POTENTIAL OF *CHRYSOMYA MEGACEPHALA* (DIPTERA: CALLIPHORIDAE) MAGGOT MEAL AS PROTEIN SOURCE IN TILAPIA (*OREOCHROMIS* SP.) FEED

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ABSTRACT

Currently, fishmeal is a common protein source in aquafeed for farmed fish. However, the demand for fishmeal is increasing but supply is stagnating or even decreasing and therefore is insufficient to meet demand. This has caused the increase of fishmeal price in global markets and thereby, incurs higher production costs. Thus, there is an urgent need to find a cheaper but suitable protein source to replace fishmeal in animal feed.

The objective of this study is to evaluate the potential of *Chrysomya megacephala* (Fabricius, 1974) maggot meal as protein source in red tilapia (*Oreochromis* sp.) feed.

Protein content of blowfly (*C. megacephala*) maggot meal extract was determined using three different colourimetric methods – Biuret method, dye-binding method and the method of Lowry *et al*. (1951). Protein estimation was performed after dissolution of maggot powder in 0.06 M sodium phosphate buffer, pH 7.0 both in the absence and presence of 1% (w/v) sodium dodecyl sulphate (SDS). Alternatively, the use of color reagent was made both before and after centrifugation of the dissolved mixture. Since dye-binding method could not be performed in the presence of SDS which its interferes with the process, both Biuret method and the method of Lowry *et al*. (1951) were employed using standard curves in presence of 1% (w/v) SDS. Two other methods, namely, Warburg-Christian method and semi-micro Kjeldhal method were also used to determine protein concentration. The inclusion of SDS and addition of color reagents before centrifugation of the dissolved sample showed a significant increase in the percentage of protein content compared to the results obtained under normal condition. A comparison of all these results supported the use of Biuret method and the method of Lowry *et* al. (1951) under specific

conditions as the substitute for semi-micro Kjeldhal method for protein estimation. SDS-polyacrylamide gel electrophoresis of maggot meal extract showed the presence of both small and medium sized proteins ranging in molecular weight from 17 — 83 kDa. Maggot meal powder derived from maggots hatched from eggs over a period of 4 days were also found to be rich in essential amino acids as proven by amino acid analysis.

A feeding trial was performed for 60 days to evaluate the potential of this blowfly maggot meal to replace fish meal in red tilapia (*Oreochromis* sp.) feed. Five isonitrogenous and isoenergy fish meal diets formulated to contain 30% of protein and 20 kJ g^{-1} of gross energy were replaced by maggot meal at 0%, 25%, 50%, 75% and 100%. Fishes that were fed with 100% showed the highest survival rate (80%), percentage weight gain (239%), specific growth rate (2.02% per day) and protein efficiency ratio (0.3), and the lowest of food conversion ratio (1.34) as compared with other experimental diets.

A selection experiment was conducted to improve the body weight of *C. megacephala*. After 10 generations of artificial selection, the body weight of *C. megacephala* maggot increased.

Taken together, all these results suggested the suitability of maggot (*C. megacephala*) meal as a protein source in red tilapia feed.

ABSTRAK

Pada masa ini, serbuk ikan adalah sumber protein umum dalam makanan aquatik untuk ikan ternak. Walau bagaimanapun, permintaan serbuk ikan semakin meningkat tetapi bekalan tidak lagi berkembang malahan menurun justerus tidak dapat memenuhi permintaan. Ini telah menyebabkan kenaikan harga serbuk ikan di pasaran global dan dengan itu, penternak ikan perlu menanggung kos yang lebih tinggi. Oleh itu, terdapat keperluan segera untuk mencari sumber protein yang lebih murah tetapi sesuai untuk menggantikan bahan berkaitan ikan dalam makanan haiwan.

Objektif kajian ini adalah untuk menilai potensi ulat *Chrysornya megacephala* (Fabricius, 1974) sebagai sumber protein dalam makanan tilapia merah (*Oreochromis* sp.).

Kandungan protein ekstrak ulat langau (*Chrysomya megacephala*) telah diukur dengan menggunakan tiga kaedah kolorimetrik yang berbeza – kaedah Biuret, kaedah pengikat-pewarna dan kaedah Lowry *et al*. (1951). Anggaran protein telah dilakukan selepas serbuk ulat dilarutkan ke dalam 0.06 M penimbal natrium fosfat, pH 7.0 dalam ketiadaan dan kehadiran 1% (w/v) natrium dodesil sulfat (SDS). Sebagai alternatif, penambahanan reagen berwarna telah dibuat sebelum dan selepas pengemparan campuran terlarut. Oleh sebab kaedah pengikat-pewarna tidak boleh dilakukan dengan kehadiran SDS kerana mengganggu proses, maka kedua-dua kaedah Biuret dan kaedah Lowry *et al*. (1951) dijalankan menggunakan lengkuk piawai dengan kehadiran 1% (w / v) SDS. Dua kaedah yang lain, iaitu, kaedah Warburg-Christian dan kaedah separa-mikro Kjeldhal juga digunakan untuk menentukan kandungan protein yang ada pada ekstrak ulat. Penambahan SDS dan reagen pewarna ke dalam campuran sampel sebelum pengemparan menunjukkan peningkatan yang ketara dalam peratusan kandungan protein berbanding dengan keputusan

yang diperolehi di bawah keadaan normal. Perbandingan semua keputusan yang diperolehi mencadangkan penggunaan kaedah Biuret dan kaedah Lowry *et al*. (1951) di bawah syarat-syarat tertentu sebagai penggantian kaedah separa-mikro Kjeldhal dalam penganggaran kandungan protein. Gel elektroforesis SDS-poliakrailamide ekstrak ulat menunjukkan kehadiran berat molekul protein yang bersaiz kecil dan sederhana iaitu 17 - 83 kDa. Serbuk ulat yang didapati daripada ulat yang menetas dari telur sepanjang tempoh 4 hari didapati kaya dengan asid amino perlu setelah disahkan melalui penganalisisian asid amino.

Satu ujian pemberian makanan telah dilakukan selama 60 hari untuk menilai potensi serbuk ulat blowfly menggantikan serbuk ikan di dalam makanan tilapia merah (*Oreochromis* sp.) Lima isonitrogenous dan isoenergy makan ikan yang digubal mengandungi 30% protein dan 20 kJ g^{-1} tenaga kasar dengan serbuk ikan telah digantikan oleh serbok ulat pada 0%, 25%, 50%, 75% dan 100%. Ikan-ikan yang diberi makanan 100% menunjukkan kadar kebolehan hidup yang tertinggi (80%), peratusan kenaikan berat badan (239%), kadar pertumbuhan spesifik (2.02% sehari), nisbah kecekapan protein (0.3) dan nisbah penukaran makanan yang paling rendah (1.34) apabila berbanding dengan makanan yang lain.

Satu eksperimen pemilihan telah dijalankan untuk meningkatkan berat badan *C. megacephala*. Berat badan *C. megacephala* telah meningkat selepas 10 generasi pemilihan dilakukan.

Dengan keputusan yang diperolehi ia mencadangkan kesesuaian (*C. megacephala*) ulat sebagai sumber protein dalam makanan ikan tilapia merah.

