Therefore, insufficiencies of amino acid could lead to anatomical abnormalities and subsequently produced poor growth. Methionine deficiency has proven to cause lens cataract and growth depression in rainbow trout and lake trout (Cowey et al., 1992; Page et al., 1978). Arginine plays an important role in producing nitric oxide for macrophage control. Thus, deficiency in this amino acid has been reported to suppress growth, increase mortality and fin erosion in rainbow trout (Cho et al., 1992) and channel catfish (Buentello & Gatlin III, 2001; Robinson et al., 1981). Lack of tryptophan is associated with scoliosis and lordosis in sockeye salmon and rainbow trout but this infection is not observed in catfish (Poston & Rumsey, 1983; Wilson et al., 1978).

### **2.4.3 Lipid requirement**

Lipid has been commonly known to supply essential fatty acid (EFA) vitally important in animal metabolism. It is an essential energy source for fish that provide linolenic (n-3) and linoleic (n-6) types of fatty acid (Steffens, 1996). Linolenic acid (n-3) fatty acid is not produced in freshwater fish and thus, must be included in the diet. The amount of lipid needed in the formulated diet is based on the desired quality of fillet, cost, EFA requirement and feed manufacturers limitation (Li et al., 1994). Through enzymatic systems, the fish were able to produce long chain HUFA, EFA and DHA to facilitate other metabolic systems and cellular membrane components (Craig & Helfrich, 2009).

A practical diet for catfish only requires 0.5 % to 0.75% n-3 fatty acid with less than 5 to 6 percent lipid level (Li et al., 2006). Since protein is an expensive source of energy, elevated level of dietary lipid up to 15% will reduce cost of the diet and spare protein without giving any adverse effect to the fish (NRC, 2011). However, higher lipid level will promote excessive fat deposition in the liver resulting to major health problems and affecting market quality (Oliva-Teles, 2012). Other than that, Li et al. (1994) highlighted that reduced disease resistance and immune function were observed in channel catfish fed high n-3 PUFA diet particularly in high temperature.

### **2.4.4 Carbohydrate and fiber requirement**

Carbohydrate is the least efficient energy sources compared to protein and lipid. However in omnivorous fish, especially the warmwater species such as channel catfish, *Pangasius* catfish, African catfish and Nile tilapia, carbohydrate is essential for the energy source other than lipid (Hung et al., 2003; Wilson, 1994). It is stored in fish

tissues and muscle as glycogen and will be utilized during unfavorable condition such as hypoxic condition, food scarcity, and high stocking density (Bonga, 1997). Fish utilize carbohydrate depending on many factors including carbohydrate source, complexity of the molecule, processing treatment and dietary inclusion level (Enes et al., 2009; Krogdahl et al., 2010; Stone, 2003; Wilson, 1994). Commercial feed for catfish commonly contains significant amounts of carbohydrate feedstuff such as corn meal, rice bran and wheat grain that are rich in starch (Wilson & Poe, 1985).

Approximately, 25% of digestible carbohydrate and less than 3 to 4% crude fiber are normally fed to fish (Jafri, 1998). On the contrary, Pantazis (2005) reported that African catfish showed more tolerance to dietary carbohydrate whereby they can utilize up to 32% carbohydrate. In addition, Jantrarotai et al. (1994) have proposed that hybrid catfish (*C. macrocephalus* x *C. gariepinus*) could tolerate semi-purified feed containing up to 50% carbohydrate. Nevertheless, Hien et al. (2010) indicated that striped catfish fed with high dietary carbohydrate resulted in negative growth and requires longer time to reach marketable size. Crude fiber or cellulose is not well digested by catfish hence lowering its level in their diet is sufficient for growth (Li et al., 2006).

#### **2.4.5 Energy requirement**

Energy requirement in fish is species-specific and is stimulated by water temperature and physiological status of the fish (Guillaume, 2001). According to Henken et al. (1986), African catfish requires gross energy and digestible energy of 19 and 14 kJ kg<sup>-1</sup> respectively with protein to energy ratio (P/E) of 27 mg kg<sup>-1</sup>. However the P/E increases with increasing temperature from 25.4 mg kJ<sup>-1</sup> in 24°C to 34.7 mg kJ<sup>-1</sup> at 29°C. Physiological fuel values with the average of 23.9, 17.6 and 39.8 kJ/g of

protein, carbohydrate and lipid respectively are commonly used to measure available energy values in formulated diets (Schulz et al., 2005). Because fish acquire their energy through diet consumed, high energy level may lead to reducing feed intake and insufficient nutrients for optimal growth. Likewise, if the dietary energy is too low, the feed will be inadequate for the fish to satisfy their energy requirements.

#### **2.4.6 Vitamin, minerals and trace elements**

Vitamins are generally small amount of organic compounds required by animal to cater their normal growth, health and reproduction (Robinson et al., 2006). Some vitamins are produced by the body and not needed to be supplemented in the diet. In normal condition, catfish feed are generally supplied with vitamin premix that contain sufficient amount of vitamins and minerals to meet the requirement for the fish and make up the losses from feed processing and storage. The values recommended for channel catfish requirement are commonly recommended for other catfish species including African catfish (Wilson & Moreau, 1996).

The dietary level with normal growth performance and absence of any vitamins deficiency signs were considered to be the minimum requirement for that specific requirement (Wilson & Moreau, 1996). The common deficiency signs such as anemia for the lack of Vitamin B12 and Folic acid (Duncan et al., 1993) as well as Exophthalmia and edema has been recorded due to insufficient Vitamin A in catfish (NRC, 1993).

Minerals are needed for fish metabolism and bone development besides balancing between body fluids and their environment (Robinson et al., 2006). Some of the minerals can be absorbed from the water. However, there are fourteen minerals that are considered as being essential in catfish diets. Similar to vitamins, catfish feeds are

typically supplemented with trace mineral premix and adequate supply of all essential minerals to meet the requirement of catfish (Robinson et al., 2006).

**Table 2.5: Nutritional requirement of catfish**

Catfish species	Nutrients	Recommended level	Major source	References
African catfish	Protein	35 - 40%	Fishmeal	(Giri et al., 2003)
		35%	Casein + Gelatine	(Farhat & Khan, 2011)
	Lipid	> 8%	Palm oil	(Lim et al., 2001)
		10%	Sunflower oil	(Hoffman & Prinsloo, 1995)
	Digestible energy	18.56 kJ g <sup>-1</sup>	Casein, Dextrin, fish oil	(Pantazis, 2005)
Channel catfish	Protein	26 -32%	Various source	(Robinson et al., 2006)
	Lipid	4-6 %		
	Digestible energy	8.5 -9.5 kcal/g protein		
Asian catfish	Protein	36%	Fishmeal, Soybean	(Singh et al., 2012)
	Lipid	8%	Cod liver oil,	(Jafri, 1998)
	Digestible energy	14.1 MJ kg <sup>-1</sup>	Corn oil	

## 2.5 Use of fishmeal in aquaculture industry

Fishmeal is one of the most important and highly utilized protein sources in animal feed production. Its excellent nutrient properties together with the current laws prohibiting the use of most meat meals due to food security has made fishmeal the most used protein source in animal feed manufacturing (Sánchez-Muros et al., 2014). Fishmeal quality varies due to many factors; among them are the conditions of raw fresh fish, temperature of cooking and drying.

Anderson et al. (1993) noted that low temperature during cooking and drying will produce fishmeal with higher quality, classified as low-temperature (LT) type meals but the standard of fishmeal also varies with this type of condition.

Fish used for meal production can be divided into three categories (Ariyawansa, 2000). The first category is fish caught for the sole purpose of fishmeal production and not suitable for direct human consumption due to the high percentage of bones and oil. Major groups of fish in this category include anchovies, sardines, menhaden and smelts (Miles & Chapman, 2015). This group of fishmeal produces high quality of essential nutrients especially well-balanced amino acids and fatty acids. Hence, the price of this high-quality fishmeal (65% crude protein) ranged from approximately \$385 to \$554 per tonnes since the year 2000 which is equivalent to 2.5 to 3.5 times the price of soybean (Miles & Chapman, 2015).

The second category of fishmeal was produced from by-catch or trash fish. Trash fish is commonly caught by trawling where it comprised almost 60% of total catch in inshore water. Among trash fish that have been identified and commonly used for fishmeal productions are goatfish (*Upeneus* sp), short mackerel (*Rastrelliger brachisoma*) and silver belly (*Leiognathus bindus*) (Ramalingam et al., 2014). Generally, trash fish is commonly used for fishmeal production in Malaysia. However, the quality of trash fish is a major concern. According to Edwards et al. (2004), although trash fish contains high protein content, the quality declines without proper storage in ice or chilled water prior to processing. Problems in storage facilities commonly occurred in offshores fisheries, as the boat would be stationed at the sea for 1 to 4 weeks. Hence, trash fish is commonly used as fishmeal in medium to small scales fishmeal plant due to the lower price of fish.

The third category of fishmeal was produced from fish offal from the consumption industries (Ariyawansa, 2000). The protein content of skin, connective tissue and bone is lower than the whole fish. Consequently, the proportion of essential amino acids such as methionine and lysine is approximately 10% lower in fish offal

than that in whole oily fish such as anchovy and pilchard (Hempel, 1993). Crab, scallop and shrimp waste meal are sometimes included in the fishmeal, which then produced higher ash content (Hempel, 1993).

In 2013, the global production of fishmeal from the five top producers plummeted by 11% for the first nine months of 2013 compared to the same period in 2012. This is due to the drop in Latin American production as a result of the El Nino effects and quota restrictions (FAO, 2014). The total production of Peru and Chile as the main fishmeal producers declined to 799,000 tonnes, the worst level over the last five years. Unfavorable weather in Denmark and Norway also drew a set back in fishing, thus limiting its production especially for high quality fishmeal in northern Europe. The limited supply of fishmeal resulted in the hiking of prices of aquaculture feed and is disadvantageous for small-scale fish producers. Consequently, they opted for lower quality fishmeal with lower cost, at the expense of producing poor growth rates (FAO, 2014).

However, besides the uncertainty in the production of fishmeal, there are studies relating to the health effects of consuming fishmeal. Several researches have revealed that fishmeal is capable of causing bovine spongiform encephalopathy (BSE) as it could degrade meat meal and other by-products involved in this disease (Easton et al., 2002). In addition, previous research has been done to determine if fishmeal can be correlated with polychlorinated biphenyls (PCBs) and results from 37 fishmeal and fish feed samples from 6 countries confirmed the presence of PCB contamination (Jacobs et al., 2002).

## **2.6 Nutritive potential of insects for aquaculture diets**

In recent years, there has been an increasing interest in the utilization of insect meals for animal feeding. To compensate the unstable production of fishmeal,

numerous studies have been conducted to find alternative protein sources, which include grains and materials from livestock and poultry remnants. Insects can be considered as a good candidate for alternative protein source in the fish diet since they are well known for their part in natural feed for freshwater and marine fish (Howe et al., 2014). They can be cultured under different environmental conditions as to optimize their nutritional values apart from being a sustainable resource (Premalatha et al., 2011).

Due to their nutritious value (high protein content and sufficient amino acid) and potential to reduce carbon footprint, insects can be considered as a prospective replacement for fishmeal in fish diet. Some of the insects that have been previously studied are listed in Table 2.4. To produce a significant amount of fish feed, insects could be reared in mass production to sustain the demand. They also have different feeding habits and can be fed with by-products acquired from agriculture and other business industries to optimize the economic and environmental expenses (Makkar et al., 2014; Tran et al., 2015; Van Huis et al., 2013).

Culturing insects requires less maintenance and is highly efficient, as they do not require much energy to support high body temperature. In addition, space requirement is one of the most prominent advantages because they do not require large areas or much water to grow compared to crops (Rumpold & Schlüter, 2013). When comparing with other protein source such as plant protein (soybean), insect culturing generally utilize smaller space and require less water for large-scale production (Makkar et al., 2014). Thus, insect production could avoid the expansion of land for soybean cultivation and reduce deforestation. In terms of nutritional value, a study by Yi et al. (2013) has reported that the level of essential amino acid (EAA) of five insects species including cricket (*Acheta domesticus*) were comparable with soybean protein. Soy has been used as fish feed owing to its cheap price but it is not part of carnivorous and

omnivorous fish diet. Insects have the advantage over soy as animal-based protein. However, further research is needed on cost price reduction and investing in up-scaling productivity. Recently, although the production of insects for human consumption has attracted much attention, studies relating to utilization of insects in fish feed have been relatively scanty as compared to poultry.

The mass rearing of insects in quality control substrates will enable the farmers to monitor closely the potential for bioaccumulation of insecticides and natural toxic materials in insects to provide harmless sources for fish feed (Spiegel et al., 2013). Ogunji et al. (2007) have demonstrated the surge of Glutathione S-transferase (GST) activity in the liver of Nile tilapia fed higher level of maggot meal suggesting the potential of having toxic residue or pesticides from the maggot itself or in hen manure originally used in the maggot meal substrate. However, the elevated level of GST does not result in growth deficiency of the fish.

Several studies conducted using insect meals as protein sources claimed the ineffectiveness of insects in producing high feed efficiency due to the presence of chitin. It is commonly presumed that inclusion of chitin in fish diet could lead to reduced protein and lipid digestibility. However, Finke (2007) suggested that insect meal would transport only a small amount of chitin to the fishes and thus, will not affect growth. The growth deficiency is more likely to be stimulated by disproportion of essential amino acid rather than the presence of chitin. On the other hand, Henry et al. (2015) gave a comprehensive review on numerous studies that show the efficiency of chitin in improving fish growth performance.

**Table 2.6: Nutritional value of insects studied for the production of formulated fish diet (Henry et al., 2015)**

Order / Latin name	Common name	Fish tested	Ether extract (% in DM)	Crude protein (% in DM)	References
<b>Orthoptera /</b>					
<i>Zonocerus variegatus</i>	Variegated grasshopper	African catfish	6.87	61.50	(Alegbeleye et al., 2012)
<i>Acheta domesticus</i>	House cricket	Walking catfish	8.5 ± 3.1	57.3 ± 11.8	(Johri et al., 2011)
<i>Locusta migratoria</i>	Migratory locust	Nile tilapia			(Emehinaiye, 2012; Van Huis et al., 2013)
<b>Isoptera</b>					
<i>Macrotermes spp.</i>	Termites	Vundu catfish, African catfish	28.2	20.4	(Sogbesan & Ugwumba, 2008; Solomon & Tiamiyu, 2007)
<b>Lepidoptera</b>					
<i>Bombyx mori</i>	Domesticated silkworm	Rohu, common carp, walking catfish, Nile tilapia	25.7 ± 9.0	60.7 ± 7.0	(Begum et al., 1994; Boscolo et al., 2001; Nandeeshia et al., 2000; Venkatesh et al., 1986)
<b>Diptera</b>					
<i>Hermetia illucens</i>	Black soldier fly	Channel catfish, rainbow trout, Atlantic salmon, turbot	15.6 ± 0.1	40.7 ± 0.4	(Bondari & Sheppard, 1981; Kroeckel et al., 2012; Lock et al., 2014; Sealey et al., 2011)
<i>Musca domestica</i>	Common housefly	Black carp, gibel carp, mudfish, African catfish	31.3 ± 1.6	46.9 ± 4.1	(Achionye-Nzeh & Ngwudo, 2003; Aniebo et al., 2008; Dong et al., 2013; Yixiang et al., 2013)
Order / Latin name	Common name	Fish tested	Ether extract (% in DM)	Crude protein (%)	References

in DM)					
<b>Coleoptera</b>					
<i>Tenebrio molitor</i>	Yellow mealworm	African catfish, common catfish, gilthead seabream, European seabass, rainbow trout	$30.1 \pm 0.7$	$58.4 \pm 0.4$	(Piccolo et al., 2014; Roncarati et al., 2015) (Gasco et al., 2014a; Gasco et al., 2014b)
<i>Zophabas morio</i>	Superworm	Nile tilapia	$38.0 \pm 0.3$	$58.4 \pm 0.4$	(Jabir et al., 2012)

## 2.7 Potential of cricket meal in animal diet

House cricket, *Acheta domesticus* is one of the most important species involved in pet trade. They have been kept domestically as pets since the 12<sup>th</sup> century by the Chinese people who used them in cricket fights (Suga, 2006). At present, they are the most abundant cricket species in pet stores, supplied as fish baits or supplementary feed for ornamental fish and reptiles due to their high nutritional value. However, entomologists have reported that *Acheta domesticus* is susceptible to the cricket paralysis virus called *Acheta domesticus* Densovirus (AdDNV) which affects major tissues of the cricket nymph resulting in paralysis and eventual death when they enter adult phase (Szelei et al., 2011).

In addition, Mormon cricket has been studied in broiler feed for the past three decades. This species is widely distributed in western North America particularly in rangeland areas (Lorch et al., 2005). Ramos-Elorduy (2008) has demonstrated that Mormon cricket (*Anabrus simplex*) could replace fishmeal and soybean entirely in broiler diets without affecting growth. Likewise, prior to that, DeFoliart et al. (1982) and Nakagaki et al. (1987) also reported significantly better growth in broiler fed Mormon cricket-based diet than corn diet. To date, studies investigating crickets as potential fish feed in animal feed is scarce although they can be found to be widespread globally.

*Gryllus testaceus* is a species that is abundantly found in China and has been studied as poultry feedstuff. According to Wang et al. (2004), broiler growth did not show any adverse effect when up to 15% *Gryllus testaceus* was partially replaced in the diet. On the other hand, *Gryllus bimaculatus* or black field cricket (Figure 2.2) is commonly distributed in a wide variety of environmental condition. They can tolerate a

wide range of diets, making them a hardy species and is well known for the chirping noise from the males to attract females.



**Figure 2.2: Black field cricket (*Gryllus bimaculatus*)**

## **2.8 Digestibility of feed by fish**

Digestibility value is an important factor in measuring the degree to which the nutrients from the ingested food, which can be digested by the fish. It also provides balance ingredients for the formulation of the diet. Feeding approaches, method for collecting faeces, diet formulation and digestibility measurement are the essential elements in determining digestive value of nutrients in any ingredients (Glencross et al., 2007). Hepher (1988) disclosed the three main factors that influence the digestion process in fish. The first factor is food ingested by the fish and the level to which it is affected by the effect of digestive enzymes. Second factor is the activity of the digestive enzyme and the third is duration of the feed susceptible to the action of digestive enzymes.

## **2.8.1 Methods used for evaluating digestion in fish**

### **2.8.1.1 Direct method**

A direct estimation method involved measuring all of the nutrients consumed and all excreted in faeces. This method has an advantage of high accuracy but care must be taken when collecting the faeces to prevent any leaching of nutrients into the water. Besides, fish are more prone to stress because the faeces are collected by stripping and this will affect the digestive and metabolic process leading to unreliable estimates of digestible value (Lovell, 1989; NRC, 2011).

This technique relies on quantitative measurement of ingested (feed) and egested (faeces) material by using this equation:

$$D\% = \frac{(I - E)}{I} \times 100$$

Where D% = % of apparent digestibility

I = Amount of nutrient ingested

E = Amount of nutrient egested

### **2.8.1.2 Indirect method**

The indirect method involves the collection of faecal sample that is unaffected by feed materials and the use of indigestible marker for the assessment of digestibility (NRC, 2011). This method is frequently used by fish producers to evaluate digestibility for most fish species. The indigestible marker is commonly included in the feed at low concentration or it could be one of the feed components. Typical marker used in fish feed that is non-toxic are chromic oxide (Cr<sub>2</sub>O<sub>3</sub>), yttrium oxide (Y<sub>2</sub>O<sub>3</sub>) and titanium dioxide (TiO<sub>2</sub>) (NRC, 2011).

The amount of feed and faeces marker is presumed to remain constant during the experimental period and the indigested marker will appear in the faeces excreted by the fish. The ratio of the marker in the feed and faeces determines the digestibility of diet and energy. The indirect method is less stressful to the fish as the faecal matters were collected in holding or rearing tank environment (Bureau & Cho, 1999).

### **2.8.2 Protein and amino acid digestibility**

An extensive amount of research has been directed to estimate the digestibility of protein in many ingredients in animal and plant feedstuff. McGoogan and Reigh (1996) stated that protein digestibility is excellent in high dietary protein (> 60%) and low fiber (< 2%) content. The digestion coefficient for crude protein for high protein feedstuff in channel catfish is in the range of 75% to 95% (NRC, 1993). Protein digestibility of ingredients such as poultry offal meal, feather meal, blood meal and gluten meal is comparable to fishmeal ranging in between 85 - 99% apparent digestion coefficient (ADC) (Allan et al., 2000).

Shahzad et al. (2006) reported that crude protein ADC in plant ingredients (corn and wheat) is higher than animal ingredients (feather meal) when tested in *Labeo rohita*. However, the ADC of animal and plant origin in catfish are similar as reported by Fagbenro (1996). Pantazis and Neofitou (2004) recorded protein digestibility (70 – 86.6%) for fingerling African catfish weighing approximately 21 g fed with diets consisting the mixture of algal and blood meal.

Silkworm meal has been proposed to be a suitable replacement for fishmeal in *Clarias batrachus* diet. Crude protein digestibility of silkworm pupae meal was found to be comparable to fishmeal (Borthakur & Sarma, 1998). However, reduced

growth was observed when fishmeal was totally replaced by grasshopper in both African and walking catfish, which, could be attributed to low protein and lipid digestibility (Alegbeleye et al., 2012; Johri et al., 2011).

### **2.8.3 Lipid digestibility**

Lipid is a source of energy that is almost digestible by fish due to the presence of 2.25 times higher energy compared to carbohydrate (Robinson & Li, 2007). Hossain et al. (1992) suggested that water temperature and dietary lipid are the factors that stimulate the lipid digestibility value. Increasing ratio of saturated fatty acid will negatively influence lipid digestibility in warmwater and coldwater fish species (NRC, 2011).

A range of 76 – 97% of lipid ADC has been reported for channel catfish fed various sources of fat and approximately 83 – 88% of lipid ADC was observed in African catfish fed cooked sesame-based diet (Jimoh et al., 2014). In Rohu, lipid digestibility did not show much difference in the results for animal and plant diet, which was similarly reported in African catfish (Hossain et al., 1997; Mohanta et al., 2006).

Lipid utilization is species-specific. This can be observed in turbot whereby growth performance and lipid ADC was reduced as dietary lipid increased up to 15%. Nevertheless, a different outcome surfaced in trout and Atlantic salmon fed with higher than 30% lipid, where positive growth and high lipid ADC were observed (Guillaume, 2001).