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Abs.	Absorbance
CI	Confident interval
cm	Centimeter
conc.	Concentration
Cu^{+}	Cuprous
ºC	Degree Celsius
Da	Dalton
DNA	Deoxyribonucleic acid
et al.	Latin phrase *et alia* (and other)
FCR	Food conversion ratio
g	Gram
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
i.e.	Latin phrase *id est* (that is)
J	Joule
kg	Kilogram
m	Meter
M	Molar
mg	Milligram
ml	Milliliter
N	Normality
N.D.	Not determined
nm	Nanometer
PER	protein efficiency ratio
PMI	Post-mortem interval
R_m	Relative mobility
RNA	Ribonucleic acid
S.D.	Standard deviation
S.E.	Standard error
SDS- PAGE	Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
SGR	Special growth rate
sp.	Species
TEMED	N,N,N’,N’-tetramethylethylenediamine
µg	Microgram
µl	Microlitre
UV	Ultraviolet
v/v	Volume per volume

w/v	Mass per volume
~	Approximate
%	Percentage
±	Plus-minus

CHAPTER 1

GENERAL INTRODUCION

The world's population grew more than 10 times from 600 million people in 1700 to 7 billion people in 2011 and is still growing rapidly. As such, an ever increasing population has exerted enormous pressure on food producers to step up food production to meet increasing demand and this has led to the overhauling of the world's agricultural systems. Previously, farmers relied on their own traditional cultures and knowledge to cultivate animals and plants. However, these traditional self-subsistence farming methods can no longer cater to the huge increase in demand for food. Hence, large-scale monoculture employing the latest technologies is now being practiced in modern farming to maximize food production cost-effectively (Cohen, 2003).

Over the years, the aquaculture industry in Malaysia has shown a steady growth due to active participation of local farmers. However, most of the raw ingredients for feedstuffs are imported because they are not currently produced in Malaysia: soybean meal, fishmeal, cereal grains, corn gluten meal, mineral sources and various micro-ingredients. Thus, feedstuffs constitute a large part of the cost of production. Soybean meal in Malaysia is usually obtained after the process of making soybean curd and soybean. Therefore, its quality is not as good as the imported soybean meal (Loh, 2002).

One of the alternative protein sources used as feedstuff in Malaysia is palm kernel meal, a by-product from the oil palm industry. Palm kernel meal is a moderate quality feed for ruminants in terms of digestibility with 16% crude fiber. However, it contains low crude

protein (15 – 17%), lacks some amino acids and has very low lysine content (O'Mara *et al.*, 1999).

Currently, fishmeal is a common protein source in aquafeed for farmed fish. However, the demand for fishmeal is increasing but supply is stagnating or even decreasing and therefore is insufficient to meet demand. This has caused the increase of fishmeal price in global markets and thereby, incurs higher production costs (Tidwell & Allan, 2001). Thus, there is an urgent need to find a cheaper but suitable protein source to replace fishmeal in animal feed.

A suitable alternative protein source for aquafeed should be sustainable and nutritious. This protein source should be easily obtainable and in sufficient amounts to meet demand. In the nutritional aspect, the essential amino acids derived from the alternative protein source should meet the basic amino acid requirements of fish; the quality of protein is determined by the composition and ratio of amino acids (Watanabe, 2002).

The ability of the fish to digest the alternative protein after feeding should be taken into account as this will directly affect the absorption of proteins by the fish and consequently the growth rate. In addition, the palatability of the protein source should be the same as the fishmeal to avoid rejection by the fish.

The use of insects as a protein source in animal food is not a new idea and many scientific papers have been published regarding this approach. In Japan and China, farmers used silkworm (*Bombyx mori*) pupae to culture carp fish (Begum *et al.*, 1994). Other insects such as mealworm beetle (*Tenebrio molitor*) and house fly (*Musca domestica*)

larvae have also been studied as alternative protein source in fish diets (Fasakin *et al.*, 2003; Ng *et al.*, 2001). Insect-based diets have been recognized as one of the cheaper alternatives to fishmeal by researchers (Yen, 2009).

The *Chrysomya megacephala* (Fabricius) blowfly maggots can be found on carcasses and are able to decompose the carcasses with their enzyme (Greenberg, 1991). During the decomposition process, maggots are converting the waste (carcasses) into a good quality form of protein. As such, the maggots are of high protein content and thus there is great potential to use the *C. megacephala* maggot as an alternative protein source in animal feed.

Hence, the following objectives were set for this study:

i. To study the suitability of *Chrysomya megacephala* maggot meal as a protein source in animal feed with a comparative study of different methods used in protein determination.

ii. To evaluate the effect of partial substitution of fishmeal by *Chrysomya megacephala* maggot meal on growth performance and feed utilization of red tilapia (*Oreochromis* sp.).

iii. To produce a robust and high protein strain of *Chrysomya megacephala* maggots to be used as animal feed by artificial selection.

CHAPTER 2

LITERATURE REVIEW

2.1 Aquaculture

Fish is an important source of protein for humans. As the human population expands beyond 7 billion and the quantity of wild-caught fish declines, large scale fish farming methods are being used to cope with the increased demand for fish.

Farmed fish and shellfish contribute to more than one quarter of the total fish directly consumed by humans and fish production is expected to increase in the future. Nowadays, cages, ponds and tanks are the common systems used in fish farming to increase fish production and contribute directly to the global fish supply (FAO Fishery Information Data and Statistics Unit, 1999).

There are many aspects to consider when expanding aquaculture because most of the fish farms are built on the natural habitats of wild fish such as mangroves and coastal ecosystems. As such, there should be proper management of resources and wastes, pathogen transmission, biological pollution and ecology impact, so as to minimize negative effects on the immediate environment. Therefore, sound aquaculture practices will be the key in balancing farmed and wild-caught fish for human consumption in the future (Naylor *et al.*, 2000).

2.2 Tilapia

Tilapia is the common name for the three genera: *Oreochromis*, *Sarotherodon*, and *Tilapia*. Among these three genera, *Oreochromis* is the most important group to

aquaculture. Examples are the Nile tilapia, *O. niloticus*, the Mozambique tilapia, *O. mossambicus*, the blue tilapia, *O. aureus*, and the Wami tilapia *O. urolepis hornorum*. In addition, they readily hybridize in captivity and many hybrid strains are now available to growers (Fitzsimmons, 2000).

Tilapia is the second most common farm-raised food fish in the world and the worldwide production has reached 3.7 million tons at 2011 (Watanabe *et al.*, 2002). Tilapia farming has expanded rapidly with the development of new strains and hybrids, monosex male culture, a variety of semi-intensive and intensive culture system. Tilapia is well liked by consumers as the flesh of fish is tasty. Thus, tilapia has become the second most important food fish in the world (Fitzsimmons, 2000).

Tilapia can tolerate a wide range of environmental conditions such as low dissolved oxygen, high ammonia level and a wide range of pH (5–11) (Watanabe *et al.*, 1997). However, cold weather and the streptococcus bacterial did contribute to a low production of tilapia in the United States in 2010.

Demand of tilapia in the global market is growing especially in the United States. Hence, there is an urgency to increase the production of tilapia in order to meet demand. Moreover, the genetically improved strain of tilapia may have different nutritional requirements as compared to existing strain (Maugle & Fagan, 1993; Watanabe, 2002). Therefore, research on establishing the most nutritious and suitable feed for the tilapia is being carried out so as to improve the growth of fish and increase profitability for fish farmers as well.

2.3 Fishmeal

Fishmeal is a nutrient-rich protein source in feed ingredients for the domestic animal's diet. However, as the demand for fishmeal exceeds supply, the market price of fishmeal has increased sharply. Since fishmeal is an important raw ingredient in animal feed, it accounts for a major portion of the production costs of aquaculture farms (Tacon & Metian, 2008).

The world's largest fishmeal consumer is the poultry and swine farm industry followed by the aquaculture industry. However, the protein content in aquafeed is much higher than in the livestock feed. An estimated one-third of total 30 metric tons fish caught is used to produce fishmeal for aquafeeds (Alverson *et al.*, 1994; Tacon, 1998) with the remainder converted to fishmeal for poultry and other animal feed.

A global survey reported a significant increase in fishmeal use associated with the marked increase in shrimp and fish production (Tacon & Metian, 2008). The expanding aquaculture industry has increased the demand for fishmeal and this has placed the pelagic fishes which are a source of fishmeal, in endangered status. The rapid growth of the aquaculture industry has changed the current fish capture pattern from large piscivorous fishes toward smaller invertebrates and planktivorous fishes but does not alleviate pressure on wild fisheries stocks (Pauly *et al.*, 1998). This would clearly threaten marine ecosystems as well as constrain the long-term growth of the aquaculture industry itself.