### **2.8.4 Carbohydrate digestibility**

Starch is the most abundant and least expensive non-protein energy source available in catfish diet. Studies have proven that freshwater warmwater fish were able

to digest higher level of carbohydrates than the coldwater or marine fish due to the increasing level of intestinal amylase activity in warmwater fish species (Stone, 2003). An experiment conducted by Cho and Slinger (1979) has proven that channel catfish has the capability to digest up to 65% uncooked starch compared with rainbow trout which only tolerated less than 50% uncooked starch. Wilson and Poe (1985) suggested that cooked corn starch was beneficial for channel catfish as it is 38% more digestible than corn prepared using pellet mill.

It appears that starches are not as digestible as lipid in channel catfish. Increased dietary carbohydrate will reduce carbohydrate digestibility. Grains including corn, rice bran and wheat grain are the most utilized source of carbohydrates, which contribute to a carbohydrate digestibility ranging from 60 to 70 percent in channel catfish (Cruz, 1975).

Starch digestibility varies according to species and eating habits. Some fish such as Atlantic halibut cannot tolerate high level of starch, hence reduce carbohydrate digestibility from 84% to 53% with increasing dietary carbohydrate inclusion from 8% to 17% (Grisdale-Helland & Helland, 1998). Krogdahl et al. (2005) stated that herbivorous fish could digest non-starch carbohydrates as opposed to carnivorous fish owing to the presence of sufficient gut microbiota.

## **2.9 Nutritional strategies in maintaining health**

The importance of nutrition playing a main role in preventing hazardous health effects on human and other animals including fish has been long established over the years. The past three decades has seen increasing interest in studying the connection between nutrition, immune response and disease resistance in fish (Lim & Webster, 2001). Due to the initial lack of understanding on the immune system of fish, initially,

very little success was achieved in fish nutrition research. However, in some studies, there are evidence that could relate the connection of dietary nutrition to immune function and disease resistance. In addition, the appearances of immunostimulants, nutrient bioavailability and interactions as well as feeding regulation are also important factors that could affect fish health (Lim & Webster, 2001).

The growing production of commercial aquaculture from a high demanding market has also resulted in increasing casualty due to infectious diseases. This is one of the major issues associated with economic loss in aquaculture industry (Barman et al., 2013; Lovell, 1996). Primarily, the method of combating disease was focused on treatments whereby antibiotic-resistance strains of bacteria were used for the infected fish. However, problems arise when the administration of antibiotics were not carried out in a proper way. The infected fish did not consume the medicated feed during the period of their illness, hence a proper dose is not well administered. Besides, the use of chemotherapeutics is costly for use in ponds and excessive usage will contaminate the environment (Anderson, 1992).

For this reason, scientists have promoted preventive measures rather than treatments to preserve animal health and disease resistance (Ogier de Baulny et al., 1996). The development of vaccines have shown positive result for some fish species but due to its high cost, it is not an effective solution since there are many other commercial diseases in the aquaculture industry (Raa et al., 1992). Therefore, other methods such as the establishment of immunostimulants through modification of the diet and feeding system was introduced (Jadhav et al., 2006; Sakai, 1999).

### **2.9.1 Health control of African catfish**

The earliest work on African catfish health control have been evaluated by numerous researchers since 1987 through Huisman and Richter (1987) and Boon et al. (1987). During the early years major infestation by protozoan and metazoan such as *Costia*, *Chilodonella* and *Dactylogyrus* are commonly found in African catfish cultured in tropical pond. Treatments by organic phosphate esters (Bromex, Dipterex and Masoten) (Viveen et al., 2013) are normally applied through the fish to control the spreading of the infections. However, in cultured hatchery condition, antibiotics such as chloramphenicol and oxytetracycline are commonly supplied in the diets as curative agents. Those infections are predominantly associated with environmental changes such as temperature, water quality and fish handling.

Another symptom that could be destructive to fingerlings African catfish is Ruptured Intestine Syndrome (Boon et al., 1987) or open belly disease where it affected the fingerlings during 5 to 8 weeks post-hatch when given at high feeding level. The disease would cause ruptures in the caudal part of intestinal part and consequently lead to mortality. Besides, other disease such as broken head disease is frequently observed in broodfish kept in high-density tank. It will lead to inflammation of skull due to lateral skull break. The high waste production from feed due to low appetite of fish was the main cause for the outbreak of this disease (Huisman & Richter, 1987).

### **2.9.1 Immunostimulant in fish diet**

Immunostimulant is a favorable non-nutritive dietary supplement that could enhance disease control in fish by increasing innate immune response against pathogenic bacteria (Jadhav et al., 2006; Lim & Webster, 2001). In comparison to

mammals, fish is more dependable towards nonspecific defense mechanisms, in which immunostimulant plays a major role in managing the health strategies of fish (Barman et al., 2013). Beta glucans ( $\beta$ -Glucan), levamisole, peptidoglycan, chitin, chitosan yeast and vitamins are among the most studied products and have proven to be effective as an immunostimulant in fish (Dautremepuits et al., 2004b; Kawakami et al., 1998; Villamil et al., 2003). They can be applied to the fish through injection, bathing and orally of which the latter method is the most practical and commonly used (Philip et al., 2001).

Dalmo et al. (1996) highlighted that diet with inclusion level of 50 - 200 $\mu$ g/ml  $\beta$ -Glucan increased survival of Atlantic salmon following challenge with *Vibrio anguillarum* and *Vibrio salmonicida*. A similar result was observed in gilthead sea bream with oral administration of 500mg/kg  $\beta$ -Glucan while fish fed with levamisole increased phagocytosis, respiratory burst, lymphokine production and complement activation (Mulero et al., 1998). The use of levamisole; an antihelminthic chemical compound in fish as possible modulator to enhance nonspecific immune activity and resistance towards pathogenic bacteria has also been positively observed in carp and rainbow trout (Findlay et al., 2000; Gannam & Schrock, 1999; Siwicki, 1990).

Chitin is one of the most abundant biopolymer mostly found in the shells of crustacean, insect's cuticle and cell wall of fungi. Chitosan is the deacetylated product derived from chitin (Knorr, 1984). Both polymers have proven to have significant effect in boosting immune response for some fish species. According to Vahedi and Ghodrati-zadeh (2011), administration of 10 – 50 mg/kg chitin in rainbow trout diet enhanced their immune system. It could also increase respiratory burst, phagocytic and cytotoxic activity in gilthead sea bream (Esteban et al., 2001). Chitosan has been utilized as an immunostimulant in rainbow trout, brook trout and gilthead sea bream to

protect them against pathogenic bacteria and improve nonspecific immunity (Anderson & Siwicki, 1994; Cuesta et al., 2003; Siwicki et al., 1994).

### **2.9.2 Pathogenicity of *Aeromonas hydrophila* fed immunostimulant diet in fish.**

Motile aeromonad septicemia (MAS) is a disease commonly infecting warmwater freshwater fish species and also could occur in brackish water. *Aeromonas hydrophila*; a gram-negative bacteria is one of the three species besides *Aeromonas sobria* and *Aeromonas caviae*, that are associated with MAS (Lim & Webster, 2001). These organisms, which are well known as facultative opportunists, normally infect fish that are exposed to the environmental stressor, other parasitic infection, skin injury and even stress during transport or handling. Pathologic conditions associated with *Aeromonas hydrophila* include dermal ulceration, hemorrhagic septicaemia, fin rot disease and scale protrusion disease (Cipriano et al., 1984).

Numerous studies on immunostimulant feed supplement in plant extract, animal origin and other biological substances have been recorded over the years. Dietary intake of chitosan has shown the highest level of survival in common carp compared to levamisole and chitin when challenged with *Aeromonas hydrophila* (Gopalakannan & Venkatesan, 2006). The same effect was observed in rainbow trout with the supplementation of diets containing chitosan-oligosaccharides (Lin et al., 2009).

Many studies have been done to investigate the use of plant as immunostimulant. The incorporation of medicinal plants, *Ficus benghalensis* and *Leucanea leucocephala* in African catfish diet has proven to have antibacterial activity against *Aeromonas hydrophila* (Verma et al., 2013). Inclusion of fucoidan in the diets of African catfish has proven to enhance humoral and cellular immunity as well as increasing the resistance against pathogenic bacteria under the condition of heavy metal pollution (El-Boshy et

al., 2014). Barros et al. (2002) has demonstrated that supplementation of soybean meal with 203 - 283 mg /kg of iron is sufficient to maintain normal immune response and enhance protection against *Edwardsiella ictaluri* bacteria in Channel catfish

However, very few studies on immunostimulant have been done by using animal source of diets. Animal extracts such as tunicate has demonstrated to be efficient in improving survival rates and increasing phagocytosis level of eel following injection with *Aeromonas hydrophila* (Davis & Hayasaka, 1984).

## **2.10 Oxidative stress biomarkers and antioxidant activity in fish**

A living organism is susceptible to a wide variety of stress when in contact with diverse environmental condition. The presence of stress in environmental conditions will normally activate reactive oxygen species (ROS), generally initiating as side products of tissue respiration. Continuous exposure to stressful states will subsequently elevate the ROS-mediated oxidative damage (Stoliar & Lushchak, 2012). Lushchak (2011) has proposed that oxidative stress occurs when increased ROS concentration disturbs the cellular metabolism as well as its regulation, and continuously impairs the cellular components.

The trigger of oxidative expression could cause antioxidant response leading to the expression of genes encoding antioxidant enzymes and rising of ROS scavengers (Stoliar & Lushchak, 2012). Despite that, in some situation, level of ROS exceeds that of their depletion causing oxidative stress (Halliwell & Gutteridge, 1999; Livingstone, 2001). Starvation has been described as one of the factors that are responsible for the increasing ROS generation in animal due to unaccustomed feed leading to food deprivation (Domenicali et al., 2001). It appears that there are several antioxidants that play significant roles in modulating antioxidant defense in animals. The antioxidant

enzymes such as superoxide dismutase, catalase and glutathione S-transferase are amongst the enzymes that are important in protecting the living organism from ROS through their defense mechanism.

### **2.10.1 Catalase**

Catalase (CAT) enzyme commonly found in peroxisome, is responsible for catalyzing the decomposition of hydrogen peroxide ( $H_2O_2$ ), producing water and molecular oxygen in addition to defending cell against oxidative damage (Sun, 1990). In the mitochondria particularly in the liver of rat, catalase activities were reported to decrease with increasing age (Tiana et al., 1998). To date, studies related to antioxidative enzymes in fish nutrition are still lacking especially in determining CAT activities.

Ogunji et al. (2011) reported an elevated level of CAT activities in the liver of carp fed with maggot meal. This is attributable to the high lipid content in maggot meal and also correlated with higher growth rates and good feed efficiency. This is consistent with the result found by Rueda-Jasso et al. (2004) in which they found that high dietary lipid resulted in increasing CAT activities. On the other hand, moderate increases in CAT activities were reported in starved dentex liver (Morales et al., 2004) but no significant effects were found in rainbow trout (Hidalgo et al., 2002). Nevertheless, starvation studies on sea bream shows that CAT activities decreases on partial food restriction and fasting fish (Pascual et al., 2003).

### **2.10.2 Superoxide dismutase**

It is well established that superoxide dismutase (SOD) rapidly converts superoxide anion ( $O_2^-$ ) into less harmful  $H_2O_2$ , and later catalyzed by CAT and

Glutathione peroxidase (GPx) into water and oxygen. According to Camougrand and Rigoulet (2001), increasing mitochondrial oxygen consumption enhanced the production of  $O_2^-$ . Hence, it is considered as one of the most responsive enzymes for acknowledging oxidative stress in animals (Winston & Di Giulio, 1991). Superoxide dismutase activities in rat liver is comparable to that of CAT whereby decreasing levels were found in aging rats (Tiana et al., 1998).

The activity of many antioxidant enzymes including CAT and SOD is influenced by the presence of copper in the diet. Copper deficiency in the diet will lead to a decline in SOD and cytochrome oxidase activity resulting in the formation of cataract in fish (Guillaume, 2001). Shao et al. (2014) observed higher levels of CAT and SOD in black sea bream fed on high levels of soybean and protein concentrate diet than the control diet. Furthermore, studies with gibel carp fed with selenium supplement has shown to decrease the amount of serum SOD (Han et al., 2011).

### **2.10.3 Glutathione S- transferase**

Glutathione S-transferase (GST) plays a crucial role in detoxifying endogenous toxic metabolites and many environmental contaminants together with tripeptide glutathione (GSH). Additionally, it also functions in phase II lipid peroxidase detoxification (Leaver et al., 1993; Nimmo, 1987). It is important as a protection for living organisms against peroxidative damage and useful in detoxification of toxics such as pesticides, oil and other hydrocarbons (Fisher & Burggren, 2007).

Fish has been commonly used in monitoring the biomarker of water and contaminant due to their sensitivity towards polluted environment (Amado et al., 2006a; Amado et al., 2006b). Ogunji et al. (2007) reported that the utilization of maggot meal in tilapia and common carp did not show any significant changes in GST activities

corresponding to fishmeal fed fish suggesting that maggot meal did not contain any compound that could stimulate the generation of ROS. In order to ensure that the feed materials given to the fish do not contain any harmful substances, studies in determining GST activities in nutritional analysis is essential.

#### **2.10.4 Studies on oxidative stress in catfish**

Numerous studies have attempted to investigate oxidative stress in catfish species. Considerable amount of studies primarily concentrated on the effect of feed additive to enhance antioxidant enzyme have been studied extensively. Several feed additives such as Quercetin has shown to increase antioxidant status in silver catfish due to the presence of flavonoid which contain antioxidant properties and prevent lipid peroxidation (Pês et al., 2016). Furthermore, the inclusion of 0.3 g per kg of organic selenium in environmental copper toxicity could increase hepatic Glutathione peroxidase (GPX) thereby protecting cell membranes against oxidative damage in African catfish (Abdel-Tawwab et al., 2007).

Besides feed, studies on environmental contaminant by looking at the oxidative stress and antioxidant defense enzymes in African catfish have also attracted much attention. Studies by Ibrahim and Harabawy (2014) examined the impact of carbofuran in *Clarias gariepinus* and reported the disturbance in antioxidant defense system. The result shows that exposure to carbofuran induced significant decrease in antioxidant enzyme (CAT, SOD, GST and GPX) in African catfish organs.

## **CHAPTER 3: GROWTH PERFORMANCES AND AMINO ACID ANALYSIS OF AFRICAN CATFISH FED VARYING LEVELS OF CRICKET MEAL DIETS.**

### **3.1 Introduction**

The increasing world population boosted the demand for protein sources which had inevitably impacted the aquaculture industry to produce high-yielding fish with lower cost. Fish feed constitutes 80% of operating cost in aquaculture whereby protein is the limiting factor that influences the market price of fish feed (Shepherd & Jackson, 2013; Tacon et al., 2011).

Simultaneously, as aquaculture industry has been considered as the fastest-growing industry in the animal food sector (FAO, 2014), studies on sustainable resources to expand this industry are essential. Apart from the aquaculture sector, terrestrial animal production is also a major consumer of fishmeal (Mallison, 2013), which led to its high cost of production due to declining amount of wild-caught fish available in its production process.

Insects meal particularly crickets can be used successfully as sustainable sources for animal feeds, while they are readily available around the world. Since there is a dearth of information on cricket meal as feed resources for fish, this study aims to experimentally investigate the effect of varying level of cricket meal (CM) as fishmeal (FM) replacement in African catfish diet on their growth performances, feed utilization, body composition and survival rate.

### 3.2 Literature review

The use of other sustainable sources as alternatives to FM in relation to the production of formulated feed has reduced the dependency on FM. Due to the high price of FM and increasing demand from aquaculture production has led to the establishment of research in insect protein for aquaculture and livestock (Barroso et al., 2014; Henry et al., 2015). Insect meal such as grasshopper meal (Ojewola et al., 2005), termites meal (Fadiyimu et al., 2003), superworm meal (Jabir et al., 2012) and maggot meal as well as molluscs such as garden snail, and plants feedstuff (Francis et al., 2001) have been identified as successful alternatives for FM replacement.

Crickets have been commonly used as complementary food source for ornamental fish, reptiles as well as in poultry industry. Previous studies have reported that field crickets gave a promising result as a soybean replacement in broiler diet (Ramos-Elorduy et al., 2002). Although these investigations reported interesting results in the poultry industry, no attempt was done to explore the potential of crickets as formulated diet for fish.

Nevertheless, there are some trials that have already been done using Orthoptera insects in fish feeding. According to Alegbeleye et al. (2012), 13% dietary inclusion of variegated grasshopper could improve growth performances of catfish. However, total replacement of grasshopper meal from fishmeal reduced growth of African and walking catfish (Alegbeleye et al., 2012; Johri et al., 2011). Other insects that have been tested in catfish are mealworm which could substitute up to 40% of fishmeal (Ng et al., 2001) while maggot meal were able to replace 75 -100% of fishmeal in African catfish diet without affecting their nutrient efficiency (Fasakin et al., 2003; Madu & Ufodike, 2004) Cricket can be found in abundance in the tropics, easily cultured and mass harvested in

controlled environments with the benefit of low cost of production, therefore, it can be a suitable candidate for alternative protein resources for animal diet.

African catfish is regarded as one of the most cultivated and popular freshwater fish in Malaysia and is increasing in demand by the growing local market. Thus, many researches have highlighted the study on different aspects of this particular fish in order to enhance its growth and increase productivity.

### **3.3 Material and method**

#### **3.3.1 Experimental diet**

Adult live field crickets (*Gryllus bimaculatus*) used in the formulated diet were purchased from a local field crickets farm. The crickets were fed chicken feed in the form of mash throughout their life cycle. They were then transported to the laboratory and refrigerated at -20°C before being dried in an oven at 60°C. The dried crickets were then grounded with dry feed grinder and kept in a cold room (4°C) prior to proximate analysis. All the raw materials for the ingredients including fishmeal, corn meal, rice bran, soybean, vitamins, mineral and Di calcium Phosphate (DCP) were purchased from a local livestock feed center.

Formulation and chemical composition of all the experimental diets and feed ingredients were tabulated in Table 1. Various inclusion percentage of CM were formulated as 0% (control), 25%, 50%, 75% and 100% inclusion level to yield an isonitrogenous content of 28% crude protein dry matter and isoenergetic content with approximately  $\pm 19$  kJ/g. Winfeed 2.8 version software was used to formulate the feed. All dry ingredients were grounded in a hammer mill (Disk Mill, FFC 454). Vitamins, minerals and DCP were mixed thoroughly with the dry ingredients and water was added to the mixture prior to being pelleted into sizes of 1mm diameter using a mini pelleting

machine (KCM, Y132M-4). The wet pellets were then dried in an oven at 70°C for 24 hours and later stored in a cold room (4°C) until used for feeding.

### **3.3.2 Experimental Fish and set-up**

African catfish were purchased from local farmers and transported to the Freshwater Aquarium Laboratory located in the Institute of Biological Sciences, Faculty of Science, University of Malaya. Two hundred and twenty five (225) African catfish fingerlings were randomly divided into five groups, each in triplicates of 15 fishes per tank with average weight of  $4.00 \pm 0.8\text{g}$ . All the fishes were acclimatized to natural environment condition for 2 weeks prior to the feeding trials and fed with a commercial diet (Dindings) twice per day at 0900h and 1500h during the acclimatization and throughout the experiment, and uneaten food was collected and dried after feeding to determine the feed intake. Water quality was monitored regularly and any mortality was recorded. Fifteen plastic tanks (3' x 2' x 1') with the capacity of 100 liters of water with closed re-circulation system were used in these feeding activities. The tanks were equipped with top filter pump at a flow rate of  $20 \text{ L min}^{-1}$  and aeration with the air-stone diffuser was provided in each tank for circulation of dissolved oxygen. Tap water, which was treated with anti-chlorine, was used and 20-30% of water was replaced once in two days to maintain water quality.

The feed were given at a rate of 10% of their Body weight (BW) ratio at the beginning of the feeding trial. The level was adjusted according to the BW after weighing them once in two weeks and the final feeding rate was at 5% of their BW ratio. Total feed intake was measured by adding up total feed intake per fish and deducted by the uneaten feed. The feeding trials were conducted over 56 days. At the end of the experiment, all fishes were weighed, sacrificed for body composition and frozen at -20°C for further analysis

The water qualities for all tanks were measured according to the method of American Public Health Association, 1992. Water temperature was maintained at 28-29°C, pH at 6.0-6.8 and dissolved oxygen (DO) above 5.0 mg/L. Ammonia and nitrate were determined weekly and the levels were maintained below 0.8 mg/ml and 1.9 mg/ml respectively (Marion, 1998).

### **3.3.3 Proximate and chemical analysis of diets and body composition**

The experimental diets, ingredients and body composition were analysed for proximate composition according to Association of Official Analytical Chemist method (AOAC, 2003)

#### **3.3.3.1 Crude protein**

Kjeldahl method was used to analyse crude protein after acid digestion. Briefly, 150 mg of sample were weighed into Kjeldahl digestion tube and 1 tablet of 100 mg Selenium Kjeltabs Catalyst and 6 ml concentrated sulphuric acid were added. The tubes were digested in FOSS Tecator Digestor Auto at 420°C for 1 hour. Then, they were left to cool down for 15 minutes before beginning the distillation process. An 80 ml of de-ionized water and 50 ml of sodium hydroxide were added to each digestion tube, mixed thoroughly and distilled with 25 ml of 4% boric acid and titration indicator by using Kjeltac semi auto analyser. For the titration indicator, 100 mg bromocresol green were dissolved in 100 ml methanol before adding with 70 ml methyl red solution in 100 ml methanol. About 7 to 8 drops of bromocresol green + methyl red indicator were added to the conical flask beaker containing boric acid. Finally, 0.01 M hydrochloric acid was used to titrate the distillation product in the conical flask and the titration values were recorded. All samples and blanks were analysed in triplicates.

The protein content of the samples and blanks were calculated as:

$$\% \text{ Nitrogen} = \frac{(S-B)(N)(14.007) \times 100}{(B) \times 1000}$$

$$\% \text{ Protein} = (\% \text{ Nitrogen}) \times (6.25)$$

Where S = Titrate of HCl (ml)

B = sample / blank (g)

N = HCl molar (0.0994)

### 3.3.3.2 Crude lipid

Soxhlet method with petroleum ether extraction (FOSS Soxtec 2055) was used to measure crude lipid content of diets and fish body composition. Briefly, the extraction cups were weighed after drying in an oven. Then, 2 g of samples were weighed and added into the cellulose thimble while the extraction cups were filled with 80 ml petroleum ether. Both thimbles and extraction cups were placed in the Foss Tecator Extraction Unit and the extraction process was performed for 1 hour. Subsequently, the extraction cups were dried in an oven with 120°C for 2 hours and later cooled off in a desiccator before weighing. The samples in the thimbles were kept in dry environment to be used in crude fiber analysis. All samples were analyzed in triplicates.