2.4 Alternative protein sources

In recent years, researchers have been studying the potential of algae, fungi, bacteria, feed peas, insects and earthworms as a replacement protein source in animal feed. However, thus far, no promising results have been obtained due to several limitations as listed below.

2.4.1 Algae

The algae protein is a byproduct from the treatment of sewage effluents for water clarification (Calvert, 1979). Feeding trials using algae for chicks showed that the Chlorella group could provide water-soluble vitamins and carotene but contains low levels of methionine (Combs, 1952). Another limitation of algae is that the cell walls are difficult to digest by animals (Hintz *et al.*, 1966).

2.4.2 Yeast

In 1975, Yoshida, a Japanese researcher, reported yeasts grown on n-paraffins are a good protein source for poultry. In addition, yeasts produced on alkanes by a British company presented no safety hazards to livestock and human (Shacklady & Gatumel, 1972). Generally, yeasts are considered as a satisfactory protein source but are rather deficient in sulfuric amino acid content (cysteine and methionine).

2.4.3 Bacteria

Production of bacterial protein on methanol for feed production is feasible. On the other hand, producing bacterial protein via fermentation of animal wastes and petroleum-

derived hydrocarbons is limited. A study of the effects of bacterial protein on the health of rats showed adverse effects on the rats in the laboratory (Agren *et al.*, 1974).

2.4.4 Fungi

Litchfield (1968) summarized that the protein content of 20 species of fungal mycelium ranged from 12.0% to 53.5%, indicating that many species from this fungal group can compete with fishmeal that is used in animal feed in term of protein content. However, fungal protein is low in sulfuric amino acid content and contains fungitoxins.

2.4.5 Plant protein

Different groups of scientists have been studying the potential practicability of plant protein to replace fishmeal in aquafeed. Most of the results showed that plant protein can only partially replace fishmeal because the inclusion of plant protein in substantial amounts has adverse effects on fish growth. A common problem of plant protein is their relatively low sulfuric amino acid content which are essential amino acids for fish (Carter & Hauler, 2000; Eusebio & Coloso, 2000).

2.4.6 Animal protein

Earthworms and insects are the two common animal protein sources used in substitution of fishmeal experiments because they can be mass produced. The nutritional value of both earthworms and insects are well studied by scientists and these animals contain high protein content. The quality of these proteins appeared to be good with 10 essential amino acids present (Sing *et al.*, 2012). Thus, they are a potential sustainable protein source in animal nutrition. Earlier studies showed that high replacement of fishmeal

by these animal proteins shared the same limitation as plant protein where slow growth of fish was observed without adverse effect such as high mortality rate. This limitation may be due to deficiencies in essential nutrients or the presence of high concentrations of saturated fat in animal proteins.

2.5 Amino acid

Amino acids are building blocks for protein and play an important role in regulating metabolism in animals. Some of the processes which require amino acids include protein synthesis, stress response, reproduction, growth and development, behavior, pigmentation, osmoregulation, immunity and survival, cell signaling and ammonia removal (Li *et al.*, 2009).

Nutritionally, the amino acids are classified into the essential amino acid group (indispensable) and the nonessential amino acid group. Those amino acids that cannot be synthesized by animals are from the essential amino acid group and can only be obtained from food whereas the nonessential amino acids can be synthesized adequately by animals in their own tissue.

The quality of protein depends on the composition and ratio of amino acids rather than protein concentration. A good protein source should contain adequate levels of protein and sufficient essential amino acids to fulfill the essential amino acid requirements of animal. However, in order to produce cheap animal feed, poor quality protein sources, for example palm kernel cake, are widely used in the manufacture of animal feed. As they contain inadequate nutrients and essential amino acids, excessive amounts of such protein

sources are used in order to meet the essential amino acid requirements. This may actually result in an oversupply of protein and subsequently lead to nitrogen pollution of the environment.

Formerly, there was a lack of supportive growth data on the quantitative amino acid requirements of tilapia. The exact amino acid requirements for several species of tilapia were unclear and this has prompted researchers to work on this area as the results obtained would help to formulate the optimum tilapia diet. In 1988, Santiago and Lovell determined the quantitative requirements of the 10 essential amino acids for the growth of young Nile tilapia (*Oreochromis niloticus*) and this is summarized in Table 2.1.

Table 2.1: The 10 essential amino acids and their optimum dietary levels (%) for juvenile Nile tilapia.

Essential amino acids	Optimum dietary level (%)
Histidine	0.48
Threonine	1.05
Valine	0.78
Methionine	0.75
Isoleucine	0.87
Leucine	0.95
Phenylalanine	1.05
Lysine	1.43

2.6 Protein Estimation

Various methods have been employed to determine protein concentration in biological samples. Sensitivity, presence of interference substance in the sample and personal preference are some of the criteria used for selecting a particular method. In general, Warburg-Christian (1942) method and coomassie blue dye-binding assay of Bradford (1976) are the commonly used methods for protein quantification in different samples (Walsh, 2004).

A single protein solution would probably yield different results if measured with different methods. This is because different methods use different principles to determine the protein content. Actually, there is no absolute method for protein estimation; every method has its own advantages and disadvantages. The colourimetric methods are simple, fast and easy to carry out in the laboratory but some of these methods suffer from the interference by certain compounds (Kamizake *et al.*, 2003).

2.6.1 Spectrophotometric Method (Warburg & Christian, 1942)

Proteins absorb light in the UV range with an absorption maximum around 280 nm. The aromatic amino acids, namely tyrosine and tryptophan mainly contribute to the absorption peak at 280 nm. However, the presence of nonprotein chromophores in nuclei acids (which absorb strongly at 260 nm) will produce higher readings at the absorbance level around 280 nm (Walker, 2002). Nevertheless, this problem can be solved by eliminating the contribution of nuclei acids using the formula below (Warburg & Christian, 1942):

$$\text{Protein concentration} = 1.55\ \text{Abs}_{.280\ \text{nm}} - 0.76\ \text{Abs}_{.260\text{nm}}$$

This method is simple and results can be obtained in a short time. Moreover, it is non-destructive to samples (Walsh, 2004). The disadvantage of this method is interference from other chromophores; thus a small amount of nucleic acid can greatly influence the results (Walker, 2002).

2.6.2 Biuret Method (Gornall *et al*., 1949)

The biuret method involves the use of alkaline copper sulfate solution which forms copper tetradentate coordination complexes with protein peptide groups. These complexes absorb maximally at 550 nm (Drochioiu *et al.*, 2006; Walsh, 2004) which can be read on spectrophotometer.

The reagent used in this method is easy to prepare and inexpensive. Furthermore, this assay is less susceptible to many interference substances as compared to other methods. Yet, the sensitivity of biuret method is low (Walsh, 2004).

2.6.3 Dye-Binding Method (Bradford, 1976)

Coomassie blue dye-binding method was devised by Bradford in 1976 and became one of the most preferred methods for determining protein concentration in many laboratories. The principle of this assay is based on the binding of coomassie blue dye to protein. The dye does not bind to free amino acids (Bradford, 1976; Wei *et al*., 1997). Thus, only protein is measured in this method (Kamizake *et al*., 2003).

Bradford's method is moderately sensitive, easy to carry out and produces results quickly. On the other hand, this assay is more susceptible to interference by other chemicals and detergents such as sodium dodecyl sulfate (SDS) (Walker, 2002; Walsh, 2004).

2.6.4 Method of Lowry *et al.* (1951)

The method of Lowry *et al*. (1951) is based on two reactions. The first reaction is similar to the biuret assay in which the peptide bond of proteins reacts with copper under alkaline condition to produce cuprous (Cu^{+}). Cuprous ion reacts with Folin-Ciocalteu reagent to form phosphomolybdotungstate which then will be reduced to heteropolymolybdenum blue after binding to the proteins. The blue color is read at 750 nm (Walker, 2002; Walsh, 2004).

Sensitivity of the method of Lowry *et al*. (1951) is moderately constant from protein to protein; thus, it is an acceptable assay to determine protein content under various conditions involving crude extracts or protein mixtures.

2.6.5 Semi-micro Kjeldahl Method (Helrich, 1990)

The semi-micro Kjeldahl method (Helrich, 1990) determines percentage of total nitrogen in a sample. This method involves the conversion of organic nitrogen into ammonium by boiling with sulphuric acid and distilling with an alkali in order to liberate ammonia for titration (Nelson & Sommers, 1973). It is a standard reference method internationally recognized especially in the food industry but the experiment requires a lengthy time period (Kamizake *et al*., 2003).

2.7 *Chrysomya megacephala*

Chrysomya megacephala (Fabricius) belongs to the class Insecta and order Diptera with a pair of wings. It is also known as "oriental latrine fly". The body size of adult *C. megacephala* is about the size of a house fly (*Musca domestica*) or slightly bigger with greenish-blue metallic thorax and abdomen, and a pair of large conspicuous red eyes at the head part. Eggs of *C. megacephala* are asymmetric because it is oval in shape with one flat face and another convex (David *et al.*, 2008) and the mature third instar is muscoid-shaped with pointed anterior and blunt posterior ends (Sukontason *et al*., 2008). The pupa is formed by the contraction and hardening of the larval skin with a true pupa inside. Puparium is typically coarctate and cylindrical in shape (Siriwattanarungsee *et al.*, 2005). The adult fly emerges by breaking the front end of the puparium and working its way to the surface by alternately expanding and contracting a blood-filled sack in the front of the head. Adult flies are only active in daylight; in darkness or in artificial light, they only move slowly or rest (Reid, 1953). Activities of adult flies are influenced by temperature, humidity, wind, light and color.

Blowflies are widely distributed across vast regions of the world including the Oriental regions, Australasia, Palearctic, South African and Afrotropical Islands (Smith, 1986; Zumpt, 1965). Among the blowflies, *C. megacephala* is the most common in Brazil (Gabre *et al.*, 2005) and Egypt (Gabre, 1994). The success of *C. megacephala* invasion and colonization in most parts of the world is probably because of the low mortality rate during its fertility period and also its survivorship strategy (Reigada & Godoy, 2005).

Chrysomya megacephala females need protein ingestion before laying eggs because it is an anaotugenous blowfly species (Spradbery & Schweizer, 1979). An egg batch contains an average of 224 eggs and is hatched within one day. The developmental time for larva and pupa, on average, is 5 days under 26°C (Gabre *et al.*, 2005). However, the developmental rate of *C. megacephala* is temperature dependent where larvae develop more rapidly at higher temperatures (Sukontason *et al*., 2008).

2.7.1 Importance of *Chrysomya megacephala*

Blowflies are forensically important because many parts of the world have used the size and the developmental stages of blowflies on corpses to estimate the post-mortem interval (PMI) of a person who has died (Lee *et al*., 2004).

Mango is one of the most cultivated fruits and commonly used in cuisine. The worldwide mango production was estimated at nearly 35 metric tons in 2009 by the Agriculture Organization of the United Nations. *C. megacephala* is a very common pollinator for mango. In Australia (Anderson *et al*., 1982) and Taiwan, farmer rear the *C. megacephala* in their mango farms to pollinate the mango flowers. A mass rearing of *C. megacephala* employing convenient and efficient methods have been successfully developed in the laboratory for the pollination of mango trees in Taiwan (Hu *et al*., 1995).

CHAPTER 3

PROTEIN ANALYSIS OF *CHRYSOMYA MEGACEPHALA* MAGGOT MEAL

3.1 Introduction

Fishmeal is the primary protein ingredient of choice in animal feeds. However, high demand of this product in the market has escalated its cost and added further to the production cost in livestock industry. Therefore, the current mission in agriculture sector is to seek for the substitution of fishmeal in animal feed (Brinker & Reiter, 2011). Selection of a good protein source to replace fishmeal in animal feed is based on the quality of protein and the presence of essential amino acids. Therefore, a good protein source must be solubilized and digested easily within the animal body. Furthermore, it should contain sufficient amount of essential amino acids, which are required for the growth of animals (Kerr & Kidd, 1999).

The potential of maggot proteins as a protein supplement for poultry and fish has been reported by many researchers (Fasakin *et al*., 2003; Oyelese, 2007). However, a significant variation in the protein content for the same maggot meal has been noticed in the data published by various research groups (Adenji, 2007; Awoniyi *et al.*, 2003; Ogunji *et al.*, 2008). Such variation can be attributed to the use of different methods for protein estimation in maggot meal by different groups (Ogunji *et al*., 2008; Zuidhof *et al*., 2003). Kjeldahl method (Helrich, 1990) is the most popular method among various methods available for protein estimation in a sample (Kamizake *et al*., 2003). However, the availability of Kjeldhal apparatus in various laboratories remains an obstacle and can be easily correlated with the use of other colourimetric methods such as biuret method

(Gornall *et al*., 1949), dye-binding method (Bradford, 1976) and the method of Lowry *et al*. (1951).

In view of the presence of other nitrogenous compounds (nucleic acids) in maggot sample, results obtained with Kjeldahl method (Helrich, 1990) seem to be towards the higher side as protein determination is based on nitrogen estimation in the sample. On the other hand; release of membrane-bound proteins and their quantitation by different colourimetric methods in the absence of any detergent remains questionable. Thus, both overestimation and underestimation of protein content in Kjeldhal method (Helrich, 1990) and colourimetric methods respectively calls for further research in the determination of total protein content in complex subjects (maggot meal). Here, we present our data on a comparative study of different methods used in protein determination and suitability of maggot meal as a good protein source in animal feed.

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Proteins

Bovine serum albumin (Lot 015K0591) was purchased from Sigma-Aldrich Inc., USA. Prestained SDS-PAGE standards (catalog No. 161-0318) containing different markers (ß-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, soyabean trypsin inhibitor and lysozyme) were supplied by Bio-Rad Laboratories, USA.

3.2.1.2 Reagents used in protein estimation

Sodium potassium tartrate, copper sulphate, sodium carbonate, sodium hydroxide and ethanol were purchased from SYSTERM®, Malaysia. Folin-Ciocalteu's phenol reagent (Lot HC942709) and sulphuric acid were the products from Merck, Germany. Coomassie brilliant blue G (Lot 117K0796) was procured from Sigma-Aldrich Inc., USA.

3.2.1.3 Reagents used in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Acrylamide (Lot 059K1523), N,N'-methylenebisacrylamide (Lot 106K0158), N,N,N',N'-tetramethylethylenediamine (TEMED) (Lot 068K0714), 2-mercaptoethanol (Lot 09524MH), coomassie brilliant blue R (Lot 99F5035) and bromophenol blue (Lot 63H3607) were obtained from Sigma-Aldrich Inc., USA. Trizma base [tris (hydroxymethyl aminomethane)] (Lot 1247B029) was the product of AMRESCO, USA. Sodium dodecyl sulphate (SDS), ammonium persulphate, glycine, glycerol, acetic acid and methanol were purchased from SYSTERM®, Malaysia.

3.2.1.4 Other reagents

Disodium hydrogen phosphate, sodium dihydrogen phosphate, buffer reference standards (pH 4.0 and 7.0) and hydrochloric acid were purchased from SYSTERM®, Malaysia.

3.2.1.5 Miscellaneous

Hydrophilic PVDF (0.45 μm) membranes and Millex HV syringe driven filter units were purchased from Millipore Corporation, Ireland. Filter circles were supplied by Whatman®, Schleicher & Shhuell, England. Parafilm 'M' was the product of Pechiney Plastic Packaging, USA.