The lipid content of the samples were calculated as:

$$\% \text{ Lipid} = \frac{(W3 - W2)}{W1} \times 100$$

Where W1 = weight of sample (g)

W2 = weight of extraction cup initial (g)

W3 = weight of extraction cup final (g)

### 3.3.3.3 Dry matter

Empty dried crucibles were weighed and approximately 4 g of samples were placed in the crucibles. The weight for both crucible with samples were recorded prior to drying in the oven at 105°C for 24 hours to a constant weight. Then, the crucibles were cooled off in a desiccator before reweighing to obtain the final weight.

The dry matter content of the samples was calculated as:

$$\text{Dry matter} = \frac{(W3 - W1)}{(W2 - W1)} \cdot 100$$

Where W1 = Weight of empty crucible

W2 = Weight of crucible + sample

W3 = Weight of crucible + sample after drying in 105°C

### 3.3.3.4 Ash

The ash content of diets and body composition was determined by drying the samples used in the dry matter determination in muffle furnace (Naberthem) at 600°C overnight. Then, the samples were cooled in a desiccator before reweighing to determine the ash content.

The ash content was calculated as:

$$\text{Ash \%} = \frac{(W4 - W1)}{(W3 - W1)} \cdot 100$$

Where W1 = Weight of empty crucible

W3 = Weight of crucible + sample after drying in 105°C

W4 = Weight of crucible + sample after drying in 600°C

### 3.3.3.5 Crude fiber

Crude fiber was determined after an alkali and acid digestion by using defatted samples from crude lipid extraction analysis. Fiber capsules and the lids were weighed together. Approximately 50 – 100 mg of samples were weighed, put into the capsules and the lids secured. Extraction vessel with 350 ml of 1.25% (v/v) sulphuric acid was placed on a hot plate and heated to boil. The capsule tray with fiber capsules containing samples was placed in the carousel and put on the stopper to lock the capsules in place. Extraction carousel was partially lowered into the boiling reagent sufficient to immerse the samples. Gentle boiling was carried out for 30 minutes and after 5 minutes of boiling; the carousel was removed from the extraction vessel. The extraction carousel was washed with boiling water 3 times with fresh hot water each time. Then, the extraction vessel was filled with 350 ml 1.25% (w/v) sodium hydroxide on the hot plate and boiled. The same procedures as the sulphuric acid were repeated and the washing procedures were performed 3 times. Later, the sample were washed once in 1% (v/v) hydrochloric acid and finally in boiling water. The capsules were dried in an oven at 130°C for 2 hours. They were then cooled off in a desiccator and weighed. The weighed capsules were placed in pre-weighed and pre-dried crucibles for ashing procedure at 600°C for 4 hours. They were then cooled off in a desiccator before reweighing to determine the crude fiber content

The crude fiber content was calculated as:

$$\% \text{ Crude fiber} = \frac{W3 - (W1 \times C) - (W5 - W4 - D)}{W2} \times 100$$

Where W1 = Initial capsule weight (mg)

W2 = Sample weight (mg)

W3 = Capsule + residue weight (mg)

W4 = Empty ashing crucible (mg)

W5 = Total ash (g)

C = Blank correction for capsule solubility

D = Capsule ash (mg)

#### **3.3.3.6 Gross energy**

Gross energy for every diets and body composition was calculated using the following factors: crude protein = 23.9 kJ/g, crude lipids = 39.8 kJ/g and NFE = 17.6 kJ/g (Schulz et al., 2005).

#### **3.3.3.7 Nitrogen free extract**

Nitrogen free extract (NFE) or carbohydrate is calculated as =  $100 - (\% \text{ crude protein} + \% \text{ crude fat} + \% \text{ crude fiber} + \% \text{ crude ash})$

#### **3.3.4 Amino acid analysis**

Amino acid profiles were conducted using the High Performance Liquid Chromatography (HPLC) (JASCO CO-2065 Plus, Intelligent Column Oven) by using the Pico-Tag method (Heinrikson and Meredith, 1984).

##### **3.3.4.1 Sample preparation**

All samples were ground, dried and assayed for crude protein prior to amino acid analysis. The crude protein of the samples determined the amount of samples used in the amino acid analysis. The weight of each sample was measured by dividing 4 with the crude protein content (i.e. crude protein for soybean meal is 39 %, thus  $4/39 = 0.10$  g of the sample were used for the analysis). The weighed samples were hydrolyzed with 6 N hydrochloric acid and vortexed. Then, they were flushed with nitrogen gas for 1

minute and dried in an oven at 140°C for 24 hours and cooled off at room temperature. A 10 ml internal standard,  $\alpha$ -amino-N butyric acid (AABA) was added to the samples and made up with 60 ml de-ionized water. Finally, the samples were filtered and kept in -20°C for further analysis. The internal standard was prepared by dissolving 0.2578 g AABA and made up to 1 L of 0.1 N hydrochloric acid.

#### **3.3.4.2 Drying and derivatization procedure**

Hydrolysis samples were filtered with 0.20  $\mu$ m cellulose nitrate membrane filter and 10  $\mu$ l of the samples were placed in a vial. The redrying agent was prepared, which consisted of methanol, water and triethylamine (2:2:1 v/v) and derivatization reagent, which was composed of methanol, triethylamine, water and phenylisothiocyanate (PITC) (7:1:1:1 v/v).

Hydrochloric acid from the samples was removed by vacuum and dried at room temperature for 30 minutes. The samples were then vacuumed with 20  $\mu$ l redrying agents and later derivatization reagent was added to the mixture and vortexed before being left at room temperature for 20 minutes. The samples were then re-vacuumed again for 30 minutes to ensure the reagents are completely dried at this stage.

#### **3.3.4.3 Chromatographic procedures**

Separation of PITC derivatives occurred in high performance liquid chromatography (HPLC). The column used is from Agilent Technologies. The mobile phase consisted of two eluants labeled as A and B. Solvent A is composed of 0.1 M ammonium acetate while solvent B consisted of 440 ml solvent A mixed with 460 ml acetonitrile and 100 ml methanol. Both eluants were filtered, degassed and kept under room temperature. The samples and standard were mixed with 100  $\mu$ l of solvent A and vortexed for 15 minutes before being injected into the HPLC machine. The amino acid

contents of the samples were determined by comparison of peak retention times to the known standard.

### **3.3.5 Tryptophan determination**

Tryptophan from the samples was hydrolyzed by using alkaline, which is Lithium hydroxide (LiOH. H<sub>2</sub>O), 4.3 N and determined by using fluorescence detector using excitation and emission wavelength given. Briefly, tryptophan standard was prepared by weighing 0.05 g of tryptophan into 50 ml volumetric flask. Hydrochloric acid (0.1 N) was added into the flask and placed into ultra-sonicator to dissolve the salute. A total of 50 ml of distilled water was added to the solution to make a 1000 µg/ml tryptophan concentration. Then, 50 µl of the solution was added to 10 ml mobile phase to make 50 µg/ml. Tryptophan standard of 10 µL was injected into HPLC system.

Lithium hydroxide was prepared by dissolving 36.09 g of LiOH. H<sub>2</sub>O in 200 ml distilled water to measure up to 10 samples. Samples were prepared by adding 0.2 g of samples and 15 ml of LiOH. H<sub>2</sub>O together into a screw-capped tube. The solution was flush with liquid nitrogen and heated at 120°C for 16 hours. The hydrolysate was transferred into a beaker and 9 ml of 6 N HCl with distilled water was added to make a total volume of less than 100 ml. The pH was adjusted to 4.5 by using HCl and dilute to 100 ml with water into a volumetric flask. The reagent was then filtered through filter paper and finally small aliquot was filtrated through a syringe filter (0.2 µm cellulose acetate membrane) before 10 µL of the samples were injected into the HPLC system.

### 3.3.6 Analysis of experimental data

From the experimental data obtained, specific growth rate (SGR), feed conversion ratio (FCR), body weight gain (BWG), protein efficiency ratio (PER), total feed intake (TFI) and survival rate (SR) were calculated as follows:

- 1) Body weight gain = final weight – initial weight
- 2) FCR = food fed / live weight gain
- 3) SGR =  $(\ln \text{ final weight of fish} - \ln \text{ initial weight of fish} / \text{time of experiment})$
- 4) PER = live weight gain (g) / protein fed (g)
- 5) TFI = Average of total feed intake per fish (g)
- 6) SR (%) =  $(\text{final number of fish} / \text{initial number of fish}) \times 100$

All calculations were measured according to triplicates tanks treatments.

### 3.3.7 Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) using SPSS version 21.0 (SPSS Inc., Chicago IL, USA). The differences between means were compared by Duncan's post hoc test at 5% ( $P < 0.05$ ) probability level. Data are presented as means  $\pm$  standard error of mean (SEM).

### 3.4 Results

The inclusion level of CM (0%, 25%, 50%, 75%, 100%) in the diets with isonitrogenous crude protein (28%) and chemical composition of the experimental diets are shown in Table 3.1. The fishmeal and cricket meal were formulated according to the percentage inclusion level of the diets and their nutrient compositions are shown in Table 3.2. However, soybean, rice bran and corn meal were changed and evaluated to make sure the overall composition were the same amongst all the diets. The results of the present study clearly indicated that growth performances of African catfish fingerlings were affected by the different experimental diets of CM. Daily water temperature, pH and DO for every tank were observed and maintained to the standard requirement. Generally, there were no significant difference between treatments and remained in the suitable range for African catfish growth indicating that the experimental diets did not affect water quality of the fish.

**Table 3.1: Formulation (g kg<sup>-1</sup>) and nutritional profile (%) of experimental diets**

<b>Ingredients (g kg<sup>-1</sup>)</b>	<b>Replacement (%)</b>				
	<b>(0%)</b>	<b>(25%)</b>	<b>(50%)</b>	<b>(75%)</b>	<b>(100%)</b>
Fishmeal	300.0	225.0	150.0	75.0	0.0
Cricket meal	0	75.0	150.0	225.0	300.0
Soybean meal	58.6	9.8	19.2	33.3	96.2
Corn starch	324.6	142.4	146.6	165.9	341.7
Rice bran	301.8	532.8	519.1	485.8	247.1
Vitamin premix <sup>1</sup>	2.0	2.0	2.0	2.0	2.0
Mineral premix <sup>2</sup>	3.0	3.0	3.0	3.0	3.0
DCP	10	10.0	10.0	10.0	10.0
Total	1000	1000	1000	1000	1000
Nutrient level determined by as is basis (% Dry matter basis)					
Dry matter	94.36	94.42	94.06	93.26	93.14
Crude protein	28.0	28.00	27.50	27.70	27.90
Crude fat	8.43 <sup>a</sup>	8.51 <sup>a</sup>	9.71 <sup>b</sup>	9.42 <sup>b</sup>	9.45 <sup>b</sup>
Crude ash	9.00 <sup>b</sup>	8.55 <sup>ab</sup>	8.24 <sup>ab</sup>	7.42 <sup>a</sup>	7.55 <sup>a</sup>
Crude fiber	1.36 <sup>a</sup>	1.86 <sup>a</sup>	2.90 <sup>ab</sup>	3.15 <sup>b</sup>	3.24 <sup>c</sup>
Gross Energy <sup>3</sup>	18.42	18.44	18.48	18.39	18.35
NFE <sup>4</sup>	47.57 <sup>b</sup>	47.50 <sup>b</sup>	45.71 <sup>ab</sup>	45.57 <sup>ab</sup>	45.00 <sup>a</sup>

**Table 3.1 continued**

<sup>1</sup>The vitamin premix supplied the following per 100 g diet: Vitamin A, 500 IU; Vitamin D3, 100 IU; Vitamin E, 0.75 mg; Vitamin K, 0.02 mg; Vitamin B1, 1.0 mg; Vitamin B2, 0.5 mg; Vitamin B3, 0.3 mg; Vitamin B6, 0.2 mg; Vitamin B12, 0.001 mg; Vitamin C, 0.1 mg; Niacin, 0.2 mg; Folic Acid, 0.1 mg; Biotin, 0.235 mg; Pantothenic acid, 1.0 mg; Inositol, 2.5 mg

<sup>2</sup>The mineral premix supplied the following per kg diet: Selenium, 0.2 mg; Iron, 8 mg; Manganese 1.0 mg; Zinc, 8.0 mg; Copper, 0.15 mg; Potassium Chloride, 0.4 mg; Magnesium Oxide, 0.6 mg; Sodium Bicarbonate, 1.5 mg; Iodine, 1.0 mg; Cobalt, 0.25 mg.

<sup>3</sup>Gross Energy was calculated as 23.9, 39.8, 17.6 kJ/g for protein, fat and NFE respectively (Schulz et al., 2005).

<sup>4</sup>NFE= 100- (% crude protein + % crude lipid + % crude ash+ % crude fiber).

**Table 3.2: Chemical composition of fishmeal, cricket meal and soybean meal used in the trial diet**

<b>Component</b>	<b>Fishmeal</b>	<b>Cricket meal</b>	<b>Soybean meal</b>
Dry matter (%)	85.59	95.18	90.01
Crude protein %	53.61	57.02	39.34
Crude lipid (%)	2.69	13.90	2.23
Crude ash (%)	19.30	4.83	7.25
Crude fiber (%)	4.64	9.21	2.80

Ten amino acids, (methionine, arginine, threonine, tryptophan, histidine, isoleucine, lysine, leucine, valine and phenylalanine) are not synthesised in fish, therefore, need to be supplied in their feeding materials (NRC, 1993). All compounds of essential amino acids (EAA) were present in experimental diets (Table 3.3). The values of amino acid in the diets containing CM were equal or slightly higher compared to the control (0%) which can be considered negligible. All Essential amino acid (EAA) tested in this study were compared with the EAA requirements for catfish as reported by Jimoh et al, (2014) and Fagbenro & Nwanna, (1999).

The amino acid profiles for fishmeal and cricket meal are shown in Table 3.3. Compared to fishmeal, the cricket meal is relatively rich in limiting amino acid; methionine, lysine and cysteine as well as greater in histidine and tyrosine. On the other hand, fishmeal is comparatively higher in threonine, valine, isoleucine and leucine.

**Table 3.3: Essential amino acid (EAA) and non-essential amino acid (NEAA) composition of fishmeal, cricket meal (*Gryllus bimaculatus*) and practical diets used in this study (g 100g<sup>-1</sup> crude protein)**

EAA	Fishmeal	Cricket meal ( <i>G. bimaculatus</i> )	Replacement (%)				
			(0%)	(25%)	(50%)	(75%)	(100%)
Histidine	1.14 ± 0.02	2.20 ± 0.01	1.62 ± 0.01 <sup>a</sup>	1.63 ± 0.03 <sup>a</sup>	1.93 ± 0.11 <sup>ab</sup>	2.19 ± 0.04 <sup>b</sup>	2.18 ± 0.03 <sup>b</sup>
Arginine	6.64 ± 0.01	6.20 ± 0.03	5.66 ± 0.13 <sup>a</sup>	5.66 ± 0.06 <sup>a</sup>	6.13 ± 0.12 <sup>ab</sup>	6.23 ± 0.03 <sup>b</sup>	5.56 ± 0.05 <sup>a</sup>
Threonine	4.35 ± 0.13	3.88 ± 0.11	4.03 ± 0.27 <sup>b</sup>	3.36 ± 0.02 <sup>a</sup>	3.61 ± 0.03 <sup>a</sup>	3.54 ± 0.02 <sup>a</sup>	3.09 ± 0.19 <sup>a</sup>
Valine	5.91 ± 0.02	4.24 ± 0.05	4.9 ± 0.11 <sup>a</sup>	5.21 ± 0.03 <sup>b</sup>	5.20 ± 0.03 <sup>b</sup>	5.26 ± 0.05 <sup>b</sup>	5.02 ± 0.03 <sup>b</sup>
Methionine	1.83 ± 0.01	2.02 ± 0.04	1.16 ± 0.02 <sup>a</sup>	1.18 ± 0.17 <sup>a</sup>	1.35 ± 0.26 <sup>a</sup>	1.45 ± 0.02 <sup>b</sup>	1.45 ± 0.02 <sup>ab</sup>
Isoleucine	4.32 ± 0.04	3.75 ± 0.01	3.67 ± 0.09 <sup>a</sup>	3.64 ± 0.03 <sup>a</sup>	3.74 ± 0.02 <sup>a</sup>	3.87 ± 0.02 <sup>a</sup>	3.55 ± 0.10 <sup>a</sup>
Leucine	7.27 ± 0.11	6.70 ± 0.07	6.5 ± 0.15 <sup>ab</sup>	6.30 ± 0.04 <sup>a</sup>	6.61 ± 0.04 <sup>ab</sup>	6.75 ± 0.03 <sup>b</sup>	6.45 ± 0.06 <sup>ab</sup>
Phenylalanine	4.40 ± 0.01	4.00 ± 0.02	5.58 ± 0.39 <sup>c</sup>	4.84 ± 0.67 <sup>b</sup>	4.42 ± 0.02 <sup>ab</sup>	3.81 ± 0.11 <sup>a</sup>	3.83 ± 0.02 <sup>a</sup>
Lysine	3.09 ± 0.10	5.14 ± 0.04	2.46 ± 0.13 <sup>a</sup>	2.33 ± 0.06 <sup>a</sup>	3.08 ± 0.06 <sup>b</sup>	3.50 ± 0.08 <sup>c</sup>	3.23 ± 0.82 <sup>b</sup>
Tryptophan	0.60 ± 0.15	0.85 ± 0.12	0.73 ± 0.002	0.76 ± 0.003	0.84 ± 0.005	0.89 ± 0.001	0.94 ± 0.001
<b>NEAA</b>							
Cystein	1.01 ± 0.02	2.02 ± 0.01	4.40 ± 0.53 <sup>a</sup>	4.89 ± 0.50 <sup>a</sup>	7.02 ± 0.31 <sup>b</sup>	6.93 ± 0.03 <sup>b</sup>	6.38 ± 0.03 <sup>b</sup>
Tyrosine	3.15 ± 0.03	7.63 ± 0.02	2.63 ± 0.06 <sup>a</sup>	3.01 ± 0.03 <sup>a</sup>	3.51 ± 0.04 <sup>ab</sup>	4.30 ± 0.16 <sup>b</sup>	3.94 ± 0.03 <sup>b</sup>

Continue from Table 3.3			Replacement (%)				
NEAA	Fishmeal	Cricket meal ( <i>G. bimaculatus</i> )	(0%)	(25%)	(50%)	(75%)	(100%)
Aspartic acid	6.05 ± 0.02	6.93 ± 0.02	1.95 ± 0.006 <sup>b</sup>	1.78 ± 0.004 <sup>a</sup>	1.99 ± 0.01 <sup>bc</sup>	2.15 ± 0.04 <sup>d</sup>	2.00 ± 0.006 <sup>c</sup>
Glutamic acid	12.08 ± 0.11	10.92 ± 0.06	3.24 ± 0.06 <sup>a</sup>	3.16 ± 0.003 <sup>a</sup>	3.30 ± 0.01 <sup>a</sup>	3.56 ± 0.09 <sup>b</sup>	3.17 ± 0.008 <sup>a</sup>
Serine	8.16 ± 0.02	4.11 ± 0.01	0.02 ± 0.003 <sup>a</sup>	1.46 ± 0.02 <sup>d</sup>	1.36 ± 0.06 <sup>c</sup>	1.30 ± 0.002 <sup>c</sup>	1.04 ± 0.05 <sup>b</sup>
Glycine	7.41 ± 0.15	5.00 ± 0.11	0.01 ± 0.001 <sup>a</sup>	1.49 ± 0.01 <sup>d</sup>	1.40 ± 0.01 <sup>d</sup>	1.41 ± 0.008 <sup>c</sup>	1.22 ± 0.008 <sup>b</sup>
Alanine	4.77 ± 0.04	0.58 ± 0.03	1.13 ± 0.01 <sup>a</sup>	1.37 ± 0.006 <sup>b</sup>	1.56 ± 0.007 <sup>c</sup>	1.82 ± 0.008 <sup>d</sup>	2.07 ± 0.02 <sup>e</sup>

<sup>1</sup>Essential Amino Acid requirement (g/100g crude protein) according Jimoh et al, (2014): arginine 3.6, histidine 1.2, isoleucine 2.0, leucine 3.5, lysine 4.8, methionine 2.4, phenylalanine 4.0, threonine 2.8, valine 2.4, tryptophan 1.1

<sup>2</sup>Values are means ± S.D of duplicates samples

<sup>3</sup>Mean values in the same row with different superscript are significantly different ( $P < 0.05$ )

**Table 3.4: Amino acid profiles (g 100g<sup>-1</sup> crude protein) of field cricket (*Gryllus testaceus*), house cricket (*Acheta domesticus*) and good quality fishmeal.**

<b>EAA</b>	<sup>1</sup> <i>G. testaceus</i>	<sup>2</sup> <i>A. domesticus</i>	<sup>3</sup> Good quality fishmeal
Histidine	1.9	2.3	3.6
Arginine	3.7	6.1	6.14
Threonine	2.8	3.6	4.4
Valine	4.4	5.1	5.2
Methionine	1.9	1.4	2.5
Isoleucine	3.1	4.4	4.8
Leucine	5.5	9.8	7.8
Phenylalanine	2.9	3.0	4.1
Lysine	4.8	5.4	7.9
Cystein <sup>1</sup>	1	0.8	1
Tyrosine <sup>1</sup>	3.9	5.2	3.2

<sup>1</sup> Non-essential amino acid

Source: <sup>1</sup>(Wang et al., 2005), <sup>2</sup>(Finke, 2002), <sup>3</sup>(Lall & Anderson, 2005)

During the 56 days of feeding trial, all experimental diets were well accepted by African catfish fingerlings. The effect of growth performance and survival of the fish fed the experimental diets are presented in Table 3.5. Growth performance and feed utilisation of fish fed 100% CM were significantly improved compared to the lower percentage of CM replacement. Reduced weight gain was observed in the group fed with 25% CM although it does not differ significantly with 0% and 50%CM. Lower weight gain was observed in fish fed diet 0% until 75%, which suggested that diet with increased replacement of fishmeal by crickets meal from 50% up to 100% enhanced the growth performance, feed efficiency and survival rate of African catfish fingerlings.

Feed conversion ratio was significantly lower in 100% CM inclusion level (average: 2.20) as compared to the lowest inclusion level. Similarly, this result is confirmed by a significant increase protein efficiency ratio (PER) and specific growth rate (SGR) of fish fed 100% CM (average: 1.69 and 2.32 respectively) than those fed with 0% to 50% CM. Values of SGR and PER increased gradually with increasing amount of CM inclusion level from 50% up to 100%. In general, these growth parameters were positively parallel with increasing CM level from 50% upwards, which suggested that African catfish could well utilize cricket meal as protein sources in their diet

**Table 3.5: Growth performance of fish fed the experimental diets.**

Parameters	Replacement (%)				
	(0%)	(25%)	(50%)	(75%)	(100%)
TFI (g fish <sup>-1</sup> )	21.76 ± 0.88 <sup>a</sup>	21.19 ± 0.82 <sup>a</sup>	25.44 ± 0.39 <sup>ab</sup>	24.08 ± 2.04 <sup>ab</sup>	32.23 ± 0.86 <sup>c</sup>
BWG (g)	6.23 ± 0.25 <sup>a</sup>	6.13 ± 1.10 <sup>a</sup>	7.43 ± 0.54 <sup>ab</sup>	8.38 ± 0.14 <sup>b</sup>	14.68 ± 0.78 <sup>c</sup>
SGR (%/day)	1.49 ± 0.10 <sup>a</sup>	1.24 ± 0.23 <sup>a</sup>	1.42 ± 0.12 <sup>a</sup>	1.69 ± 0.03 <sup>ab</sup>	2.32 ± 0.15 <sup>b</sup>
FCR	3.50 ± 0.82 <sup>c</sup>	3.48 ± 1.77 <sup>c</sup>	3.43 ± 1.05 <sup>c</sup>	2.87 ± 0.53 <sup>b</sup>	2.20 ± 0.17 <sup>a</sup>
PER	1.11 ± 0.08 <sup>a</sup>	1.11 ± 0.17 <sup>a</sup>	1.15 ± 0.09 <sup>a</sup>	1.36 ± 0.09 <sup>ab</sup>	1.69 ± 0.17 <sup>b</sup>
SR (%)	93.30 ± 3.84 <sup>a</sup>	86.70 ± 2.22 <sup>a</sup>	86.70 ± 2.22 <sup>a</sup>	93.30 ± 3.84 <sup>a</sup>	93.30 ± 6.66 <sup>a</sup>

<sup>1</sup>Values are the mean ± SEM of triplicate groups of 15 fish per tank.

<sup>2</sup>Mean values in the same row with different superscript are significantly different ( $P < 0.05$ )

Full replacement of CM up to 100% of FM clearly affected the whole body composition of African catfish. The crude protein for initial fish were significantly different with the final body composition of experimental fishes fed with diet 50%, 75% and 100% CM inclusion level but did not differ significantly with diet 0% and 25% CM inclusion level (Table 3.6) An increasing trend of crude protein level was observed with higher inclusion of cricket meal despite the numerically lower level of 100% CM compared to 75% CM

On the other hand, crude lipid body composition exhibited a decreasing trend from 0% to 100% CM but did not differ significantly between the initial group, 0%, 25% and 50% CM. Crude ash was found to be highly significantly different in final body of fish fed diet above 50% CM level when compared to the initial group. The highest level was observed in the group that received 100% CM level.



**Figure 3.1: Fish fed with fishmeal diet after 56 days of feeding trial**



**Figure 3.2: Fish fed with cricket meal diet after 56 days of feeding trial**

**Table 3.6: Initial and final body composition (g kg<sup>-1</sup>) of fish fed the experiment diets (% Dry matter basis)**

Components	Replacements (%)					
	Initial	(0%)	(25%)	(50%)	(75%)	(100%)
Crude protein	46.18 ± 0.51 <sup>a</sup>	46.34 ± 0.11 <sup>a</sup>	46.87 ± 1.00 <sup>a</sup>	47.53 ± 2.08 <sup>b</sup>	48.88 ± 1.50 <sup>b</sup>	48.33 ± 0.62 <sup>b</sup>
Crude lipid	16.38 ± 2.28 <sup>c</sup>	16.82 ± 0.84 <sup>c</sup>	16.99 ± 2.24 <sup>c</sup>	16.92 ± 1.07 <sup>c</sup>	14.71 ± 0.19 <sup>b</sup>	13.90 ± 1.56 <sup>a</sup>
Crude ash	16.64 ± 0.03 <sup>a</sup>	17.69 ± 0.49 <sup>a</sup>	19.24 ± 1.26 <sup>ab</sup>	20.36 ± 0.52 <sup>b</sup>	22.78 ± 0.91 <sup>bc</sup>	25.29 ± 1.11 <sup>c</sup>
Dry matter	91.37 ± 1.00 <sup>a</sup>	92.00 ± 1.76 <sup>a</sup>	95.46 ± 0.57 <sup>b</sup>	94.63 ± 2.15 <sup>b</sup>	97.69 ± 0.31 <sup>c</sup>	97.61 ± 0.24 <sup>c</sup>

<sup>1</sup>Values are the mean ± SEM of triplicate groups of 15 fish per tank.

<sup>2</sup>Mean values in the same row with different letters are significantly different ( $P < 0.05$ )

### 3.5 Discussion

The results of the present study clearly indicated that growth performances of African catfish fingerlings were affected by the different experimental diet of cricket meal. The crude protein for field cricket, *Gryllus bimaculatus* in this study was analyzed as 57% on a dry weight basis (Table 3.2), which is slightly lower than the percentage of other species such as *Gryllus testaceus*, 58.3% (Wang et al., 2005), mormon crickets *Anabrus simplex* Haldeman, 59.8% (DeFoliart et al., 1982) and house crickets *Acheta domesticus*, 62% but somewhat higher than the variegated grasshopper, *Zonocerus variegatus*, 26.8 (Alegbeleye et al., 2012) and cornfield grasshopper, *Sphenarium purpurascens*, 52.6% (Ramos-Elorduy et al., 1997) from Orthoptera species.

Fishmeal used in this study is locally made and utilized by most local farmers here in Malaysia. This fishmeal has higher ash content (19%), lower crude protein (53.6%) and crude fat (2.96%) than the high quality fishmeal commonly used in commercial fish feed that contains more than 66% crude protein and around 8% to 11% crude fat along with ash that is lower than 12%. It is also more economical than the imported, higher quality fishmeal. Hence, the amino acid profiles for the fishmeal used in this study also shows the lower level of histidine, methionine and lysine compared to higher quality of fishmeal reported by Lall and Anderson (2005).

Higher levels of arginine, leucine and phenylalanine were recorded in *Gryllus bimaculatus* tested in this study in comparison to *Gryllus testaceus* as reported by Wang et al. (2005). In addition, studies by Finke (2002) on house cricket, (*Acheta domesticus*) amino acid profile shows a comparable result as *Gryllus bimaculatus* but somewhat higher level of leucine appeared in *A.domesticus*.

Lysine and methionine are the most limiting amino acid in fish feed particularly in plants and animal byproducts. Thus many commercial diets include lysine and methionine supplementation to satisfy the amino acid requirement for their fish feed (Nunes et al., 2014). Similarly, in this case, all experimental diets are deficient in lysine, methionine and phenylalanine. However according to Robinson and Li, (2006), the non-essential amino acid such as cysteine can replace about 60% of methionine requirement while tyrosine can spare up to 50% of phenylalanine in catfish diet.

Robinson and Li (2007) have proposed that crude protein level as low as 26% is still accepted for growing catfish and in their revised publication, they have suggested that crude protein of 28% provide good growth at stocking density of less than 10,000 per acre per day with 1000 fish growing up to 100 pounds (Robinson & Li, 2007).. However, it was observed that FCR levels were extremely high for Diet 0% until 100%. This situation may be attributed to insufficient crude protein level for the practical diet formulation in African catfish fingerlings. As a result, future studies on higher crude protein level will be beneficial to generate lower FCR value and consequently increase growth rate.

Other insects that have been tested in catfish diet have shown poor growth in total replacement for fishmeal. Previous studies by Roncarati et al. (2015) highlighted the inclusion of mealworm (*Tenebrio molitor*) (50.8% crude protein) in common catfish could enhance their growth although fishmeal fed fish (51.6% crude protein) showed significantly higher weight gain than mealworm group. As for survival, the control diet (0%) and 75% and 100% CM inclusion level showed the highest rate (average: 93.3%) compared to 25% and 50% CM (average: 86.7) although the differences observed were insignificant.

Body composition was affected by many factors including growth, ingredient used in the diets and water temperature. Apart from that, feed rate also could affect body composition of fish (Ahmed, 2007; Kim et al., 2012). The results showed that gross body composition of fish fed with 50% to 100% CM inclusion level showed significantly higher crude protein content than those fed with lower inclusion of cricket meal. However, higher fat content in fish fed with lower inclusion level of CM might be resulted due to lower feed intake and thereby possibly impair growth (Johansen et al., 2003). Increasing body lipid deposition must be carefully considered as it could affect carcass quality, storage characteristics and flavor (Hillestad & Johnsen, 1994).

Since cricket meal in this study was acquired from local supplier and the amount of local farmers producing them is limited, the price was fairly still expensive compared to fishmeal. In tropical areas, crickets can be produced in large amount and relatively within a short period of time. Therefore, although the price is the limiting factor here, further research to develop a lower cost, practical method, as well as sustainable manner for mass production culture of cricket will be beneficial to reduce production cost.

### 3.6 Conclusion

The study of insects as substitutes for fishmeal has been growing over the years in order to find sustainable resources to replace a very volatile and expensive fishmeal. However, unlike other insects such as maggot meal, flies and silkworm, the information about crickets as protein sources for fish feed is very limited compared to poultry feed. Field crickets, *Gryllus bimaculatus* as an animal protein source is a very good candidate since they contain sufficient amount of EAA for fish requirement. Besides, this species can be harvested in a considerable amount with mass rearing under controlled condition

In conclusion, the use of a practical diet containing 100% CM is appropriate for growth and nutrition utilisation of African catfish fingerlings. With a view to reduce feed cost for the aquaculture and other livestock industry, further studies on cricket meal as alternatives protein source should continue to be carried out since it holds great potential.

## **CHAPTER 4: NUTRIENT AND AMINO ACID DIGESTIBILITY OF CRICKET MEAL AND FISHMEAL IN AFRICAN CATFISH, *Clarias gariepinus* DIET**

### **4.1 Introduction**

The study of fisheries nutrition started since the early 1930s. The evaluation of fish feed efficiency by using digestibility, metabolizability and growth are similarly utilized as in warm-blooded animals (Belal, 2005).

Digestion coefficient is crucial in determining fish diets in terms of growth (Lovell, 2012). The apparent digestibility can be measured by using two methods. The first method is regarded as 'direct method', which relies on quantitative measurement of ingested (feed) and egested (faeces) materials. The second method is called 'indirect method' (Lovell, 2012) or 'indicator method' by determining the percentage of the marker (i.e., chromic oxide, barium oxide, etc.) in the feed and sample of the faeces to estimate the digestibility coefficient.

Recently, there has been increasing interest in the use of insect meals as a protein ingredients. However, there are still uncertainties on the digestibility of insects meal in fish feed due to the presence of chitin in some insects. Little is known about the efficiency of cricket meal as an ingredient in formulated feed as well as its effect on digestibility by fish. Therefore, this study was conducted to assess the growth performance, nutrient digestibility and amino acid digestibility of cricket meal and fishmeal fed to *Clarias gariepinus*.

## 4.2 Literature review

Insect meals have captivated interest among researchers due to their high protein content and ease of culturing within a relatively short period of time. However, the digestibility of fish when consuming insects were the concern of the researchers due to the presence of chitin that could diminish the apparent digestibility, hence reduce growth performance. Despite that, many studies have proven that fish performance were either improved or not affected by the dietary inclusion of insects, crustaceans or benthic invertebrate in which chitin is one of the compound present (Henry et al., 2015).

Apparently, insects contain lower levels of carbohydrate than plants with approximately lesser than 20% in which most considerably consists of chitin from the exoskeleton (Barroso et al., 2014). However, the amount of chitin varies according to species and developmental stage. The fish's ability in digesting chitin is also debatable since some fish can digest chitin better than others. Incorporating chitin has immunostimulatory effect in marine fish diet but generally it is one of the limiting factors present in insects that could reduce its digestibility by fish (Barroso et al., 2014; Ng et al., 2001).

## **4.3 Materials and method**

### **4.3.1 Experimental Diet**

Adult live cricket and all raw ingredients were purchased and processed according to the experimental diet procedure elaborated in Chapter 3, paragraph 3.3.1. Chromic oxide ( $\text{Cr}_2\text{O}_3$ ) at 0.5% concentration was incorporated into the diet as an inert marker.

Table 1 shows the composition and nutrition profiles of the diets involved in this feeding trial. Two iso-nitrogenous diets with 30% crude protein content were used in this experiment. The cricket meal diet contained 30% inclusion of cricket meal while the fishmeal diet contained 30% fishmeal. For both diets, the remaining 70% consisted of other ingredients, including soybean, rice bran, corn meal, vitamin, minerals and Di-calcium phosphate, (DCP).

### **4.3.2 Experimental Fish and Set-Up**

Ninety (90) *Clarias gariepinus* weighing  $51.70 \pm 0.8$  g were randomly divided into two groups, each in triplicates of 15 fishes. All the fishes were obtained from a local supplier and acclimatized to natural environmental conditions for two weeks prior to the feeding trials.

Faecal material was collected once daily in the morning from the bottom of filtration box prior to feeding. Faeces from each replicated treatment were dried and stored for chemical analysis.

Six plastic tanks (3' x 2' x 1'), which contained 100 liters of water in closed re-circulation system, were used for the feeding activities. The tanks were equipped with top filter pump with a flow rate of 20 L/min for the circulation of dissolved oxygen. Tap

water, which was treated with anti-chlorine, was used and 20-30% of water was replaced once in two days to maintain water quality.

The commercial diets (Dindings) were fed twice per day at 0900 and 1500 hour during the acclimatisation period. After two weeks, the experimental diet was fed twice daily at the same times throughout the experimental period. The feeds were given at a rate of 3% of Body weight (BW) ratio at the beginning of the feeding trial and the final feeding rate was at 1% of their BW ratio. Uneaten food was collected and dried in an oven and then weighed to determine the total feed. The level was adjusted according to the BW after weighing them once in two weeks. The feeding trials were conducted over 56 days duration.

The water quality for all tanks was measured according to the method by APHA (1992) as described in Chapter 3, paragraph 3.3.2

#### **4.3.3 Proximate and Chemical Analysis**

The experimental diets and ingredients were analyzed for the proximate composition according to the Association of Official Analytical Chemist methods (AOAC, 2003) as described in Chapter 3, paragraph 3.3.3. Kjeldahl method was used to estimate crude protein after acid digestion using FOSS Tecator Digestor Auto. Moisture and dry matter were determined by drying in an oven at 105°C to constant weight. Ash was measured by combustion in a muffle furnace (Naberthem) at 600°C. Crude lipid content was measured with petroleum ether extraction by using Soxhlet method (FOSS Soxtec 2055). Nitrogen-free extract (NFE) is calculated as = 100 - (% crude protein + % crude fat + % crude fiber + % crude ash + % moisture). Gross energy for every diet was calculated using the following factors: crude protein = 23.9 kJ/g, crude lipids = 39.8 kJ/g and NFE = 17.6 kJ/g (Schulz et al., 2005).

#### 4.3.4 Chitin determination

Chitin content in raw cricket meal was estimated by measuring the acid detergent fiber (ADF) and protein residue of ADF according to Marono et al. (2015) and AOAC (1990). The ADF solution was prepared by using 10 L of distilled water, 360 g of hexadecyltrimethylammonium bromide, 500 ml of sulphuric acid and the solution was made up to 18 L with distilled water. All samples were defatted and ground to pass a 1 mm forage mill prior to use. A total of 1 g of sample ( $W_0$ ) was added into 600 ml Berzelius beaker with 100 ml of ADF solution. Then, the reagent was boiled for one hour using a refluxing apparatus. On the other hand, 50 ml crucibles were dried at 100°C overnight before weighing ( $W_1$ ). The crucibles were placed in vacuum unit and the reagents were poured through the crucible prior to vacuum process. Hot water was used to rinse the crucibles until all the traces were removed. After hot water is filtered off, acetone was used to rinse repeatedly and finish the rinsing portion until the drained liquid is cleared. The samples collected were placed in small crucibles and dried in an oven overnight at 105°C and later cooled to room temperature in a desiccator before weighing ( $W_2$ ) on the next day. The samples residue were then analysed for ash ( $W_3$ ) and crude protein to determine chitin composition in the cricket meal.

The ADF content of the samples was calculated as:

$$\% \text{ ADF} = \frac{[(W_2 - W_1)]}{W_0} \cdot 100$$

$$\% \text{ ADF}_{\text{ash}} = \frac{(W_3 - W_1)}{(W_2 - W_1)} \cdot 100$$

Chitin in raw cricket meal was calculated as follows:

$$\text{Chitin (\%)} = \text{ash free ADF (\%)} - \text{ADF crude protein (\%)}$$

#### **4.3.5 Chromic oxide determination**

Chromic oxide indicator in faeces and feed were measured according to the method of Furukawa (1966). Briefly, 50 to 100 mg of samples were weighed and dried in an oven at 60°C overnight. Then, the samples were transferred into borosilicate digestion tube with 6 ml of nitric acid and incubated overnight at room temperature. The next day, all the samples were digested in 150°C for approximately one hour and later cooled off at room temperature. A volume of 3 ml of 70% perchloric acid was added to the tube and digested at 220°C for 75 minutes until the appearance of strong red colour and later cooled off at room temperature. The cooled samples were decanted from the tube into a volumetric flask and made up to 50 ml with distilled water. Then the absorbance of each sample of feed and faeces were recorded from atomic spectrophotometer at 452.4 nm.

#### **4.3.6 Amino acid digestibility**

Amino acid profiles for diets and faeces were determined using the High Performance liquid Chromatography (HPLC) (JASCO CO-2065 Plus, Intelligent Column Oven) and the contents were determined by comparison peak retention times to a known standard by using the Pico-Tag method by Heinrikson and Meredith (1984) while Tryptophan was determined after alkaline hydrolysis according to (Nielsen & Hurrell, 1985) as described in Chapter 3, paragraph 3.3.4.

### 4.3.7 Analysis of Experimental Data

#### 4.3.7.1 Growth performance analysis

From the data obtained, specific growth rate (SGR), food conversion ratio (FCR), body weight gain (BWG), protein efficiency ratio (PER) and survival rate (%) were calculated according to Chapter 3, paragraph 3.3.5.

#### 4.3.7.2 Apparent digestibility coefficient

The Apparent digestibility coefficient (ADC) for the crude protein, lipid, dry matter, carbohydrate and amino acid availability coefficient (AAC) of fish fed with cricket meal and fishmeal were calculated according to (Cho et al., 1982)

ADC for protein, lipid, carbohydrate, ash and amino acid availability coefficient (AAC)

$$ADC / AAC = 1 - \left( \frac{DC}{FC} \right) \cdot \left( \frac{FN}{DN} \right)$$

The ADC for dry matter

$$ADC = 100 - \left[ 100 \cdot \left( \frac{DC}{FC} \right) \right]$$

Where DC= % dietary  $\text{Cr}_2\text{O}_3$

FC = % faecal  $\text{Cr}_2\text{O}_3$

FN = % faecal nutrient

DN = % dietary nutrient

### 4.3.8 Statistical Analysis

All growth data and ADC values were subjected to Independent sample T-Test using SPSS version 21.0. The differences between means were determined at 5% ( $P < 0.05$ ) probability level. Confidence intervals were calculated as mean  $\pm$  SE (standard error).

**Table 4.1: Formulation (g kg<sup>-1</sup>) and nutritional profile (%), of fishmeal and cricket meal diet for the digestibility study**

Ingredients	Fishmeal diet	Cricket meal diet
Fishmeal	300	0
Crickets meal	0	300
Soybean meal	117.7	117.8
Corn meal	297.0	243.9
Ricebran	269.8	318.3
Vitamins premix	2.0	2.0
Minerals premix	3.0	3.0
DCP	10	10
Chromic oxide	5.0	5.0
Nutritional profile (%)		
Crude Protein	29.92	29.30
Crude lipid	8.63	9.67
Dry matter	95.57	96.42
Ash	9.67	9.53
Crude fiber	1.50	2.36
Chitin	-	2.27
NFE / Carbohydrate	45.85	45.56
Gross Energy (kJ g <sup>-1</sup> )	18.65	18.87

<sup>1</sup>The vitamin premix supplied the following per 100 g diet: Vitamin A, 500 IU; Vitamin D3, 100 IU; Vitamin E, 0.75 mg; Vitamin K, 0.02 mg; Vitamin B1, 1.0 mg; Vitamin B2, 0.5 mg; Vitamin B3, 0.3 mg; Vitamin B6, 0.2 mg; Vitamin B12, 0.001 mg; Vitamin C, 0.1 mg; Niacin, 0.2 mg; Folic Acid, 0.1 mg; Biotin, 0.235 mg; Pantothenic acid, 1.0 mg, Inositol, 2.5 mg

<sup>2</sup>The mineral premix supplied the following per kg diet: Selenium, 0.2 mg; Iron, 8 mg;

**Table 4.1 continued**

Manganese 1.0 mg; Zinc, 8.0 mg; Copper, 0.15 mg; Potassium Chloride, 0.4 mg;

Magnesium Oxide, 0.6 mg; Sodium Bicarbonate, 1.5 mg; Iodine, 1.0 mg; Cobalt, 0.25 mg.

**4.4 Results**

Diet compositions and nutritional profiles of cricket meal and fishmeal diet fed to the African catfish were reported in Table 4.1. Chromic oxide was included in the diets as inert marker. Chitin was estimated in the cricket meal diet based on chitin composition in raw cricket meal. The raw cricket meal contains approximately 7.15% chitin after ADF analysis while the cricket meal diet used in this experiment was formulated to have 2.27% chitin content.

Table 4.2 summarized the chemical composition of faecal materials collected from the fish during the feeding trials. Higher crude protein and ash content were observed in faeces from fishmeal group. On the contrary, the crude fiber content of the cricket meal-fed fish was higher than that of the fishmeal group (12.56, 11.23 respectively). Carbohydrate or NFE was also higher in cricket meal than fishmeal group (43.04 and 38.45 respectively) while no significant differences ( $P > 0.05$ ) were observed in gross energy in both groups of fish.