All glass distilled water was used throughout these studies and all experiments were performed at room temperature (~ 25˚C) unless otherwise stated.

3.2.2 Methods

3.2.2.1 pH measurements

A Mettler Toledo pH meter, Delta 320 attached with a BNC's combined electrode, type HA405-K2/120 consisting of glass and reference electrodes in a single entity was used in pH measurements. The least count of the pH meter was ± 0.01 pH unit. It was calibrated with the help of standard buffers (pH 4.0 in acidic range and pH 7.0 in neutral to alkaline range) at room temperature before pH measurements.

3.2.2.2 Absorption measurements

Absorption measurements were carried out in both the ultraviolet (UV) and visible regions on a Shimadzu double beam Spectrophotometer, UV-2450. Quartz and glass cuvettes of 1 cm path length were used in the ultraviolet (UV) and visible range respectively. Scattering corrections, if required, were made by extrapolation of absorbance values in the wavelength range, 360–340 nm to the desired wavelength.

3.2.2.3 Fluorescence spectroscopy

Fluorescence measurements were made on a Hitachi Fluorescence Spectrophotometer, model FL-2500. The excitation and emission slits were set at 10 nm each. After exciting the protein sample at 280 nm, the fluorescence spectrum was recorded in the wavelength range, 300–400 nm, using a quartz cuvette of 1 cm path length.

3.2.2.4 Sample collection

Adult *C. megacephala* were collected from a local wet market (Sungai Way, Petaling Jaya, Selangor, Malaysia) and brought to the laboratory (Pesticide Toxicology Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya) for colonization. They were reared in dried plastic containers (24 × 28 cm) supplied with granulated sugar, water and small pieces of fresh beef liver (as a protein source as well as egg collecting medium) placed in separate petri dishes. Beef liver pieces containing egg deposits were transferred to an open plastic box (6 × 9 cm) supplied with fresh beef liver pieces. This was placed in another plastic container (24 × 28 cm) covered with muslin cloth.

Roaming maggots (third instar larvae) were collected from the food medium into another plastic container (10 × 15 × 6 cm) and killed by adding hot water. They were separated using sieve, dried in an oven at 100˚C for 24 hours and grounded into powder form.

3.2.2.5 Preparation of maggot (*C. megacephala*) meal extract

Dried maggot powder (0.125 g) was dissolved in 25 ml of 0.06 M sodium phosphate buffer, pH 7.0 taken in a 50 ml beaker. The mixture was stirred using a magnetic stirrer, at 37˚C for 6 hours followed by centrifugation at 3645×g in order to get a clear solution. Supernatant was collected and used as a maggot (*C. megacephala*) meal extract in subsequent studies. Alternatively, 0.06 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) SDS was used to dissolve dried maggot powder and treated in the same way to prepare the extract.

3.2.2.6 Determination of protein concentration

Following treatments were made to prepare different samples of maggot (*C. megacephala*) meal extract for the determination of protein concentration using various colourimetric methods.

i. Treatment 1. Sample was prepared in the same way as described above by dissolving dried maggot powder (0.125 g) in 25 ml of 0.06 M sodium phosphate buffer, pH 7.0, followed by centrifugation at 3645×g. The supernatant was collected for protein content estimation.

ii. Treatment 2. Dried maggot powder (0.125 g) was dissolved in 25 ml of 0.06 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) SDS, followed by centrifugation at 3645×g and the supernatant was collected for protein estimation.

iii. Treatment 3. Dried maggot powder (0.125 g) was dissolved in 25 ml of 0.06 M sodium phosphate, pH 7.0 and reagents for different colour reactions were added separately. It was followed by centrifugation at 3645×g and the absorbance of the supernatant solution was read at respective wavelengths.

iv. Treatment 4. Dried maggot powder (0.125 g) was dissolved in 25 ml of 0.06 M sodium phosphate, pH 7.0 containing 1% (w/v) SDS and reagents for different colour reactions were added separately. It was followed by centrifugation at 3645×g and the absorbance of the supernatant solution was read at respective wavelengths.

Total protein content in maggot meal extract was determined using 5 different analytical procedures *i.e.* biuret method (Gornall *et al*., 1949), dye-binding method (Bradford, 1976), method of Lowry *et al*. (1951), spectrophotometric method (Warburg & Christian, 1942) and semi-micro Kjeldhal method (Helrich, 1990). Bovine serum albumin (BSA) was used as the standard for different colourimetric methods and standard plots were obtained both in the absence and presence of 1% (w/v) SDS.

In standard colourimetric assays, analytical reagents were added in sequence to a constant volume of maggot meal extract (supernatant obtained from Treatments 1 and 2) and the colour intensity was measured against a suitable blank (prepared in the same way but without maggot sample) on spectrophotometer. Alternatively, reagents were added first to the crude maggot meal extract (refer to Treatments 3 and 4) to develop colour followed by centrifugation at 3645×g and colour intensity measurement in the modified assays. Sodium phosphate buffer (0.06 M), pH 7.0 was used in these experiments.

Each experiment was performed at least three times and results were analyzed using Games-Howell post-hoc test (1976).

3.2.2.6.1 Spectrophotometric method (Warburg & Christian, 1942)

Different volumes (0.6, 1.2 and 1.5 ml) of maggot meal extract were pipetted into three different tubes and the final volume was made up to 3.0 ml with the same buffer. The solution was vortexed, filtered through millipore filter and absorbance values were recorded at 260 and 280 nm against buffer.

3.2.2.6.2 Biuret method (Gornall *et al*., 1949)

This method involves the use of biuret reagent which was prepared by dissolving 1.5 g cupric sulphate and 6.0 g sodium potassium tartrate in 500 ml water, taken in a volumetric flask. It was followed by the addition of 300 ml of 10% (w/v) sodium hydroxide solution with constant stirring. The reagent was stored at 8˚C for two weeks.

Suitable volumes of the buffer were added to different volumes (0.3, 0.6 and 0.9 ml) of the protein (maggot meal extract) solution, taken in three different tubes in order to make the final volume to 1.0 ml. It was followed by the addition of 4.0 ml of biuret reagent to each tube. The mixture was vortexed for 1 minute and the absorbance of the solution was read at 540 nm against suitable blank after 30 minutes of incubation at room temperature.

For the preparation of standard plots, increasing volumes (0.1–0.8 ml) of the stock protein (BSA) solution (4.0 mg/ml) were taken in a series of tubes and the final volume in each tube was made to 1.0 ml with buffer. It was followed in the same way as described above.

3.2.2.6.3 Dye-binding method (Bradford, 1976)

Bradford's reagent was prepared by dissolving 100 mg coomassie brilliant blue G in 50 ml of 95% (v/v) ethanol followed by the addition of 100 ml of 85% (v/v) phosphoric acid. The final volume of the reagent was made up to 1 litre with water. It was filtered through Whatman No. 1 filter paper before storage in an amber coloured bottle at room temperature. The reagent was stored for four weeks.

Different volumes (0.5, 0.6 and 0.7 ml) of maggot meal extract, taken in three different tubes were diluted to 1.0 ml with buffer followed by the addition of 5.0 ml of Bradford's reagent. The contents were shaken well and incubated for 30 minutes at room temperature. The absorbance of the coloured solution was recorded at 595 nm against a suitable blank, prepared in the same way but without protein.

For the preparation of the standard plot, increasing volumes (10–80 µl) of the stock protein (BSA) solution (1.0 mg/ml) were taken in a series of tubes and the final volume in each tube was made to 1.0 ml with buffer. Remaining procedure was the same as described above.