The apparent digestibility coefficients for both diets are shown in Table 4.3. Generally, both diets showed significant differences in all ADC components. The cricket meal had significantly higher ( $P < 0.05$ ) ADC for crude protein ( $81.21 \pm 0.03\%$ ), crude lipid ( $89.82 \pm 0.02\%$ ), dry matter ( $73.97 \pm 0.01\%$ ) and gross energy ( $64.42 \pm 0.01$ ) whereas fishmeal showed lower level of ADC with crude protein level of ( $78.22 \pm 0.03\%$ ), crude lipid ( $82.03 \pm 0.02\%$ ) dry matter ( $62.26 \pm 0.02\%$ ) and gross energy

(56.52  $\pm$  0.03). Hence, cricket meal can be considered as highly digestible compared to fishmeal.

**Table 4.2: Chemical composition (g kg<sup>-1</sup>) of faeces collected from fish fed cricket meal diet and fishmeal diet (% dry matter basis)**

Components	Fishmeal	Cricket meal
Crude protein	14.52	11.30
Crude lipid	1.7	1.6
Ash	4.5	2.9
Dry matter	70.4	71.4
Crude fiber	11.23	12.56
Gross energy (kJ/g)	10.91	10.91
NFE	38.45	43.04

**Table 4.3: Mean ( $\pm$  S.E) Apparent Digestibility Coefficient (ADC %) of crude protein, crude lipid, dry matter and gross energy in the experimental diets.**

Components	Fishmeal	Cricket meal
Crude protein	78.22 $\pm$ 0.03 <sup>a</sup>	81.21 $\pm$ 0.03 <sup>b</sup>
Crude lipid	82.03 $\pm$ 0.02 <sup>a</sup>	89.82 $\pm$ 0.02 <sup>b</sup>
Dry matter	62.26 $\pm$ 0.02 <sup>a</sup>	73.97 $\pm$ 0.01 <sup>b</sup>
Gross Energy	56.52 $\pm$ 0.03 <sup>a</sup>	64.42 $\pm$ 0.01 <sup>b</sup>

<sup>1</sup> Values are means of triplicate groups of 15 fish. Mean values in the same row with different superscript are significantly different ( $P < 0.05$ )

Generally, all essential amino acids (EAA) were present in both diets based on the amino acid composition of the experimental diets presented in Table 4.4. Leucine was the most abundant indispensable amino acid in both diets. With the exception of Isoleucine and Phenylalanine, the values of EAA compounds for the cricket meal were found to be slightly higher than the fishmeal, but not statistically significant at ( $P > 0.05$ ). However, the essential amino acid of methionine and tryptophan in both diets with the addition of phenylalanine in cricket meal did not fulfill the requirement of African catfish as reported Jimoh et al. (2014).

All compounds of amino acid availability coefficient (AAC) in cricket meal exhibited higher level than fishmeal. Apparently, amino acid availability reflected crude protein digestibility. The level of amino acid availability coefficient in cricket meal group (AAC CM) ranges from 0.906 to 0.961 while fishmeal (AAC FM) was between 0.812 to 0.938 with lysine as the most digestible AAC compound for both diets. On the other hand, methionine (0.81) in fishmeal and (0.90) in cricket meal was the least digested indispensable amino acid.

**Table 4.4: Amino acid composition and availability coefficient (AAC) of cricket meal diet and fishmeal diet (g/100g) crude protein**

<b>EAA</b>	<b>CM</b>	<b>FM</b>	<b>AAC CM</b>	<b>AAC FM</b>
Histidine	2.20 ± 0.05	1.61 ± 0.01	0.93	0.85
Arginine	5.20 ± 0.01	5.33 ± 0.01	0.96	0.91
Threonine	3.04 ± 0.04	3.04 ± 0.03	0.92	0.83
Valine	4.53 ± 0.03	4.46 ± 0.03	0.94	0.91
Methionine	1.44 ± 0.03	1.38 ± 0.01	0.90	0.81
Lysine	4.74 ± 0.07	4.88 ± 0.02	0.96	0.94
Isoleucine	3.67 ± 0.03	4.72 ± 0.02	0.94	0.91
Leucine	6.65 ± 0.03	6.26 ± 0.03	0.94	0.90
Phenylalanine	3.77 ± 0.03	4.14 ± 0.01	0.93	0.85
Tryptophan	0.91 ± 0.02	0.89 ± 0.03	0.95	0.90
<b>Non-EAA</b>	<b>CM</b>	<b>FM</b>	<b>AAC CM</b>	<b>AAC FM</b>
Tyrosine	2.84 ± 0.02	2.05 ± 0.01	0.92	0.81
Cystein	1.20 ± 0.03	1.11 ± 0.01	0.90	0.81

<sup>1</sup> Essential Amino Acid requirement for African catfish (g/100g crude protein) according to Jimoh et al. (2014): arginine 3.6, histidine 1.2, isoleucine 2.0, leucine 3.5, lysine 4.8, methionine 2.4, phenylalanine 4.0, threonine 2.8, valine 2.4

<sup>2</sup> Tryptophan requirements for African catfish is 1.1 g/100g crude protein according to Fagbenro and Nwanna (1999).

<sup>3</sup> Values are mean of duplicates groups

The growth performance and feed utilization parameters of *Clarias gariepinus* are shown in Table 4.5. During the experimental period, no mortality was recorded. At the end of the feeding trials, fish that consumed cricket meal diet increased their weight gain significantly ( $P < 0.05$ ) ( $50.13 \pm 0.49$  g/fish) compared to the fishmeal diet ( $37.11 \pm 0.50$  g/fish). Feed conversion ratio (FCR), for cricket meal-fed fish demonstrated a significantly lower level ( $P < 0.05$ ) ( $1.68 \pm 0.004$ ) while protein efficiency ratio (PER) had higher ( $P < 0.05$ ) ( $2.02 \pm 0.004$ ) than those fed with fishmeal. However, the specific growth rate was not significantly affected by both diets ( $P > 0.05$ ).

**Table 4.5: Growth performance of fish fed the experimental diets.**

Variables	Fishmeal	Cricket meal
TFI (g/day)	$82.76 \pm 0.6^a$	$84.22 \pm 1.5^a$
BWG (g fish <sup>-1</sup> )	$37.11 \pm 0.50^a$	$50.13 \pm 0.49^b$
FCR	$2.23 \pm 0.003^a$	$1.68 \pm 0.004^b$
SGR (%/day)	$1.01 \pm 0.001^a$	$1.25 \pm 0.002^a$
PER	$1.50 \pm 0.002^a$	$2.02 \pm 0.004^b$
SR (%)	100 <sup>a</sup>	100 <sup>a</sup>

<sup>1</sup> Values are means of triplicate groups of 15 fish.

<sup>2</sup> Mean values in the same row with different superscript are significantly different ( $P < 0.05$ )

## 4.5 Discussion

Generally, there are three systems to collect faeces that could produce meaningful estimates of nutrient digestibility in ways that could minimize the leaching (Bureau & Cho, 1999). The first system was proposed by Ogino et al. (1973) where fish faeces are collected through a filtration column from the effluent water in the fish tank. The second system is called Guelph system in which a settling column is used to separate the faeces from the effluent water (Cho et al., 1985). The third system was introduced by Choubert et al, 1979 where faecal material was filtered out by mechanical rotating screen and it is called St-Pee system. In this study, the former system by Ogino et al. (1973) was used to collect fish faeces. The uneaten feed were collected 10 to 15 minutes after feeding session and faeces were gathered prior to feeding every day.

The results presented above shows that the cricket meal, which was composed of 30% crude protein cricket meal, was more digestible than fishmeal to *Clarias gariepinus* in terms of crude protein, lipid, dry matter and gross energy. The ADC crude protein value for cricket meal (76.08%) and fish meal (63.92%) were within the range of crude protein ADC levels (58-92%) in various animal and plant-based diets fed to *Clarias isheriensis* (47.5-51.2 g) as reported by Fagbenro (1996).

Other insects such as non-defatted and defatted silkworm pupae resulted in higher protein digestibility than fishmeal when fed to rohu. Mozambique tilapia could utilize silkworm pupae with high apparent protein digestibility up to 86% (Hossain et al., 1992) while walking catfish showed similar digestibility of CP to fishmeal (Borthakur & Sarma, 1998). Besides that, migratory locust also has demonstrated comparable digestibility as that of fishmeal when substituted up to 25% (Abanikannda, 2012). However, other insects such as superworm meal and grasshopper meal have shown

significantly reduced digestibility rates in tilapia and African catfish respectively (Alegbeleye et al., 2012; Jabir et al., 2012).

The current study shows that fishmeal contains lower dry matter and energy ADC level. The crude protein ADC for fishmeal recorded in this study was slightly lower than the 82% reported by Pantazis and Neofitou (2004) for African catfish with approximately 65 g of weight. Alegbeleye et al. (2012) also recorded similar amount of protein digestibility with fingerlings of African catfish (4 – 5 g). This difference is due to many factors including the origin of the fishmeal itself. The fishmeal used in this study was locally made and utilized by most local farmers in Malaysia. Hence, it is more economical but is lower in nutrient availability compared to the imported ones. We believe that studies with local fishmeal are essential for the references of local fish producers although future investigation with higher quality fishmeal will be beneficial as a whole.

Various studies have reported that inclusion of chitin in aqua feeds can improve growth rate in some fish species (Tibbetts & Lall, 2013) and enhances immune response and disease resistance against pathogens (Harikrishnan et al., 2012; and Zhang et al., 2012). Chitin is an integral part of invertebrate cuticle (exoskeleton) and can be used as toxin binder, which significantly diminishes the adverse effect of aflatoxin, and mycotoxin that may possess carcinogenic activity (Khajarearn et al., 2003). However, other studies have suggested that a high level of chitin in fish diet will results in a higher FCR level, thus, reducing growth performance (Goodman, 1989) (and Alegbeleye et al., 2012).

According to Longvah et al. (2011), chitin could inhibit the efficiency of protein utilization. Previous research by Ozimek et al. (1985) has claimed that removal of chitin from honey bees elevated the net protein utilization from 42 to 62, which was almost

equivalent to casein. On the other hand, according to Finke (2007), most of the nitrogen recovered in insect meal is from amino acid but only a small amount is related to chitin. The amount of chitin in black field cricket meal in the current study (7.5%) is lower than adult field cricket, *Gryllus testaceus* (8.7%) (Wang et al., 2005) but somewhat higher than mealworm larvae, *Tenebrio molitor* (5.75%) and black soldier fly larvae, *Hermetia illucens* (4.25%) (Marono et al., 2015).

The lipid ADC of the cricket meal differs significantly ( $P < 0.05$ ) compared to the fishmeal. The high ADC content of lipid from cricket meal indicated a strong ability of African catfish to utilize the lipid component of insects. Lipids are the preferable energy source than carbohydrates and are almost completely digestible in fish. Moreover, an increase in dietary lipids will increase protein digestibility (NRC, 1993).

The ADC for energy is affected by many factors including the source, composition, freshness of the source as well as processing temperature in producing the meal. The energy digestibility observed in commercial fishmeal used in this study is relatively lower than Omena (78.20%) and anchovy fishmeal (86.0%) as reported by Maina et al. (2002). In Japanese seabass, energy digestibility of non-defatted silkworm pupae meal is 75%, which is significantly higher than cricket meal in the present study. Cricket meal digestible energy is also categorised as low when compared to other animal protein sources such as fishmeal, poultry-by-product and hydrolyzed feather meal (Kitagima & Fracalossi, 2011).

Insects appear to be a good source of amino acid. The amounts of other compounds of EAA, except phenylalanine and methionine were higher than the recommended levels for African catfish (methionine - 2.4% and phenylalanine - 4.0%) as suggested by Jimoh et al. (2014). However, the deficiencies were overcome with the additional non-essential amino acids of cysteine and tyrosine.

The AAC of indispensable amino acid in the cricket meal diet was higher and showed fewer variations than that of fishmeal. However, it should be noted that all AAC in both diets was greater than 0.80. To the author's knowledge, no studies on the essential amino acid AAC of insect meal have been conducted in fish nutrition. For this reason, it is not possible to compare the values obtained in the current studies with published data from the fish diet. Nevertheless, Penkov et al. (2002) have demonstrated high amino acid digestibility (lysine 94%, methionine 95%) by using silkworm pupae meal in geese diet. On the other hand, studies with housefly meal in broiler diet reported faecal crude protein digestibility of 69% while the amino acid availability was much higher with >90% which might be attributed to indigestibility of chitin (Pretorius, 2011).

In terms of growth performance indicators, the findings of the present study were consistent with the nutrient ADC observations, whereby, weight gain, FCR, and PER values were better in the cricket meal group than the reference group. However, similar to ADC data, no significant differences were found in SGR and survival rates. The reduced growth performance of fish on the fishmeal group may be due to inefficient utilization of local commercial fishmeal used in this study by African catfish as a result of low digestibility.

#### **4.6 Conclusion**

In conclusion, the result from the present study indicates that protein from cricket meal is well digested by African catfish at the level that is markedly higher than local fishmeal. Although the level is considerably adequate for standard African catfish digestibility, the nutrient digestibility of cricket meal is comparatively less than other protein sources for alternative fishmeal replacement. Processing of cricket meal in order

to enhance fish palatability, nutrient availability, and digestibility to meet the requirements for fish nutrition is potential for future research. The utilisation of cricket meal as an alternative formulated feedstuff was suitable to *Clarias gariepinus* as the EAA present in the diet proved to be sufficient for growth performance. The present findings suggested that cricket meal can be included in African catfish diet without impairing their nutrient digestibility while simultaneously elevating their growth performance

**CHAPTER 5: THE EFFECT OF DIETARY CRICKET MEAL ON THE  
GROWTH PERFORMANCE AND RESISTANCE AGAINST  
PATHOGENICITY OF *Aeromonas hydrophila* IN AFRICAN CATFISH, *Clarias  
gariepinus*.**

**5.1 Introduction**

The main objectives of the aquaculture industry are to gain optimal growth and produce high quality fish, as it is a well-established fact that nutritional status is an essential factor in determining growth and capability of all animals including fish to withstand diseases. Disease outbreak is most common when stress appeared through a variety of factors, which included poor nutrition (Lall, 2000; Pohlenz & Gatlin, 2014). In recent years, heightened attention has been given in the development of proper diets that could improve health, immunity and prevent disease outbreak that could avoid major economic losses in farmed aquatic animals.

In view of the ineffective use of antibiotics in aquaculture, feed with immunostimulant properties has been suggested as alternatives to the antimicrobial agent for improving fish health. In the present experiment, administration of cricket meal as a protein source and recent findings on the effect of cricket meal on the immune response of catfish is discussed.

## 5.2 Literature Review

Knowledge of dietary protein is important in determining the optimum protein requirement for the development of cost-effective feed. Higher dietary crude protein level has been associated with increased growth performance. However, dietary protein has a certain threshold level where the growth development will be depressed as protein intake exceeds beyond that level (Kim & Lall, 2001; Yang et al., 2002).

Motile *Aeromonas* species (MAS) or better known as the aerobic bacteria namely *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* were associated with most hazardous infection disease, which affected African catfish (Janda & Abbott, 2010). Studies have proven that fish infected with MAS led to skin lesion and hemorrhage septicaemia (Ahamad et al., 2013; Anyanwu et al., 2015; Law, 2001) which resulted in massive mortalities and financial loss to fish farmers worldwide. Hence, the production of feed as an immunostimulant for preventing bacteria disease has been in demand especially in avoiding the use of antibiotics. Antibiotics may encourage the development of pathogens, resulting in a negative impact on the fish due to the accumulation of antibiotic residue (Depaola et al., 1995; McPhearson et al., 1991; Schmidt et al., 2000).

Chitin is a natural, common constituent polymer found in crustacean shells, insect exoskeleton and fungi cell wall. It has been widely studied in aquaculture as an immunostimulant to protect salmonid, white shrimp, rainbow trout and brook trout against bacterial disease (Siwicki et al., 1994; Vahedi & Ghodrati-zadeh, 2011; Wang & Chen, 2005). Crickets have been known to contain a significant amount of chitin and have proven to give a positive response in the growth of African catfish (Taufek et al., 2016).

## **5.3 Materials and Method**

### **5.3.1 Experimental diet**

Adult live crickets and all raw ingredients were purchased and processed according to the experimental diet procedure elaborated in Chapter 3, paragraph 3.3.1

Formulation and chemical composition of all the experimental diets and feed ingredients were tabulated in Table 5.1. Three formulated diets were used in the first feeding trial which includes 30% crude protein (30% CP), 35% CP and 40% CP cricket meal diet. The second feeding trial involved 35% and 40% CP cricket meal as well as 35% CP fishmeal as control. Winfeed 2.8 version software was used to establish the formulated feed. All dry ingredients were grounded in a hammer mill (Disk Mill, FFC 454). Vitamins, minerals and DCP were mixed thoroughly with the dry ingredients and water was added to the mixture prior to being pelleted into sizes of 1mm in diameter using a mini pelleting machine (KCM, Y132M-4). The wet pellets were then dried in an oven at 70°C for 24 hours and later stored in a cold room (4°C) until used for feeding.

### **5.3.2 Experimental Fish and set-up**

African catfish were acquired from local farmers and transported to the Freshwater Aquarium Laboratory located in the Institute of Biological Sciences, Faculty of Science, University of Malaya. The fingerlings were randomly divided into three groups for both experiments each in triplicates of 15 fishes (average weight  $15.5 \pm 0.8$  g) and 10 fishes (average weight  $22.5 \pm 0.6$  g) in the first and the second experiment respectively. All the fish were acclimatized to natural environment condition for 2 weeks prior to the feeding trials and fed with commercial diet twice per day during the acclimatization and throughout the experiment. Uneaten food was collected after

feeding and weighed to determine the total feed consumed. Water quality was monitored regularly and any mortality was recorded. Nine plastic tanks (3' x 2' x 1') with a capacity of 100 liters of water with closed re-circulation system were used in these feeding activities. The tanks were equipped with top filter pump at a flow rate of 20 L/min and aeration diffuser was provided in each tank for circulation of dissolved oxygen. Tap water, which was treated with anti-chlorine, was used and 20-30% of water was replaced once in two days to maintain water quality

The feed were given at 10% of their body weight (BW) throughout the experiment for both feeding trials. The experiments were conducted over 56 days for the first feeding trial and 40 days for the second. The water quality for all tanks were measured according to the method by APHA (1992) as described in Chapter 3, paragraph 3.3.2

### **5.3.3 Proximate and chemical analysis**

The experimental diets, ingredients and body composition were analyzed for the proximate composition according to Association of Official Analytical Chemist method (AOAC, 2000) as described in Chapter 3, paragraph 3.3.3. Kjeldahl method was used to analyze crude protein after acid digestion (FOSS Tecator Digester Auto). Moisture and dry matter were measured by drying in an oven at 105°C to constant weight. Meanwhile, ash was determined by combustion in a muffle furnace (Naberthem) at 600°C. Soxhlet method with petroleum ether extraction (FOSS Soxtec 2055) was used to measure crude lipid content. Nitrogen free extract (NFE) is calculated as = 100 - (% crude protein + % crude fat + % crude ash + % crude fiber). Gross energy for every diets and body composition was calculated using the following factors: crude protein = 23.9 kJ/g, crude lipids = 39.8 kJ/g and NFE = 17.6 kJ/g (Schulz et al., 2005).

#### **5.3.4 Haematological and biochemical parameters analysis**

After 40 days of feeding trial for the second experiment, blood samples were randomly collected from five fishes from each tank. About 500 µl of blood from each fish was sampled from caudal circulation and transferred into tubes containing heparin as an anticoagulant. Following centrifugation, plasma was collected and stored in -80°C for biochemical parameters analysis. Prior to centrifugation, 100 µl of the whole blood were separated and stored at 4°C for white blood cell count (WBC). The acetic acid (3%) (v/v), gelatin violet (1%) (w/v) and a drop of methylene blue were mixed for WBC diluting fluid. The blood samples were diluted at 1:20 with triplicate counts for each sample and counted through haemocytometry by using Neubauer haemocytometer.

#### **5.3.5 Plasma total protein determination**

Protein concentration for liver and blood plasma were determined using Bradford assay, which contains Coomassie Brilliant Blue G reagent, and bovine serum albumin (BSA) as the standard (Bradford, 1976). Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml of 95% ethanol. A 100 ml of 85% (v/v) phosphoric acid was added to the mixture and the solution was made up to a final solution of 1 L with distilled water. The solution was filtered with Whatman No.1 filter paper and left overnight before use. Bovine serum albumin (BSA) stock solution with a concentration of 1.0 mg/ml was prepared in distilled water. From the stock solution, six standards solutions were prepared from the range of 51 to 306 µg/ml. The absorbance was read at 595 nm and the amounts of BSA in the standards were plotted against their average absorbance. The protein content of the samples was estimated from the standard curve.

### 5.3.6 Albumin and globulin determination

Plasma albumin was determined by using bromocresol green according to Doumas et al. (1971). The stock solution of bromocresol green was prepared by dissolving 0.15 g (0.25 mM) of bromocresol green into 0.1 M NaOH and diluted to a 1 L with distilled water. Then, 4.5 ml of 30% Brij 35 solution (w/v), 0.004 mM of sodium azide and 150 ml of stock solution was made up into 1 L with 0.075 M succinate buffer. The solution was adjusted to pH 4.2 with 1 M HCl before being stored in 4°C. Albumin standards stock solution were prepared by using 0.05 g/ml bovine serum albumin (BSA) and diluted in ultrapure water. Six standard solutions were prepared from the stock solution ranging from 0.63 - 3.76 µg/ml. A total of 100 µl of samples were put in their respective tubes and 5 ml of bromocresol green reagent was added into each tube before incubation for 5 minutes. The mixtures were then transferred into appropriate cuvettes and absorbances were measured at 620 nm.

Plasma globulin content was calculated by subtracting albumin content from total protein content.

### 5.3.7 Lysozyme assay

Lysozyme activity was assayed spectrophotometrically according to Shugar (1952) with slight modification by using lyophilized *Micrococcus lysodekticus* (A<sub>450</sub>) as the substrate in phosphate buffer (66 mM, pH 6.24). A 100 µl of plasma sample was added to 2.5 ml substrate suspension. The absorbance was measured at 450 nm in room temperature for 10 minutes and the initial and final absorbance were recorded. A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001/min

### 5.3.8 Bacterial pathogen and experimental challenge

*Aeromonas hydrophila* used in this study was obtained from the National Fish Health Research Center (NaFish), located in Batu Maung, Penang, Malaysia. The bacterium was cultured in Tryptone Soy Broth (TSB) overnight at 30°C. Subsequently, the bacterial suspension was washed with phosphate buffer saline (PBS, pH 7.4) and diluted to get the desired concentration for the bacteria challenge.

Prior to performing the bacteria challenge to the fish under study, the LD<sub>50</sub> dose of the bacteria was established by challenging another group of fish to the unrelated but same bacteria sp. Ninety fish were divided into nine tanks, with each treatment being composed of 10 fish. The nine treatments composed of  $1.6 \times 10^4$  to  $10^{12}$  colony forming unit /ml. Mortalities were observed within 96 hours and water variables were monitored during the experiment. The LD<sub>50</sub> calculated from this experiment was  $1.6 \times 10^7$  cfu/ml. This concentration was used for the subsequent challenge.

After 40 days of the second feeding trial, 10 fish per tank were intra-muscularly injected with 0.1 ml bacterial suspension. The fish continued to receive their respective feed and mortalities were recorded daily over 12 days post-challenge.

### 5.3.9 Confirmation of pathogenicity

Freshly dead fish were collected and immediately dissected to isolate *Aeromonas hydrophila* in the liver and intestine. The fish were dissected aseptically whereby the samples were homogenized in 1:10 volume of physiological saline. The homogenized solution was diluted serially and 100 µl from each serial dilution were spread onto selective media, which contained Mueller-Hinton agar supplemented with 5% defibrinated sheep blood and 30µg/ml ampicillin (Misra et al., 1989). They were

incubated at 30°C for 12 hours and the colonies formed were calculated according to Rashid et al. (1994).

$$\text{Bacterial CFU/g of fish organ} = \text{No. of colonies counted in the plate} \times 10^n \times 100$$

Where n was the dilution factor

#### **5.3.10 Analysis of experimental data**

From the data obtained, specific growth rate (SGR), food conversion ratio (FCR), body weight gain (BWG), protein efficiency ratio (PER) and survival rate (%) were calculated after 56 days of the first feeding trial according to Chapter 3, paragraph 3.3.5.

The relative percentage of survival (RPS) of the fish was calculated after challenge with *Aeromonas hydrophila* according to (Amend & McDowell, 1983)

$$\text{RPS} = 1 - (\% \text{ mortality in cricket meal group} / \% \text{ mortality in control group}) \times 100.$$

#### **5.3.11 Statistical analysis**

All data were subjected to one-way analysis of variance (ANOVA) using SPSS version 21.0 (SPSS Inc., Chicago IL, USA). The differences between means were compared by Duncan's post hoc test at 5% ( $P < 0.05$ ) probability level. Data are presented as mean  $\pm$  standard error (SE).

**Table 5.1: Formulation (g kg<sup>-1</sup>) and nutritional profile (%) of experimental diets.**

<b>Ingredients (g kg<sup>-1</sup>)</b>	<b>Replacement (%)</b>			
	<b>Control <sup>6</sup></b>	<b>30%CP <sup>5</sup></b>	<b>35%CP <sup>5,6</sup></b>	<b>40%CP <sup>5,6</sup></b>
Fishmeal	350.0	0	0	0
Cricket meal	0	300.0	350.0	400.0
Soybean meal	184.7	117.7	195.1	287.5
Corn starch	114.3	297.5	234.6	254.7
Rice bran	336.0	269.8	205.3	42.8
Vitamin premix <sup>1</sup>	2.0	2.0	2.0	2.0
Mineral premix <sup>2</sup>	3.0	3.0	3.0	3.0
DCP	10	10.0	10.0	10.0
Total	1000	1000	1000	1000
Nutrient level determined by as is basis (% Dry matter basis)				
Dry matter	94.28	94.36	94.42	94.06
Crude protein	36.55	29.70	34.70	40.20
Crude fat	10.03	10.80	11.82	11.76
Crude ash	9.00	9.97	9.70	8.18
Crude fiber	3.50	3.02	4.90	5.77
Chitin	-	2.40	2.70	3.00
Gross Energy (kJ/g) <sup>3</sup>	18.93	18.60	18.86	19.24
NFE <sup>4</sup>	35.20	40.87	33.3	28.15

<sup>1</sup>The vitamin premix supplied the following per 100 g diet: Vitamin A, 500 IU; Vitamin D3, 100IU; Vitamin E, 0.75 mg; Vitamin K, 0.02 mg; Vitamin B1, 1.0 mg; Vitamin B2, 0.5 mg; Vitamin B3, 0.3 mg; Vitamin B6, 0.2 mg; Vitamin B12, 0.001 mg; Vitamin C, 0.1 mg; Niacin, 0.2 mg; Folic Acid, 0.1 mg; Biotin, 0.235 mg; Pantothenic acid, 1.0 mg, Inositol, 2.5 mg

**Table 5.1 continued**

<sup>2</sup>The mineral premix supplied the following per kg diet: Selenium, 0.2 mg; Iron, 8 mg; Manganese 1.0 mg; Zinc, 8.0 mg; Copper, 0.15 mg; Potassium Chloride, 0.4 mg; Magnesium Oxide, 0.6 mg; Sodium Bicarbonate, 1.5 mg; Iodine, 1.0 mg; Cobalt, 0.25 mg.

<sup>3</sup> Gross energy for every diets and body composition was calculated using the following factors: crude protein = 23.9 kJ/g, crude lipids = 39.8 kJ/g and NFE = 17.6 kJ/g (Schulz et al., 2005).

<sup>4</sup>NFE= % Dry matter - (% crude protein + % crude fat + % crude ash+ % crude fiber)

<sup>5</sup>Diets used in the first feeding trial

<sup>6</sup>Diets used in the second feeding trial

## 5.4 Results

In this study, the level of dietary protein influenced growth parameters. Significantly high levels of weight gain, specific growth rate and protein efficiency ratio were observed in fish fed with 35% crude protein diet ( $P < 0.05$ ) (Table 5.2). Food conversion ratio was lowest in the diet of 35% CP, although no significant difference was found in the diet of 40% CP. The result demonstrated that fish fed with dietary protein at 30% shows the lowest growth response but improved ( $P < 0.05$ ) as the dietary level increased to 35% CP and slightly diminished as the level reached 40% CP. However, the survival rate for all treatments remains at 100% throughout the experimental period. These indicate that diet with 35% CP level satisfied the requirement to achieve best growth performance for African catfish. In the second experiment, dietary 35% and 40% were selected due to the insignificant differences observed between both diets in terms of BWG and SGR compared to 30% group.

**Table 5.2: Growth performance of fish fed the experimental diets.**

Variable	30%CP	35%CP	40%CP
TFI (g fish <sup>-1</sup> )	56.44 ± 1.80 <sup>b</sup>	57.90 ± 1.48 <sup>b</sup>	51.93 ± 1.87 <sup>a</sup>
BWG (g fish <sup>-1</sup> )	10.70 ± 0.43 <sup>a</sup>	15.54 ± 0.67 <sup>b</sup>	13.26 ± 0.38 <sup>ab</sup>
FCR (g/g)	1.94 ± 0.81 <sup>a</sup>	1.5 ± 0.45 <sup>a</sup>	1.6 ± 0.12 <sup>a</sup>
SGR (g/g)	1.99 ± 0.11 <sup>a</sup>	2.55 ± 0.17 <sup>b</sup>	2.28 ± 0.23 <sup>ab</sup>
PER (g/g)	1.86 ± 0.04 <sup>a</sup>	2.29 ± 0.10 <sup>b</sup>	1.95 ± 0.09 <sup>a</sup>
SR (%)	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>

<sup>1</sup>Values are the mean ± SEM of triplicate groups of 15 fish.

<sup>2</sup>Mean values in the same row with different superscript letters are significantly different ( $P < 0.05$ )

White blood cell (WBC) count of fish fed control diet was significantly lower than cricket meal fed-fish (Table 5.3). However, the values did not differ among fish fed cricket meal diets. A significant reduction ( $P < 0.05$ ) in total protein was detected in control group whereas no significant differences were observed in both cricket meal groups. On the other hand, the level of albumin did not show significant differences among all diets ( $P > 0.05$ ). The globulin concentration significantly decreased in the control group although no significant difference was observed in the 40% CP cricket meal fed fish. Similarly, lysozyme activity of those fed the control diet reduced significantly ( $P < 0.05$ ) when compared to cricket meal diet.

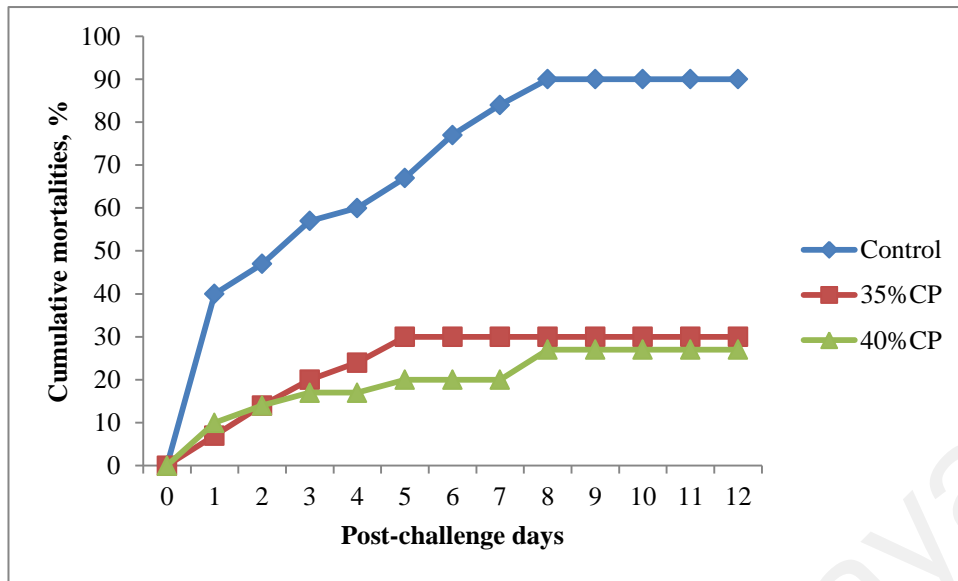
**Table 5.3: Biochemical parameters, WBC and lysozyme activity of African catfish fed with experimental diets**

Parameters	Control	35% CP	40% CP
WBC ( $10^3 \text{ mm}^3$ )	$13.56 \pm 0.86^a$	$18.46 \pm 0.75^b$	$19.17 \pm 0.13^b$
Total protein (mg/dL)	$53.42 \pm 5.59^a$	$59.71 \pm 7.81^b$	$61.14 \pm 6.15^b$
Albumin (mg/dL)	$1.90 \pm 0.12^a$	$1.98 \pm 0.16^a$	$2.19 \pm 0.22^a$
Globulin (mg/dL)	$51.52 \pm 5.49^a$	$57.73 \pm 7.76^b$	$58.95 \pm 6.08^b$
Lysozyme (U/ml)	$8.4 \pm 1.16^a$	$19.6 \pm 2.71^b$	$22.2 \pm 2.54^b$

<sup>1</sup>Values are the mean  $\pm$  SEM of triplicate groups of 10 fish.

<sup>2</sup>Mean values in the same row with different superscript letters are significantly different ( $P < 0.05$ )

The intramuscular injection resulted in 90% mortalities in fish fed with fishmeal diet (Figure 5.1) within 8 days of post inoculation whereas the fish fed with 35% and 40% dietary protein of cricket meal caused 30% and 27% mortalities after 5 and 8 days of post-injection respectively. The relative percentage of survival (Table 5.4) proved that fish fed with diet containing 35% and 40% CP cricket meal showed no significant difference ( $P > 0.05$ ) and they were more tolerant to these diets since higher survival were recorded in both diets than control.



**Figure 5.1: Cumulative mortalities of fish fed with cricket meal and fishmeal (control) in 12 days post- challenge with *Aeromonas hydrophila* infection**

**Table 5.4: Relative percentage of survival and mortality of fish fed with cricket meal and fishmeal (control) in 12 days post-challenged with *Aeromonas hydrophila***

Diets	Survival (%)	Mortality (%)	RPS (%)
Control	10	90	-
35% CP	70	30	66.7
40% CP	73	27	70

<sup>1</sup> Fish were challenged by intramuscular injection with  $1.6 \times 10^7$  cfu/ml of *Aeromonas hydrophila*

<sup>2</sup> All groups consisted of 30 fingerlings

To confirm the pathogenicity of *Aeromonas hydrophila*, the bacteria were isolated from the liver and intestines of experimental infected fish into Mueller-Hinton agar (Figure 5.2). Table 5.5 demonstrated that the highest bacterial load was found to be  $5.82 \times 10^7$  cfu/g in the intestine of the control group while the lowest was  $1.34 \times 10^5$  cfu/g in the liver of fish fed with 35% CP cricket meal diet.

**Table 5.5: Bacterial load in liver and intestine of fish challenged with *Aeromonas hydrophila***

Diets	Liver (cfu/ml)	Intestine (cfu/ml)
Control	$6.25 \times 10^5$	$5.82 \times 10^7$
35% CP	$1.34 \times 10^5$	$3.22 \times 10^6$
40% CP	$1.51 \times 10^5$	$4.05 \times 10^6$

<sup>1</sup> Liver and intestine were excised from dead and infected fish



**Figure 5.2: Bacteria isolated from intestine of infected African catfish 12 days post-challenge**

## 5.5 Discussion

Proteins are biomolecules that are composed of 50% carbon, 16% nitrogen, 21.5% oxygen and 6.5% hydrogen. Due to the fact that protein is one of the most expensive component in fish feed, protein requirement for each fish cultured need to be taken into consideration. Wastage of dietary protein due to non-quantification of accurate protein requirement by using any protein source in the fish feed will lead to profit reduction and most importantly to environmental issues. Although fish are capable of ingesting high protein diet, approximately 65% of the protein consumed might be excreted as ammonia ( $\text{NH}_3$ ) as well as solid waste (Craig & Helfrich, 2009). Hence, the optimum level of dietary protein not only will promote high growth and feed efficiency but also reduce water quality deterioration especially in intensive fish farming or recirculating aquaculture systems (Oishi et al., 2010).

The results of the present study clearly show that dietary cricket meal of 35% crude protein level enhanced the growth of African catfish. The optimum protein levels in the diet for African catfish defined by weight gain, FCR, PER and SGR was found to be 35%. This finding is consistent with a report from past studies by Farhat and Khan (2011) which recommended the inclusion of protein in the range of 34.4% - 39.6% to optimize the growth potential in African catfish fingerling. However, the protein requirement in this study is higher than the required amount suggested for *Clarias batrachus* with 30% inclusion level (Chuapoehek, 1987), although higher values (40 – 55%) were suggested for warm water fish in general (NRC, 1983).

Protein or amino acids are essential for growth, reproduction and replacement of depleted tissue. The amino acid is converted to glucose as an energy source for the brain and red blood cells. The optimum crude protein in the fish diet may vary due to different factors including water quality, diet composition, protein quality, digestibility,

fish size and genetics (Akiyama et al., 1997; De Silva et al., 2000; Fatma Abidi & Khan, 2010; Shearer, 2000). In the present study, fish fed with 30% CP shows depressed growth after 56 days of feeding. This may be attributed to insufficient digestible energy to crude protein (DE/P) ratio due to lower crude protein level (Robinson et al., 2006). At each protein level, feed consumption was reduced in diets with the highest energy levels. Growth depression was also observed as the diet reached up to 40% CP when dietary energy becomes too high, the feed consumption is reduced, resulting in less intake of essential nutrients (Farhat & Khan, 2011; Robinson et al., 2006).

In the second experiment, the white blood cell count showed a significant increased level in the fish fed cricket meal diet. White blood cells play an important role in non-specific immunity and indicator of fish health status. Hence, increase WBC's count reduce the immunosuppression caused by *Aeromonas hydrophila* diet due to the presence of chitin as immunostimulant in cricket meal. This result is also supported by other works that reported an increase in WBC count in *Labeo rohita* juveniles when fed with levamisole and ascorbic acid (Choudhury et al., 2005) and garlic peel in African catfish diet (Thanikachalam et al., 2010).

The plasma/serum protein particularly albumin and globulin play a major role in maintaining immune response of fish. In the present study, increased plasma protein and globulin of fish fed with cricket meal suggests a stronger immune response of fish fed this diet. Previous research by Siwicki (1990) has reported an elevated level of serum total protein when  $\beta$ -glucan (0.2%) and chitosan (0.5%) in common carp diet. Besides, increased in total protein, albumin and globulin were also observed in olive flounder fed with chitosan (Dautremepuits et al., 2004a).

Lysozyme activity has been primarily used as a defense mechanism of nonspecific humeral immunity that could disrupt the cell wall of harmful pathogens invaders particularly parasites, bacteria and virus. It has been reported to have antibacterial activity, which could cause lysis and stimulate phagocytosis in bacteria (Ellis, 1999). The increase in lysozyme activity of fish fed with cricket meal suggested that the presence of chitin could enhance the lysozyme activity in fish and can be considered to be a natural protective mechanism in fish. A number of studies indicate that chitin and chitosan, as well as herbal supplementation enriched diet could elevate lysozyme activity in fish. Diet enriched with 1% chitin has been reported to increase lysozyme activity in *Cirrhina mirgala* (Mari et al., 2014) while Esteban et al. (2001) reported no significant differences between 100 mg/kg chitin and control diet fed to gilthead seabream. Based on previous studies in *Cyprinus carpio*, dietary inclusion of chitosan induced significantly higher lysozyme activity in chitosan fed fish followed by levamisole and chitin (Gopalakannan & Venkatesan, 2006). On the other hand, African catfish fed supplemented indigenous plant has also been reported to increase lysozyme activity (Kumar et al., 2013).

Disease resistance was measured by determining the survival of the animal after being challenged with certain pathogen (Palti et al., 1999). Fish are most susceptible to bacteria and virus due to direct contact with the environment. The mucus and skin or scales are natural barriers to foreign substances, and act as non-specific or innate defense mechanism thus suppressing the colonization of fish pathogen. These mechanisms prevent the attachment, invasion or multiplication of the invaders on or in the tissues. In the current study, RPS of fish fed with cricket meal was higher (66.7-70%) up to 12 days post-injection in comparison to fishmeal diet. Relative percentage

survival values exceeding 50% indicates a positive effect of the vaccine (Amend & McDowell, 1983).

From the observation after the challenge, all fish developed clinical sign such as loss of balance, spreading of grayish – white lesion on the surface of the body up to caudal fin and the fin bases become reddish in color. These clinical symptoms were observed in all fish. However, the group fed with cricket meal showed wound recovery after six days post-challenge with no loss in appetite and hence consumed their respective feed up until 12 days after challenge. As a result, the mortality rate was reduced and they stop dying after 8 and 5 days post-infection for 40% CP and 35% CP respectively. This situation might be due to enhancement of the non-specific immune system of the fish by cricket meal. On the other hand, the control group exhibited highly stressed condition and thus did not consume their feed and consequently most of them died after 12 days post-challenge.



**Figure 5.3: Fish fed with cricket meal showed recovered wound (arrow) on the injected area 6 days post-challenge**

Wang et al. (2005) have reported that field crickets contain 8.7% chitin; a compound which has strong experimental evidence as an effective immunostimulant in various fishes and also has the ability to improve growth performance of snow trout and golden mahseer (Mohan et al., 2009). Some studies have also demonstrated the potency of chitin as immunostimulators that could enhance immune response, disease resistance and survival of fish and shellfish (Esteban et al., 2001; Kawakami et al., 1998; Sakai et al., 1992). Mari et al. (2014) have observed approximately 70% reduction of mortalities of *Cirrhina mrigala* against *Aphanomyces invadans* bacteria when fed 10% chitin supplemented diet. On the other hand, a diet containing 0.75% chitin shows a significantly high RPS (63.16%) in *Macrobrachium rosenbergii* compared to chitin-free diet when challenged against white tail disease viruses. However, studies using insects meal to evaluate the effect on immunostimulant is very limited. At present, housefly, *Musca domestica* have proven to provide increased protection against *Edwardsiella trada* and significantly enhanced peritoneal phagocytic activity in red sea bream (Ido et al., 2015).

The intestine and liver tissues of African catfish infected with *Aeromonas hydrophila* were cultured to confirm death as a result of *Aeromonas hydrophila* and the pathogenicity test was also conducted to determine the bacterial loads isolated in both livers and intestines of fish fed the experimental diets. Based on bacterial isolation from the fish after the challenge, the intestine showed higher bacteria load than liver. This was also observed in the climbing perch with  $9.4 \times 10^8$  cfu/g and  $2.9 \times 10^6$  cfu/g in intestine and liver respectively (Hossain et al., 2013). In addition, Asian stinging catfish, *Heteropneustes fossilis* also exhibited higher levels of bacteria accumulation in intestine ( $1.8 \times 10^9$  cfu/g) than liver ( $6.46 \times 10^8$  cfu/g) (Mostafa et al., 2008). However, a finding by Sarkar and Rashid (2012) in their study indicated that walking catfish,

*Clarias batrachus* showed higher bacterial load in the liver ( $6.5 \times 10^8$  cfu/g) compared to intestine ( $5.6 \times 10^7$  cfu/g). Thus, it was proven that *Aeromonas hydrophila* was pathogenic to African catfish, which caused 90% mortalities if the fish were only fed with fishmeal without any immunostimulant.

## 5.6 Conclusion

These findings suggested that in general, cricket meal with 35% crude protein level were able to improve growth performance and reduce disease resistance against pathogenic *Aeromonas hydrophila* as it enhances the non-specific immunity of African catfish. Antibody produced from the immunostimulatory effect on cricket meal plays a role in conferring significant protection against *Aeromonas hydrophila* infection on catfish. Thus, cricket meal can act as an immunostimulant and potential protein sources in African catfish diet. Further studies need to be carried out to isolate and characterize the active compound in cricket meal other than chitin that was responsible for antibacterial activity and determines other biochemical aspects of the fish against *Aeromonas hydrophila*.