3.2.2.6.4 Method of Lowry *et al*. (1951)

The method involves the use of Copper reagent as well as Folin-Ciocalteu's phenol reagent. Copper reagent was prepared in water by mixing 4% (w/v) sodium carbonate, 4% (w/v) sodium potassium tartrate and 2% (w/v) copper sulphate in the ratio 100: 1: 1 (v/v/v) in the sequence in order to avoid precipitation. The reagent was filtered through Whatman filter paper, No. 1 before use. Working solution of Folin-Ciocalteu's phenol reagent was prepared by diluting the stock solution supplied by the manufacturer with water in a ratio 1: 3 (v/v) and stored in an amber coloured bottle.

To different volumes (0.2, 0.4 and 0.5 ml) of maggot meal extract taken in three different tubes, suitable volumes of the buffer were added first to make the total volume to 1.0 ml. Then, 5.0 ml of Copper reagent was added to each tube and incubated for 10 minutes at room temperature after shaking well. It was followed by the addition of 1.0 ml of working Folin-Ciocalteu's phenol reagent to each tube and the contents were mixed well. After incubation of 30 minutes at room temperature, the absorbance of the coloured solution was read at 700 nm against a suitable blank.

Standard plots were prepared using increasing volumes (0.1–0.8 ml) of BSA standard solutions [4.0 mg/ml, prepared in buffer alone and 0.5 mg/ml, prepared in the

same buffer containing 1% (w/v) SDS] in a series of tubes and treating them in the same way as described above with maggot meal extract.

3.2.2.6.5 Semi-micro Kjeldahl method (Helrich, 1990)

Maggot sample (0.1 g maggot powder) was digested with 15 ml of concentrated sulphuric acid taken in the digester flask for 1 hour at 400 ˚C under fume hood using BÜCHI Labortechnik digester, model K-435. The digested product was distilled with sodium hydroxide for 5 minutes on an automatic rapid steam distillation machine (C. Gerhardt, UK Ltd.), model Vapodest 20. Subsequently, the steam was purged into a flask containing 10 ml of 2% (w/v) boric acid and 6 drops of bromocresol green as the indicator and contents were mixed well. The distilled product was titrated with 0.05 N sulphuric acid and the percentage (%) of nitrogen available in samples was calculated following the method of Helrich (1990). Protein concentration (%) in the sample was obtained by multiplying the nitrogen percentage value with the conversion factor, 6.25.

3.2.2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein profiles of maggot meal extract were obtained by running the SDS-PAGE of maggot meal extract on 10% (w/v) polyacrylamide gel containing 0.1% SDS following the method of Laemmli (1970). Following stock solutions were needed for the preparation of both stacking and separating gels.

i. Solution A 29.2% (w/v) Acrylamide and 0.8% (w/v) N,N'-methylenebisacrylamide

ii. Solution B	1.5 M Tris-HCl buffer, pH 8.8
iii. Solution C	10% (w/v) Ammonium persulphate
iv. Solution D	0.5 M Tris-HCl buffer, pH 6.8, containing 0.4% (w/v) SDS
v. Sample buffer	62 mM Tris-HCl buffer, pH 6.8, containing 2.3% (w/v) SDS, 0.01% (w/v) bromophenol blue, 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol
vi. Electrophoresis buffer	0.025 M Tris, 0.192 M glycine and 0.1% (w/v) SDS, pH 8.3
vii. Fixing solution	40% (v/v) Methanol and 10% (v/v) acetic acid in water
viii. Staining solution	0.2% (w/v) Coomassie brilliant blue R in the fixing solution
ix. Destaining solution	5% (v/v) Methanol and 7% (v/v) acetic acid in water

A small pore gel (separating gel) solution was prepared by mixing 3.0 ml of Solution A, 3.0 ml of Solution B, 3.0 ml of water, 0.05 ml of Solution C and 5.0 µl of TEMED in a conical flask. Separating gel was prepared by pouring the above solution into the space between two glass plates assembled in the gel casting unit up to three-fourth of their height followed by layering the surface of the separating gel solution with a few drops of water. The gel was left at room temperature for 45 minutes to polymerize and the water layer was removed with the help of filter paper strips after the polymerization of the separating gel. Subsequently, large pore gel (stacking gel) solution (prepared by mixing 0.7 ml of Solution A, 1.25 ml of Solution D, 3.05 ml of water, 0.1 ml of Solution C and 5.0 µl of TEMED)

was poured gently above the separating gel up to a height of 2.0 cm using a micropipette. Immediately, a comb with 10 wells was inserted into it and the solution was allowed to polymerize at room temperature for 1 hour. Then, the comb was removed from the stacking gel and the newly formed wells were rinsed twice with electrophoresis buffer. The glass plates with the polymerized gel were fitted into an electrophoresis apparatus with half-filled electrophoresis buffer.

Prestained SDS-PAGE standard proteins along with their molecular weights given in parentheses such as ß-galactosidase (116,254 Da), bovine serum albumin (84,796 Da), ovalbumin (53,896 Da), carbonic anhydrase (37,418 Da), soyabean trypsin inhibitor (29,051 Da) and lysozyme (19,809 Da) were used for molecular weight determination of major poplypeptides of maggot meal extract. Protein sample was prepared by mixing 100 µl of maggot meal extract with 100 µl of the sample buffer and the mixture was heated for 3–5 minutes in boiling water bath. About 10 µl of the sample containing either standard proteins or maggot meal extract were loaded in separate wells with the help of a micropipette. The electrophoresis was performed for about 2 hours using a voltage of 10 volts/well. The power was switched off when the tracking dye reached the bottom of the gel. The gel was removed from the glass plates by purging electrophoresis buffer in the spaces between the gel and the glass plates with the help of a syringe. The gel was stained with staining solution for 30 minutes and destained in destaining solution by repetitive process until the background was clear. Distances travelled by the protein and dye bands were measured with the help of a ruler after placing the gel on a glass plate. Relative mobilities of different standard proteins as well as major polypeptides of maggot meal

extract were determined by dividing the distance travelled by the protein band with that of the dye (bromophenol blue) band. A straight line plot between log molecular weight and the relative mobility (R_m) of standard proteins was obtained by least squares analysis and molecular weights of major protein bands in maggot meal extract were determined.

3.2.3 Determination of amino acid composition

Amino acid analysis of maggot powder was carried out by hydrolyzing 74 mg of maggot sample with 15 ml of 6 N hydrochloric acid (HCl) at 100˚C for 24 hours in a sealed tube. A fixed volume (10 ml) of α-butyl amino acid was added into the hydrolyzed sample as the internal standard. Subsequently, the final volume of the mixture was made up to 50 ml with deionized water and filtered through 0.2 µm cellulose nitrate membrane. Then, 10 µl of the hydrolyzed sample was pipetted into a vial and mixed with 10 µl of the internal standard solution. Immediately, the mixture was dried under vacuum for 30 minutes. Meanwhile, a redrying solution was prepared by mixing methanol, water and triethylamine in the ratio 2: 2: 1 (v/v/v). After mixing with 20 µl of redrying solution, the mixture was re-dried under vacuum for 30 minutes.

The mixture was allowed to react with 20 µl of PITC reagent [phenylisothiocyanate, water, triethylamine, methanol (1: 1: 1: 7) (v/v/v/v)] for 20 minutes followed by drying under vacuum for 30 minutes in order to remove the excess PITC reagent. The derivatized samples were then dissolved in sample buffer (0.1 M ammonium acetate buffer, pH 6.5) which was used as a mobile phase for high performance liquid chromatography (HPLC) and filtered through a Millipore membrane. A 20 µl sample was injected into a reversed-

phase column in HPLC system (model Md 2010 from JASCO Inc., Japan) and monitored by UV absorption (PicoTag system, Waters).