**CHAPTER 6: THE EFFECT OF DIETARY CRICKET MEAL (*Gryllus bimaculatus*) ON GROWTH PERFORMANCE, ANTIOXIDANT ENZYME ACTIVITIES AND HAEMATOLOGICAL RESPONSE OF AFRICAN CATFISH (*Clarias gariepinus*).**

**6.1 Introduction**

Today, insects meal are gaining acknowledgement from researchers for their potential as fishmeal replacement. However, a major concern associated with the use of insects is the possibility of the presence of harmful substance or compound in the insects that could cause unfavorable physiological effects on fish which could lead to a reduction in growth performance (Barros et al., 2002; Zheng et al., 2012) and occurrence of abnormalities in haematological parameters (Dabrowski et al., 2001; Yue & Zhou, 2008).

Crickets belong to the Orthoptera order of insects, which was reported to have a high crude protein level ranging from 55% to 73% and sufficient essential amino acid except for methionine and lysine, which can be supplied directly in the feed (Barroso et al., 2014; Finke, 2002). Presently, live crickets are commercially available in pet stores, supplied as fish baits or supplementary feed for ornamental fish such as Arowana and reptiles such as iguana due to their high nutritional value. Previous study by Taufek et al. (2013) have reported that cricket meal can replace up to 100% of fishmeal and produced better growth performance than the control diet in African catfish nutrition.

Hence, this study was conducted due to its importance in assessing the suitability of cricket meal in African catfish nutrition without compromising on their growth and feed efficiency. Thus, the aim of this research was to elucidate the influence of the field

cricket, *Gryllus bimaculatus* as full or partial substitute for fishmeal on growth, antioxidant response and hematological parameters in African catfish.

## 6.2 Literature Review

Reactive oxygen species (ROS) affects all aerobic organisms including fish. It is regarded to be major mediators of oxygen cytotoxicity (Buetler et al., 2004). In response, animals developed an antioxidant defense mechanism to combat the action of ROS. Lacking in antioxidant defense will increase ROS, resulting in oxidative stress (Jacob, 1995). This mechanism involves various antioxidant compounds, consisting of dietary tocopherol, vitamin C and antioxidant defense enzymes (Buettner, 1993). The oxidation of lipid present in the feed could affect palatability of the feed, therefore reducing feed intake and fish growth. Besides, amino acids, fatty acids, vitamins and minerals deficiencies can also affect feed intake (Ogunji et al., 2007).

A number of enzymes known to have major antioxidant activity in fish including superoxide dismutase (SOD) (EC 1.15.1.1) which is responsible for catalyzing the reduction of superoxide anion ( $O_2^-$ ) to hydrogen peroxides ( $H_2O_2$ ) while catalase (CAT) (EC 1.11.1.6) is accountable for reducing  $H_2O_2$  to  $H_2O$ . Another enzyme activity that has been and is continuously being studied in fish is Glutathione *S*-transferase (GST) (EC 2.5.1.18), which is in charge of the detoxification of foreign compounds. This enzyme catalyzes the conjugation of glutathione reduced form (GSH) with compound containing electrophilic center through the formation of a thioether bond between the sulphur atom of GSH and the substrate (Chasseaud, 1979; Mannervik, 1985). The activities of these enzymes may differ in organs and tissues of freshwater and marine fish (Wdzięczak et al., 1982). Other factors that determine the activities of antioxidant enzymes in fish can include feeding behavior, environmental condition and other

ecological backgrounds (Radi & Matkovics, 1988; Roche & Bogé, 1996; Winston & Di Giulio, 1991).

Haematological indices are essential tools as an indicator to evaluate the physiological and physiopathological changes in fish (Hrubec et al., 2000; Rainza-Paiva et al., 2000). Stocking density and feeding habits are among the ecological factors that influence biochemical parameters (Barnhart, 1969; Turnbull et al., 2005). Besides that, pollutant and other environmental factors could also induce the variations in haematological parameters.

## **6.3 Material and method**

### **6.3.1 Experimental diet**

Adult live field cricket and all raw ingredients were purchased and processed according to the experimental diet procedure elaborated in Chapter 3, paragraph 3.3.1.

Formulation and chemical composition of all the experimental diets and feed ingredients are tabulated as in Table 6.1. Three formulated isonitrogenous and isoenergetic diets (35% crude protein and 19 kJ/g respectively) were used in the feeding trials. They were; 100% cricket meal (100% CM), 75% cricket meal (75% CM) and 100% fishmeal (100% FM). The 100% FM acted as control. The experimental diets were chosen according to the previous result in Chapter 3. Winfeed version 2.8 software was used to formulate the diets.

### 6.3.2 Experimental Fish and set-up

African catfish, (*Clarias gariepinus*) were obtained from local farmers and transported to the Freshwater Aquarium Laboratory located at the Institute of Biological Sciences, Faculty of Science, University of Malaya. The fingerlings were randomly divided into three groups each in triplicates of 10 fishes (average weight  $13.2 \pm 0.3$  g). All the fish were acclimatized to natural environmental conditions for 2 weeks prior to the feeding trials and fed with commercial diets twice per day during the acclimatization period. The feed was given twice daily *ad-libitum* during the trials for over forty-nine days. Total feed intakes were recorded and all uneaten food was collected after feeding and weighed to determine the total feed consumed. Water quality was monitored regularly and any mortality was recorded. Nine plastic tanks (3' x 2' x 1') with a capacity of 100 liters of water with closed re-circulation system were used in these feeding activities. The tanks were equipped with top filter pumps at a flow rate of 20 L/min together with an aeration diffuser in each tank for circulation of dissolved oxygen. Dechlorinated tap water was used and 20-30% of water was replaced once within two days to maintain water quality.

The water quality for all tanks were measured according to the method by APHA (1992) as described in Chapter 3, paragraph 3.3.2

### 6.3.3 Proximate and chemical analysis

The experimental diets and ingredients were analyzed for proximate composition according to the Association of Official Analytical Chemist methods (AOAC, 2003) as described in Chapter 3, paragraph 3.3.3. Kjeldahl method was used to analyze crude protein after acid digestion (FOSS Tecator Digestor Auto). Moisture and dry matter were measured by drying in an oven at 105°C to constant weight.

Meanwhile, ash was determined by combustion in a muffle furnace (Naberthem) at 600°C. Crude lipid content was measured by using petroleum ether extraction (FOSS Soxtec 2055) according to Soxhlet method. Fiber was determined after alkali and acid digestion. Chitin was estimated by using the acid detergent fiber (ADF) and protein residue of ADF according to Marono et al, (2015). Nitrogen free extract (NFE) was calculated as = 100 - (% crude protein + % crude fat + % crude ash). Gross energy for every diets and body composition was calculated using the following factors: crude protein = 23.9 kJ/g, crude lipids = 39.8 kJ/g and NFE = 17.6 kJ/g according to Schulz et al. (2005).

#### **6.3.4 Amino acid analysis**

Amino acid profiles for diets was determined using the High Performance Liquid Chromatography (HPLC) (JASCO CO-2065 Plus, Intelligent Column Oven) and the contents were determined by comparison peak retention times to a known standard by using the Pico-Tag method by Heinrikson and Meredith (1984) while Tryptophan was determined after alkaline hydrolysis according to Nielsen and Hurrell (1985) as described in Chapter 3, paragraph 3.3.4.

#### **6.3.5 Sample preparation**

After 7 weeks of feeding trials, liver and blood samples were randomly collected from five fishes in each tank. The sacrificed fish were marked individually and their body weights, as well as liver weights were recorded accordingly. The livers were excised and weighed in order to obtain the hepatic somatic index (HSI%). A total of 0.6 g of each liver was homogenized in 6 ml buffer containing 25 mM sodium phosphate buffer (pH 7.4), 0.1 mM protease inhibitor, 1.0 mM EDTA, 0.1 mM dithiothreitol (DTT) and 0.1 mM phenylthiourea (PTU). The homogenates were

centrifuged at  $100,000 \times g$  (Beckman 80Ti) for 30 minutes at  $4^{\circ}\text{C}$  and the supernatants were stored at  $-80^{\circ}\text{C}$  for further analysis.

Prior to liver collection, blood was collected from five fish in each tank. About  $500 \mu\text{L}$  of blood from each fish was sampled from caudal circulation, which was divided into two portions; one for haematological parameters and another for plasma protein analysis. The blood was drawn using 1 ml syringe with needle size of 22G  $1\frac{1}{2}$  inch and placed in anti-coagulant tubes before being stored in a refrigerator ( $4^{\circ}\text{C}$ ) until its use for further haematological analysis. Another portion was centrifuged at 3500 rpm for 15 minutes to obtain plasma and later stored in a  $-80^{\circ}\text{C}$  freezer until plasma protein analysis.

#### **6.3.6 Haematological parameters analysis**

Red blood cell (RBC) and white blood cells (WBC) counts were done microscopically using Neubauer haemocytometer (Assistant, Germany). Isotonic saline (0.85%) was used for RBC diluent while 3% acetic acid (v/v), 1% gelatin violet (w/v) and a drop of methylene blue were mixed for WBC diluting fluid. The blood samples were diluted at 1:200 and 1:20 for RBC and WBC respectively, with triplicate counts for each sample.

Hematocrit (Ht) was analyzed using capillary tubes and spun at 12,000 rpm for 5 minutes in a microhematocrit centrifuge. The result was then measured according to microcapillary reader. Hemoglobin (Hb) was determined by calorimetric cyanmethemoglobin method as described by Baker and Silverton (1985). Human hemoglobin (Sigma-Aldrich) was used as standard and 5 ml of Drabkin's Reagent (Sigma-Aldrich) was diluted with  $20 \mu\text{L}$  of whole blood sample and incubated for 15 minutes. The samples were measured at an absorbance of 540 nm.

Red blood indices, which include mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were also calculated.

### 6.3.7 Liver protein concentration

Protein concentration of liver were determined by using Bradford assay as described in Chapter 5, paragraph 5.3.5

### 6.3.8 Oxidative stress parameters

#### 6.3.8.1 Catalase

Catalase (CAT) (EC 1.11.1.6) activity was assayed according to Claiborne (1985). The 50 mM Sodium phosphate buffer was prepared and adjusted to pH 7.0 at 25°C by using 1M hydrochloric acid. Then, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (19 mM) was prepared by using sodium phosphate buffer. In a 3 ml reaction mix, 2.65 ml of sodium phosphate buffer, 50 µl of samples and 300 µl of H<sub>2</sub>O<sub>2</sub> were added to a cuvette. The reaction was quantified at 25°C by measuring the disappearance of H<sub>2</sub>O<sub>2</sub> at 240 nm within 5 minutes. CAT activity was reported in terms of nmol H<sub>2</sub>O<sub>2</sub> consumed/min/mg/protein ( $\epsilon_{240\text{ nm}} = 0.0436\text{ mM/cm}$ ).

Calculation

$$\text{Enzyme activity: } \frac{[D_{240\text{nm}}/\text{min} \times (\text{sample} - \text{blank}) \times 3 \times df]}{0.0436 \times \text{sample}(ml)}$$

Specific activity (nmol): Enzyme activity/protein concentration of sample

#### 6.3.8.2 Superoxide dismutase

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured according to the method of McCord and Fridovich (1969). SOD assay is composed of solution A, which contains 25 ml of 200 mM sodium phosphate buffer (pH 7.8), 50 ml of 0.01 mM xanthine, 1 ml of 1.1 mM cytochrome c and 1 ml of 10.70 mM Ethylenediaminetetraacetic acid sodium salt dihydrate (EDTA). All reagents were reconstituted in 100 ml beaker and mixed well. A small amount of 1 M NaOH was added to dissolve xanthine and pH was adjusted to 7.8 with 1 M HCl at 25°C. The solution was then transferred to a volumetric flask and made up to 100 ml with purified water.

Xanthine oxidase (XOD) was prepared in ice-water containing approximately 0.08 unit/mg immediately before its use. A total of 2.8 ml solution A was pipetted into 4 cuvettes, each in duplicate samples of blank and XOD. The absorbance was monitored spectrophotometrically at 550 nm in 25°C until constant and then 200 µl of purified water was added into the blank cuvette while 100 µl of purified water and 100 µl of XOD reagent was added to the respective XOD cuvettes. The increase in absorbance was monitored for approximately 5 minutes and the change of absorbance for the XOD cuvettes versus the blank for this reaction were maintained at  $0.025 \pm 0.005$ .

All reagents were equilibrated to 25°C prior to the beginning of the assay. A total of 2.8 ml of solution A was added to the sample cuvette and 100 µl of samples were added. Each sample was monitored in triplicate. The absorbance was observed at 550 nm until constant, then 100 µl of XOD were added into all samples cuvettes. The inversions were mixed thoroughly and increases in absorbance at 550 nm within 5 minutes were observed. The fastest linear rate over 1 minute interval for the XOD

reaction was recorded. The rates for each sample and blank were obtained by using this time interval. The  $\Delta A_{550}$  nm for each sample was within 20 - 60% of the XOD rate. In the reaction mix, the final concentration consisted of 50 mM Sodium phosphate buffer, 0.1mM EDTA, 0.01 mM cytochrome c, 0.05 mM xanthine and 0.008 mM xanthine oxidase. SOD activity was calculated by its ability to inhibit 50% reduction of cytochrome c and the result is expressed as nmol/min/mg/protein.

#### Calculation

$$\text{Percent inhibition: } \frac{[DA_{550nm}/\text{min} \times (XOD - \text{sample}) \times 100]}{DA_{550nm}/\text{min} \times (XOD - \text{blank})}$$

$$\text{Specific activity: } \frac{(\% \text{inhibition} \times df)}{50\% \times \text{sample}(ml) \times \text{sample}(protein)}$$

#### 6.3.8.3 Glutathione S-transferase

Glutathione S-transferases (GST) (EC 2.5.1.18) was evaluated by measuring the activity towards 1-Chloro-2,4-dinitrobenzene (CDNB) at 340 nm as described by Habig et al. (1974). In a 3 ml cuvette, the assay contains 2.85 ml of 100 mM sodium phosphate buffer (pH 6.5), 50  $\mu$ l of 60 mM glutathione (GSH) (dissolve in sodium phosphate buffer), 50  $\mu$ l of samples and 50  $\mu$ l of 60 mM CDNB (dissolve in ethanol). Each sample was monitored in triplicate. One unit of GST activity was calculated as the amount of enzyme catalyzing the conjugation of 1 $\mu$ mol of CDNB with GSH per minute at 25°C ( $\epsilon_{340 \text{ nm}} = 9.6 \text{ mM/cm}$ ). The result is expressed as nmol/min/mg protein.

### Calculation

$$\text{Enzyme activity: } \frac{[DA_{340nm}/\text{min} \times (\text{sample} - \text{blank}) \times 3 \times df]}{9.6 \times \text{sample}(ml)}$$

Specific activity (nmol): Enzyme activity/protein concentration of sample

### 6.3.9 Analysis of experimental data

From the data obtained, specific growth rate (SGR), food conversion ratio (FCR), body weight gain (BWG), protein efficiency ratio (PER) and survival rate (%) were calculated according to Chapter 3, paragraph 3.3.5.

Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Seiverd (1983).

$$MCV (fl) = Ht / Hb$$

$$MCH (pg) = \frac{(Hb \times 10)}{RBC}$$

$$MCHC (\%) = (Hb / Ht) \times 100$$

### 6.3.10 Statistical Analysis

All data were subjected to one-way analysis of variance (ANOVA) using SPSS version 21.0 (SPSS Inc., Chicago IL, USA). The differences between means were compared by Duncan's post hoc test at 5% ( $P < 0.05$ ) probability level. Data are presented as mean  $\pm$  standard error of mean (SEM).

**Table 6.1: Formulation (g kg<sup>-1</sup>) and nutritional profile (%) of experimental diets**

<b>Ingredients (g kg<sup>-1</sup>)</b>	<b>Experimental diets (%)</b>		
	<b>100%CM</b>	<b>75%CM</b>	<b>100%FM</b>
Fishmeal	0	87.5	350.0
Cricket meal	350.0	262.5	0
Soybean meal	195.1	286.1	335.5
Corn starch	234.6	83.9	99.4
Rice bran	205.3	265.0	200.1
Vitamin premix <sup>1</sup>	2.0	2.0	2.0
Mineral premix <sup>2</sup>	3.0	3.0	3.0
DCP	10.0	10.0	10.0
Total	1000	1000	1000
Nutrient level determined by as is basis (% Dry matter basis)			
Dry matter	94.36	94.76	95.42
Crude protein	35.35	35.61	36.55
Crude fat	10.50	9.00	8.06
Crude ash	8.90	7.42	8.38
Crude fiber	5.14	4.51	3.29
Chitin	2.70	2.50	-
NFE <sup>3</sup>	40.1	42.46	41.72
Gross Energy <sup>4</sup>	19.69	19.57	19.29

<sup>1</sup>The vitamin premix supplied the following per 100 g diet: Vitamin A, 500 IU; Vitamin D3, 100IU; Vitamin E, 0.75 mg; Vitamin K, 0.02 mg; Vitamin B1, 1.0 mg; Vitamin B2, 0.5 mg; Vitamin B3, 0.3 mg; Vitamin B6, 0.2 mg; Vitamin B12, 0.001 mg; Vitamin C, 0.1 mg; Niacin, 0.2 mg; Folic Acid, 0.1 mg; Biotin, 0.235 mg; Pantothenic acid, 1.0 mg, Inositol, 2.5 mg

**Table 6.1 continued**

<sup>2</sup>The mineral premix supplied the following per kg diet: Selenium, 0.2 mg; Iron, 8 mg; Manganese 1.0 mg; Zinc, 8.0 mg; Copper, 0.15 mg; Potassium Chloride, 0.4 mg; Magnesium Oxide, 0.6 mg; Sodium Bicarbonate, 1.5 mg; Iodine, 1.0 mg; Cobalt, 0.25 mg.

<sup>3</sup>NFE= 100- (%protein + %fat + % ash+ %fiber)

<sup>4</sup>Gross Energy was calculated as 23.9, 39.8, 17.6 kJ/g for protein, fat and NFE respectively (Schulz et al., 2005)

**Table 6.2: Essential Amino acids of cricket meal diets and African catfish requirements**

Amino acids (g/100g protein)	Experimental diets		African catfish EAA
	75% CM	100% CM	
Histidine	2.25	2.23	1.2 <sup>1</sup>
Arginine	6.20	5.80	3.6 <sup>1</sup>
Threonine	3.62	3.51	2.8 <sup>1</sup>
Valine	5.29	5.32	2.4 <sup>1</sup>
Methionine	1.51	1.52	2.4 <sup>1</sup>
Isoleucine	3.93	3.86	2.0 <sup>1</sup>
Leucine	6.82	6.56	3.5 <sup>1</sup>
Phenylalanine	3.86	3.94	4.0 <sup>1</sup>
Lysine	4.53	4.79	4.8 <sup>1</sup>
Tryptophan	0.97	0.94	1.1 <sup>2</sup>
Methionine + Cystine *	2.69	2.75	2.4

\* Non-essential amino acid

<sup>1</sup> African catfish requirement according to Jimoh et al. (2014)

<sup>2</sup> African catfish requirement according to Fagbenro and Nwanna (1999)

## 6.4 Results

Chitin was estimated in the cricket meal diet based on chitin composition in raw cricket meal. The raw cricket meal contains approximately 7.15% chitin after ADF analysis, while the cricket meal diet used in this experiment was estimated to have 2.50% and 2.70% chitin for 75% CM and 100% CM, respectively. Throughout the experimental period, water parameters were monitored regularly to ensure that the results obtained in this study was not influenced by poor water quality.

The essential amino acid (EAA) tested in this study was compared with the EAA requirement for catfish as reported by Jimoh et al. (2014), as well as Fagbenro and Nwanna (1999) in Table 6.2. Generally, all essential amino acids were present in both cricket meal diets with Leucine as the most abundant amino acid in both diets. The values of amino acid in the diets containing cricket meal were higher or within the range of African catfish requirements, except methionine.

The outcomes from the growth performance assessment as shown in Table 6.3 demonstrated that cricket meal was a good substitute for fishmeal as a protein source in African catfish diet. Feed intake of fish fed with fishmeal was significantly lower ( $P < 0.05$ ) than those fed with cricket meal. Total replacement of cricket meal (100% CM) diet showed significantly higher weight gain compared to the control diet (100% FM), but no significant differences in weight gain were observed between 75% CM and 100% FM diets. Feed conversion and protein efficiency ratio values were insignificant ( $P > 0.05$ ) between all diets, although 100% CM shows to be numerically the lowest value of FCR and highest in PER. Specific growth rates were significantly higher ( $P < 0.05$ ) in fish fed with cricket meal, but fishmeal-fed fish shows better results in terms of

hepatosomatic index. Survival rates observed were more than 86.6% in all groups, which suggested that all diets did not significantly affect survival rate.

**Table 6.3: Growth performance of fish fed the experimental diets.**

Variables	Experimental diets		
	100%CM	75%CM	100%FM
TFI (g fish <sup>-1</sup> )	39.90 ± 1.43 <sup>b</sup>	38.00 ± 1.12 <sup>b</sup>	30.06 ± 1.36 <sup>a</sup>
BWG (g fish <sup>-1</sup> )	35.70 ± 5.18 <sup>b</sup>	30.63 ± 1.36 <sup>ab</sup>	19.20 ± 0.97 <sup>a</sup>
FCR (g/g)	1.17 ± 0.20 <sup>a</sup>	1.24 ± 0.05 <sup>a</sup>	1.58 ± 0.08 <sup>a</sup>
SGR (g/g)	2.76 ± 0.23 <sup>b</sup>	2.63 ± 0.10 <sup>b</sup>	1.93 ± 0.14 <sup>a</sup>
PER (g/g)	2.56 ± 0.38 <sup>a</sup>	2.30 ± 0.10 <sup>a</sup>	1.82 ± 0.09 <sup>a</sup>
HSI (g/g)	1.22 ± 0.08 <sup>a</sup>	1.20 ± 0.08 <sup>a</sup>	1.65 ± 0.09 <sup>b</sup>
SR (%)	90 ± 0.00 <sup>a</sup>	86.6 ± 3.33 <sup>a</sup>	90 ± 5.77 <sup>a</sup>

<sup>1</sup>The results represent means ± SEM of ten fish/tank with a total of thirty fish per diet

<sup>2</sup>Mean values in the same row with different superscript are significantly different ( $P < 0.05$ )

Haematological indices for all of the fish fed with the experimental diets were shown in Table 6.4. The haemoglobin, MCH and MCHC appeared to show an increasing trend with additional cricket meal in the diets and significantly higher values ( $P < 0.05$ ) were shown in 100% CM compared with the other groups. No significant difference in red blood cell count and MCV were observed in all groups ( $P > 0.05$ ), while Ht demonstrated significantly higher values ( $P < 0.05$ ) in fish fed with cricket meal diet (31.08 - 33.05%) in comparison to the control diet and initial fish. White blood cells were insignificantly different ( $P > 0.05$ ) between cricket meal groups and initial fish, but fishmeal fed-fish showed a significantly higher value compared to other groups. The plasma protein values did not differ significantly throughout the diets,

which ranged from 3.84-5.16 mg/dl. Therefore, increasing dietary cricket meal inclusion did not produce any effect on plasma protein levels in African catfish.

**Table 6.4: Haematological and plasma biochemical parameters of African catfish fed various experimental diets.**

Parameters	Initial	100%CM	75%CM	100%FM
Hb (g/dl)	6.33 ± 0.14 <sup>c</sup>	8.83 ± 0.80 <sup>a</sup>	7.92 ± 0.31 <sup>b</sup>	7.21 ± 0.11 <sup>bc</sup>
Ht (%)	26.27 ± 0.51 <sup>b</sup>	33.05 ± 0.48 <sup>a</sup>	31.88 ± 0.56 <sup>a</sup>	28.24 ± 0.88 <sup>b</sup>
RBC (10 <sup>6</sup> mm <sup>3</sup> )	1.73 ± 0.45 <sup>a</sup>	2.45 ± 0.60 <sup>a</sup>	2.35 ± 0.29 <sup>a</sup>	2.14 ± 0.005 <sup>a</sup>
WBC (10 <sup>3</sup> mm <sup>3</sup> )	7.13 ± 0.61 <sup>b</sup>	7.46 ± 0.75 <sup>b</sup>	7.54 ± 0.86 <sup>b</sup>	8.17 ± 0.13 <sup>a</sup>
MCV (fl)	69.69 ± 9.78 <sup>a</sup>	73.07 ± 9.43 <sup>a</sup>	71.92 ± 5.83 <sup>a</sup>	69.97 ± 0.95 <sup>a</sup>
MCH (pg)	11.50 ± 2.07 <sup>b</sup>	21.85 ± 3.13 <sup>a</sup>	14.12 ± 0.20 <sup>b</sup>	12.21 ± 0.25 <sup>b</sup>
MCHC (%)	16.50 ± 2.37 <sup>b</sup>	30.11 ± 3.40 <sup>a</sup>	19.73 ± 1.31 <sup>b</sup>	18.40 ± 0.11 <sup>b</sup>
Plasma protein (mg/dl)	3.84 ± 0.01 <sup>a</sup>	4.64 ± 0.27 <sup>a</sup>	5.16 ± 0.04 <sup>a</sup>	4.65 ± 0.024 <sup>a</sup>

<sup>1</sup>The results represent means ± SEM of five fish/tank with a total of fifteen fish per diet

<sup>2</sup>Mean values in the same row with different superscript are significantly different

(P<0.05)

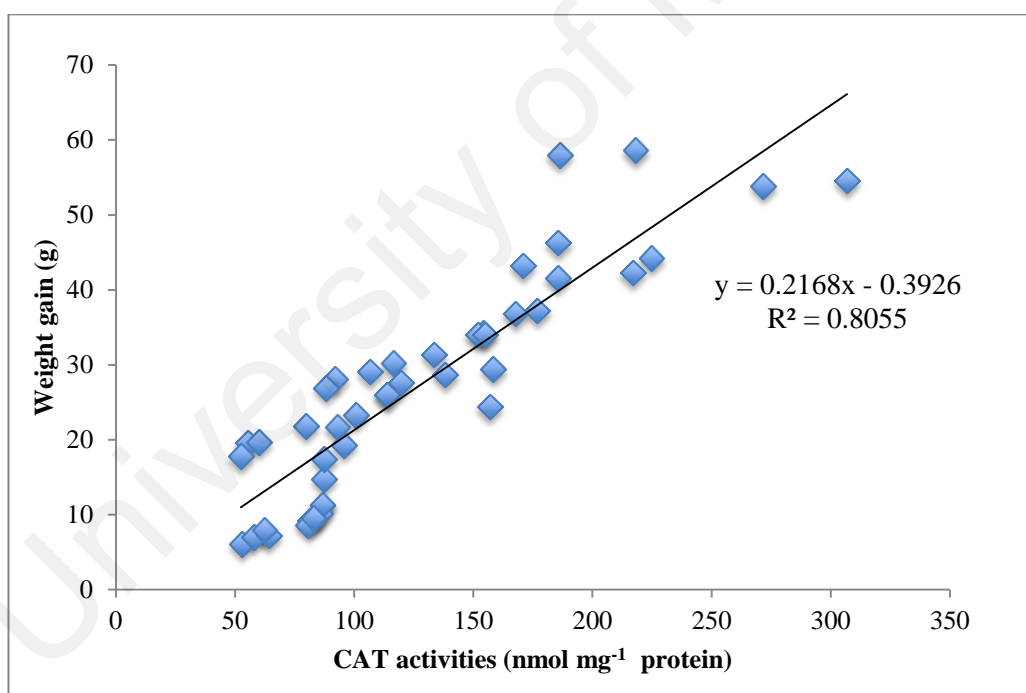
Enzyme activities were assessed in the liver of African catfish that were fed with the experimental diets. Catalase activity as well as weight gain for each fish were recorded individually. However, mean initial weight for each tank was considered as the initial weight for each fish. Catalase shows a significantly higher activity in 100% CM group (173.13 ± 16.63), but decreased with a lower inclusion level of cricket meal in the diets (Table 6.5). Increasing tendencies in African catfish liver CAT activities can be correlated with increasing weight gain during the feeding trial (Figure 6.1). Glutathione S-transferase and superoxide dismutase activities in 100% CM exhibited slightly increased activities compared to other diets, but with no significant differences observed in all groups for both enzymes.

**Table 6.5: Liver protein, Catalase (CAT), Superoxide dismutase (SOD) and Glutathione S-transferase (GST) activity of African catfish fed experimental diets.**

Enzymes and liver protein	Experimental diets		
	100%CM	75%CM	100%FM
Liver protein (mg/ml)	0.600 ± 0.01 <sup>a</sup>	0.610 ± 0.014 <sup>a</sup>	0.652 ± 0.027 <sup>a</sup>
CAT (nmol/mg protein)	173.13 ± 16.63 <sup>a</sup>	121.53 ± 14.65 <sup>b</sup>	103.69 ± 11.93 <sup>b</sup>
SOD (nmol/mg protein)	26.83 ± 0.24 <sup>a</sup>	25.14 ± 0.16 <sup>a</sup>	24.99 ± 0.22 <sup>a</sup>
GST (nmol/mg protein)	191.53 ± 13.9 <sup>a</sup>	189.36 ± 13.7 <sup>a</sup>	176.83 ± 19.5 <sup>a</sup>

<sup>1</sup>The results represent means ± SEM of five fish/tank with a total of fifteen fish per diet

<sup>2</sup>Mean values in the same row of the same experiment with different superscript are significantly different ( $P < 0.05$ )



**Figure 6.1: Correlations between catalase activity and mean weight gain of fish fed experimental diets.**

<sup>1</sup>The results represent means ± SEM of five fish/tank with a total of fifteen fish per diet

## 6.5 Discussion

To date, there is no reliable study on cricket meal as formulated feed for fish. Hence, the present study was conducted to evaluate the growth and biochemical aspects of fish when given cricket meal as the main protein ingredient in comparison to fishmeal in fish feed. However, there are numerous studies, which evaluated cricket meal as poultry feedstuff with a positive effect. Previous studies by Ramos-Elorduy (2008) highlighted that Mormon cricket meal, *Anabrus simplex* can replace fishmeal and soybean entirely in the broiler diet. Similar findings were reported by Nakagaki et al. (1987) and DeFoliart et al. (1982) who found significantly better growth of broiler in cricket meal-based diet compared with corn diet. Wang et al. (2005) also indicated that 15% of field cricket meal, *Gryllus testaceus* supplementation could replace fishmeal diet without adverse effects on broiler's weight gain.

Insects are very efficient at transforming a wide variety of organic matter into an edible mass, which was partly due to the fact that they are cold-blooded animals. Therefore, they utilize less energy to regulate body temperature. Other than their beneficial effect of sustainable and smaller ecological footprint, insect farming reduces the amount of water and land used dedicated to grow food or livestock. Insect meal was widely studied in recent years and is considered as a sustainable substitute for fishmeal in fish diet. Numerous insects had been explored to be utilized in fish feed including grasshopper (*Z. variegatus*), termites (*M. subhyalinus*), maggot (*M. domestica*), meal worms (*T. molitor*), silkworm pupae (*B. mori*), and black soldier fly (*H. illucens*) as fish feed and have proven to be good candidates for fishmeal replacement (Alegbeleye et al., 2012; Ng et al., 2001; Ogunji et al., 2011; Sogbesan & Ugwumba, 2008; St-Hilaire et al., 2007).

The inclusion of up to 80% mealworm in African catfish diet was reported to produce similar growth as fishmeal diet. On the other hand, silkworm meal could substitute up to 75% protein in Asian stinging catfish without reducing growth performance. Similarly, tilapia were able to accept silkworm pupae meal, which resulted in high protein digestibility (85-86%) (Henry et al., 2015; Makkar et al., 2014). However, in Orthoptera insects, grasshopper meal and locust meal could only replace up to 25% of fishmeal without affecting growth and nutrient utilization of African catfish (Alegbeleye et al., 2012; Balogun, 2011). According to Alegbeleye et al. (2012), a higher inclusion of grasshopper meal reduced digestibility due to the presence of chitin.

The growth and nutrient efficiency of the fishes increased as cricket meal level increased in their diets. Significant reduction in feed consumption was observed in fish fed with dietary fishmeal, which might be attributed to the lower freshness level of fishmeal compared to cricket meal. Reducing level of freshness in the feed will be less favourable to the fish which resulted in a lower feed intake (Suarez et al., 1999). Weight gain elevates considerably in full cricket meal diet compared to fishmeal that results in higher SGR value in cricket meal fed fish. Feed conversion ratio and protein efficiency ratio did not differ significantly between cricket meal and fishmeal diet, although numerically better values were observed in 100% CM fed fish, therefore suggesting the possibility that a full replacement of fishmeal with cricket meal could yield a positive growth response in African catfish juveniles. Higher hepatosomatic index (HSI) in fish fed with fishmeal diet can be attributed to high glycogen and lipid accumulation in the liver. (Chaiyapechara et al., 2003; Lie, 2001). The greater growth of African catfish fed 75% and 100% cricket meal might be attributed to the greater nutritional value and digestibility of cricket meal compared to the fishmeal diet.

Cricket meal appears to be a good source of amino acid for African catfish diet. With the exception of methionine, all EAA in cricket meal diet were higher or within the range of African catfish requirements. Although methionine level was insufficient, non-essential amino acids, such as cysteine can replace about 60% of methionine requirements in catfish diet (Lovell, 1989).

Haematological parameters play a vital role in evaluating physiological status of the fish under study. Besides, it can also be a useful tool to assess feed composition and nutritional status in relation to environmental conditions affecting fish (Svobodová et al., 2005). Haematocrit, red blood cell, and white blood cell are used as indicators in monitoring the environment, fish health, and feed toxicity in aquatic animals (Ozovehe, 2013).

A higher level of Hb, MCH, MCHC and Ht were demonstrated in the cricket meal diet. However the values in all the groups are in accordance with healthy African catfish haematological parameters (Dienye & Olumuji, 2014; Erhunmwunse & Ainerua, 2013). The level of RBC did not differ significantly in all groups whereas WBC showed a slightly increased level in the fish fed with fishmeal diet, which is usually associated with the rising numbers of antigens in the circulating system (Oyawoye & Ogunkunle, 1998). Nevertheless, the values in all groups were within the normal range recorded for African catfish (Musa & Omoregie, 1999). The haematological parameters indicated that fishes fed with cricket meal had better health status than those of fishmeal fed fish.

Oxidative stress occurs when there is a disproportion of ROS production with the volume of antioxidant systems to control their damaging effects (Monaghan et al., 2009). ROS are essential by-products of normal metabolic processes that could damage DNA in the cell nucleus and is harmful to other proteins and lipids within cell

membranes if not suppressed by the antioxidant mechanism (Monaghan et al., 2009; Pamplona & Costantini, 2011)

Catalase activity is correlated with an increasing concentration of  $H_2O_2$  (Wdzięczak et al., 1982; Wilhelm Filho et al., 2005). In the present study, we detected a higher activity of this enzyme in the liver of African catfish fed with 100% CM diet followed in descending order by 75% CM, and the lowest was observed in the control group. A positive relationship was found between CAT and weight gain of the fish,  $R_2 = 0.8017$ , which suggested that enhanced CAT activities were induced by higher growth response due to high metabolic rates. This situation was in accordance with Pascual et al. (2003) who observed that CAT activity decreased in fasting fish, and therefore, reducing their weight. However, the activities increased when the fish were fed back normally and they regained their weight back.

The response of the antioxidant mechanism could differ between fish size where smaller fish showed lower antioxidant parameters than larger fish following the same stress exposure between the two sizes (Kanak et al., 2014). The reduced growth response in the fishmeal group might be attributed to less feed intake leading to the generation of reactive oxygen species and consequently, decreased CAT activities (Pascual et al., 2003; Robinson et al., 1997).

The incorporation of cricket meal in the diets did not differ significantly with GST activity. However, cricket-based diet exhibited numerically higher activities than fishmeal, with a similar tendency in activities correlating to CAT. This was supported by Ogunji et al. (2011) who also found that CAT and GST activities in carp liver produced a similar trend when including maggot meal in their diet.

On the other hand, although superoxide dismutase activities showed no significant differences between all of the groups, the 100% CM group demonstrated higher hepatic

SOD activities compared with the 75% CM and 100% FM groups whose trend was similar as CAT activities. This result is consistent with the fact that the first line of defence against ROS is represented by the CAT-SOD enzyme mechanism. Hence, SOD catalyses the reduction of superoxide anions into hydrogen peroxide, which is later decomposed by CAT at intra- and extracellular levels (Nordberg & Arner, 2001).

The simultaneous induction of SOD and the significant increase in CAT activity in the 100% CM group are in agreement with previous studies done with other fishes, such as gibel carp fed with selenium and the inclusion of lycopene in rainbow trout (Han et al., 2011; Sahin et al., 2014). The observation of decreased activity of SOD and CAT in the liver of fishes fed with fishmeal could account for the increased lipid peroxidation as indicative of oxidative stress. We can suppose that a reduced level of SOD was attributed to the accumulation of  $H_2O_2$  due to the reducing level of CAT. As a consequence, SOD levels were also suppressed by the accumulation of superoxide anion ( $O_2^-$ ). This hypothesis was supported by Bagnyukova et al. (2006); Wilhelm Filho et al. (1993) and Wu et al. (2006).

The increasing level of GST in any substance implies that it contains compounds that could stimulate the biotransformation of xenobiotics. In this case, although GST activity is numerically higher in cricket meal treated groups, the effect is insignificant owing to the fact that the crickets used in this experiment were raised in a control substrate and were not fed with any materials exposed to harmful chemicals. The GST contributes to the detoxification of oxidative stress products by catalysing the conjugation of a variety of metabolites, including xenobiotic metabolites and lipoperoxidation products, with GSH and by transforming the toxic compounds into more easily dischargeable ones. Glutathione S-transferases biotransformation has proven to increase energy consumed, which then stimulate oxidative stress (Cazenave et

al., 2006; Wiegand et al., 2007). However, the minor effect in the biotransformation by GST does not influence growth efficiency in cricket meal-fed fish.

Crickets were known to contain a significant amount of chitin in their exoskeleton. Various aquatic animals demonstrated an increase in innate immune response when incorporating chitin in their diet (Powell & Rowley, 2007; Sakai et al., 1992; Vahedi & Ghodrati-zadeh, 2011). Based on our previous studies, crickets show a positive result as an immunostimulant in African catfish due to the presence of antimicrobial activity in chitin. In addition, chitin and its derivatives were reported to have antioxidant properties that could prevent deleterious effects in various diseases (Khoushab & Yamabhai, 2010; Ngo & Kim, 2014). However, some studies reported a reduced digestibility in fish when fed a higher inclusion of insect meal due to the presence of chitin (Alegbeleye et al., 2012; Köprücü & Özdemir, 2005).

## **6.6 Conclusions**

The data obtained during this experiment suggested that dietary cricket meal could improve growth performance of African catfish and enhance feed efficiency. Antioxidant responses of fish fed 100% cricket meal diet increased CAT activity, although only a minor effect was observed in SOD and GST activities. These facts demonstrated that cricket meal diet could boost antioxidant status when given to African catfish in a formulated diet. Moreover, the haematological findings also supported the fact that diet containing up to 100% cricket meal is possible for feeding African catfish without adverse effects. Further works concerning the impact of cricket meal on other antioxidative enzymes as well as haematological parameters in different physiological situations should be taken into consideration. Finally, a future study by using crickets that fed on unused byproducts and raised in a more sustainable way could

be taken into consideration. This could have significant implications for the aquaculture industry where insect meal is more acceptable as sustainable protein sources.

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## CHAPTER 7: GENERAL CONCLUSION AND FUTURE PERSPECTIVE

### 7.1 Conclusion

Based on the findings presented from the current study in this thesis, the following conclusions can be made:

- Field cricket could replace up to 100% of fishmeal in African catfish due to their higher growth parameters with increasing CM level from 50% upwards. However, crude protein level of 28% is insufficient to provide optimal growth for African catfish fingerlings.
- The essential amino acids for every diet were within the range of African catfish requirement except for methionine, phenylalanine and lysine (in Chapter 3). However, cysteine and tyrosine can spare up to 60% and 50% of methionine and phenylalanine respectively. Increasing crude protein level in Chapter 4 and 6 do increase lysine composition in cricket meal diet.
- The findings from digestibility study suggest that cricket meal is well digested by African catfish as it shows significantly higher ADC for protein, lipid, dry matter and gross energy compared to local fishmeal used in this study. However, the level of nutrient digestibility is still comparatively lower than other protein sources.
- The presence of chitin in cricket meal diets did not affect growth performance and feed efficiency of African catfish.
- The inclusion of 35% crude protein level in cricket meal diet could enhance growth performance as well as increase RPS up to 12 days post-challenge with *Aeromonas hydrophila*. Increasing levels of lysozyme in cricket meal groups indicated that the presence of chitin in cricket meal could be a natural protective mechanism in fish.

- Full replacement of cricket meal (100% CM) in African catfish diet enhanced CAT activity despite the minor effect in SOD and GST enzymes.
- Haematological indicators also suggested that the values of blood parameters of cricket meal and fishmeal fed fish gave no indications of abnormalities as they were within the normal range of healthy African catfish.

## **7.2 Future perspective**

The current findings add substantially to a growing knowledge on fish nutrition particularly in finding new solutions for sustainable feed. In all experimental trials, the methionine levels in cricket meal diet were lower than the required amount for African catfish nutrition although cysteine could replace the insufficient amount. However, the inclusion of synthetic amino acid particularly methionine has the potential to contribute positive effects in increasing growth performance.

In this thesis, fishmeal used was purchased from a local manufacturer, which is commonly used by farmers in Malaysia. It is more economical but lower in nutritional values compared to imported fishmeal. Hence, a further study by utilizing higher quality fishmeal is necessary to determine the full potential of cricket meal.

Digestibility is a common issue in insect meals that needs to be addressed in order to create a potential value for insect protein. Nutrient digestibility of cricket meal by removing the chitin portion of the insects (wings and legs) could be factors that can contribute to increase digestibility and thus ought to be explored further. Besides, extrusion process of the feed could also be beneficial for increasing digestibility.

Further experimental investigations are needed to estimate other immunological parameters such as phagocytic activity, respiratory burst and bactericidal activity to gain more clarification on non-specific defense mechanism.

The present study only observes CAT, SOD and GST to determine oxidative stress when fish were supplied with cricket meal. However, it is known that the oxidation of lipid in the feed could affect the feed palatability. Hence, further investigation in lipid peroxidation and other antioxidative enzymes would be very interesting.

Cricket meal showed potential as fishmeal replacement particularly as a sustainable resource. However, currently the expensive price of crickets is the major issue due to lack of up-scale production in Malaysia since the market is mainly focused on zoos and pet shops. One possibility could be to emphasize on reducing the production cost for cricket farm yet still profitable in order to be competitive with commonly used protein sources.

Finally, the fish produced need to be tested for sensory quality since it is one of the important factors that determine the marketability of the fish if these products are going to be mass produced in future fish feed. With more additional research, cricket meal could be a potential major protein source not only in aqua-feed but also for other animal diets.

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## LIST OF PUBLICATIONS AND PAPER PRESENTED

- Taufek, N.M., Aspani, F., Muin, H., Raji, A.A., Alias, Z., Razak, S.A (2016). The effect of dietary cricket meal (*Gryllus bimaculatus*) on growth performance, antioxidant activities and haematological response of African catfish (*Clarias gariepinus*). Fish Physiology and Biochemistry. 42 (4): 1143 - 1155
- Taufek, N.M., Muin, H., Raji, A.A., Yusof, H., Alias, Z., Razak, S.A (2016). Apparent digestibility coefficient and Amino acid availability of cricket meal, *Gryllus bimaculatus* and fishmeal in African catfish, *Clarias gariepinus* diet. Accepted for publication by Journal of World Aquaculture Society. DOI: 10.11/jwas.12302
- Taufek, N.M., Aspani, F., Muin, H., Raji, A.A., Alias, Z., Razak, S.A (2016). Effect of dietary cricket meal on the growth performance and resistance against pathogenicity of *Aeromonas hydrophila* in African catfish, *Clarias gariepinus*. Under review in Aquaculture Nutrition.
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- Potential value of black field cricket meal as protein replacement for African catfish, *Clarias gariepinus* fingerlings nutrition, UMTAS, 12<sup>th</sup> International UMT Annual Symposium, 8<sup>th</sup> – 10<sup>th</sup> October 2013, Permai Hotel Kuala Terengganu – Poster presentation
- Effect of varying level of dietary crude protein of black cricket meal on growth performance and body composition in fingerlings African catfish, *Clarius gariepinus* (Burchell, 1822), 16<sup>th</sup> International Symposium on Fish Nutrition and Feeding 2014, 25<sup>th</sup> -30<sup>th</sup> May 2014, Great Barrier Reef Cairns, Australia – Poster presentation
- Apparent digestibility of cricket meal of African catfish, *Clarias gariepinus*, International Conference on Waste Management, Ecology and Biological Sciences (WMEBS'15), 13<sup>th</sup> – 14<sup>th</sup> May 2015, Kuala Lumpur – Oral presentation.