3.2.4 Analysis of tryptophan

Proteins of maggot (*C. megacephala*) meal extract, prepared in 0.06 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) SDS were precipitated with 95% (v/v) ethanol in the ratio 1: 9 (v/v) and the mixture was left overnight at 8˚C. It was centrifuged at 14 000×g for 30 minutes and the precipitate was collected and incubated for 12 hours at 30˚C. It was dissolved in 10 ml of 0.06 M sodium phosphate buffer, pH 7.0. Intrinsic fluorescence was measured by exciting the protein sample at 280 nm and the fluorescence spectrum was recorded in the wavelength range, 300–400 nm.

3.3 Results and discussion

Table 3.1 shows a comparative analysis of the colourimetric methods – Biuret method (Gornall *et al*., 1949), dye-binding method (Bradford, 1976) and the method of Lowry *et al*. (1951), used in the determination of protein concentration in maggot meal extract. Standard plots obtained with these methods both in the absence and presence of 1% (w/v) SDS (Figures 3.1–3.3) yielded different linear equations and sensitivity range. Among the three methods, dye-binding method (Bradford, 1976) was found to be most effective due to its sensitivity up to a protein concentration of 7.9 µg/ml. On the other hand, biuret method (Gornall *et al*., 1949) showed poor sensitivity as the minimum protein concentration estimated was found to be 400 mg/ml. Presence of 1% (w/v) SDS in protein samples showed interference in both dye-binding method (Bradford, 1976) and the method of Lowry *et al*. (1951). However, SDS interference was more pronounced in dye-binding method (Bradford, 1976) due to inconsistencies in the results obtained at different protein concentrations (data not shown). The standard curve obtained with the method of Lowry *et al*. (1951) in presence of 1% (w/v) SDS showed significant reduction (20%) in the slope value (from 1.50 to 1.20) of linear equation compared to the one obtained in the absence of 1% (w/v) SDS. Interference of SDS with Bradford's method (1976) for protein estimation has also been reported earlier (Brooks *et al*., 1995). Therefore, Bradford's method (1976) seems to be useful for protein quantitation in normal protein samples without SDS. As several proteins in the multicellular subjects are known to be membrane-bound (Dennis, 1995; Jason *et al*., 2001), it is necessary to dissolve these proteins with a detergent like SDS for making correct estimation of total protein content. In view of this, standard plots obtained with various methods both in the absence and presence of 1% (w/v) SDS were

used. Since these methods are widely used for protein estimation in different biological samples (Crossman *et al.*, 2000; Matha *et al.*, 1983), quantification of protein in maggot meal sample was also made using all these methods under different experimental conditions.

Total protein content in maggot meal extract was determined using five different methods including above mentioned colourimetric methods under different experimental conditions as described in 3.2. Materials and methods. Warburg-Christian method (1941) was employed after dissolving the maggot powder in the buffer both in the absence and presence of 1% (w/v) SDS and the three colourimetric methods were tested using all four treatments. Results obtained with these methods in terms of protein concentration (mg/ml) as well as percentage (%) protein content are given in Table 3.2. Since the semi-micro Kjeldhal method (Helrich, 1990) is based on the total nitrogen estimation in the sample and involves complete digestion of the sample, different treatments were not employed for this method. The total protein percentage in maggot sample as obtained from semi-micro Kjeldhal method (Helrich, 1990) was found ~ 55.5% (Table 3.2). Semi-micro Kjeldhal method (Helrich, 1990) is the most popular method for protein estimation in biological samples and used extensively by many groups (Crossman *et al*., 2000; Keller & Neville, 1986; Kingsley, 1939; Zaia *et al.*, 2000).

A comparison of percentage protein content as determined by the other four methods both in the absence and presence of 1% (w/v) SDS (between Treatments 1 and 2; Treatments 3 and 4) suggested marked enhancement in the total protein content after SDS treatment. For example, 21% and 24% increase in the protein content was observed when determined in presence of 1% (w/v) SDS by the method of Lowry *et al*. (1951) and Warburg-Christian method (1941) respectively (see columns 3 and 5 of Table 3.2).

Similarly, a comparison of the data between Treatments 3 and 4 by the method of Lowry *et al*. (1951) showed ~ 19% increase in the protein content when measured in presence of 1% (w/v) SDS. This seems understandable as SDS, being anionic detergent would have solubilized the lipid bilayer (Lichtenberg *et al.*, 1983) and thus released all membrane-bound proteins into the solution. A lesser increase (~ 10%) in the percentage protein content observed with the biuret method (Gornall *et al*., 1949) upon SDS treatment can be ascribed either to the poor sensitivity of the method or the presence of interfering substances in the sample. A very high value of protein content (~ 70%) observed in Warburg-Christian method (1941) in presence of 1% (w/v) SDS may be due to the presence of other substances in the sample which absorb near 280 nm.

Application of Treatments 3 and 4 in the protein estimation was made to check the presence of any left-over protein in the residue obtained after filtration of the maggot meal extract. Results obtained with Treatments 1 and 3 verified this hypothesis as nearly 20% and 12% increase in the protein content was observed in biuret method (Gornall *et al*., 1949) and the method of Lowry *et al*. (1951) respectively when the reagents were added first for color development before the centrifugation (columns 3 and 7 of Table 3.2). These results also strengthened our hypothesis that several membrane-bound proteins remained undetected when the maggot sample was solubilized in buffer only. Surprisingly, a comparison between results obtained with Treatments 2 and 4 also showed a significant increase [17% and 10% when determined by biuret method (Gornall *et al*., 1949) and the method of Lowry *et al*. (1951) respectively, when the reagents were added first in the presence of 1% (w/v) SDS (see columns 5 and 9 of Table 3.2)].

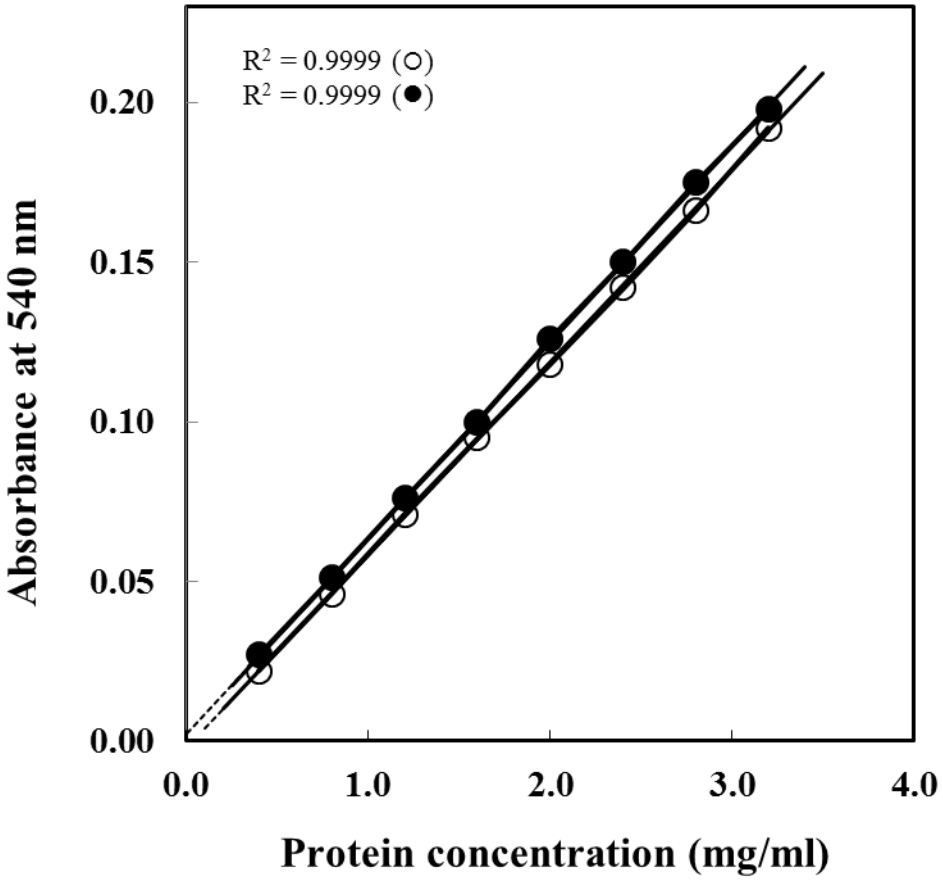

Figure 3.1: Standard curves for the determination of protein concentration by Biuret method (1949) using BSA as the standard. These curves were obtained in 0.06 M sodium phosphate buffer, pH 7.0 in the absence (○) and presence (●) of 1% SDS.

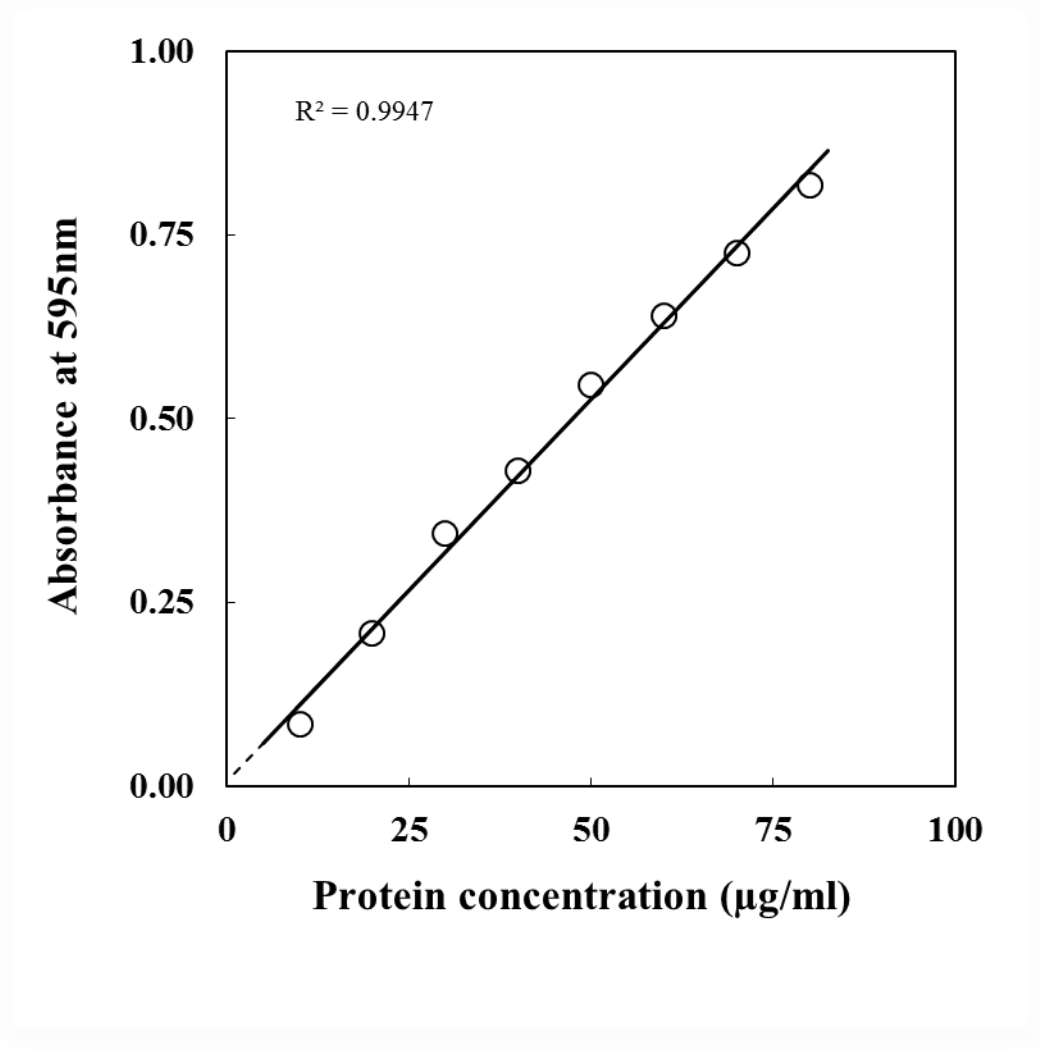

Figure 3.2: Standard curve for the determination of protein concentration by dye-binding method of Bradford (1976) using BSA as the standard. The curve was obtained in 0.06 M sodium phosphate buffer, pH 7.0 in the absence (○) of 1% SDS.

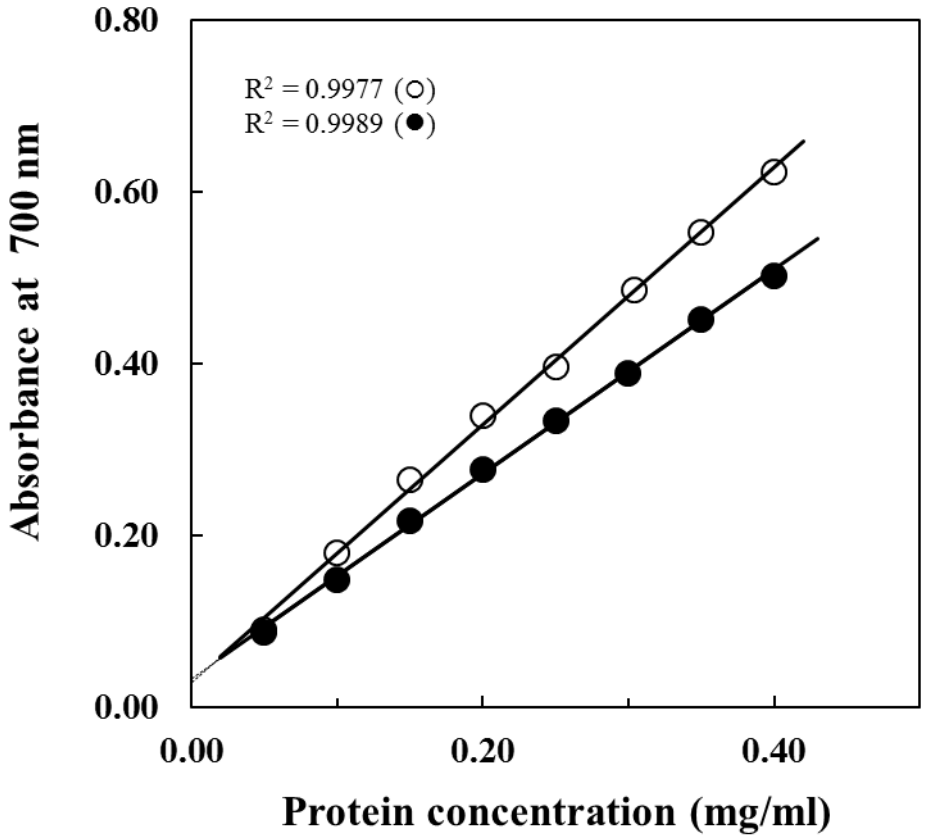

Figure 3.3: Standard curves for the determination of protein concentration by the method of Lowry *et al*. (1951) using BSA as the standard. These curves were obtained in 0.06 M sodium phosphate buffer, pH 7.0 in the absence (○) and presence (●) of 1% SDS.

Table 3.1: Regression analysis of various analytical methods used for protein estimation in maggot (*C. megacephala*) meal extract.

Methods	Regression equation	Sensitivity range
1. Biuret (1949)		
- without 1 % SDS	$(Abs.)_{540nm} = 0.06$ Protein conc. (mg/ml) – 0.002	0.40 – 3.23 mg/ml
- with 1 % SDS	$(Abs.)_{540nm} = 0.06$ Protein conc. (mg/ml) + 0.020	0.42 – 3.27 mg/ml
2. Bradford (1976)	$(Abs.)_{595nm} = 0.01$ Protein conc. (µg/ml) + 0.005	7.90 – 81.30 µg/ml
3. Lowry *et al*. (1951)		
- without 1 % SDS	$(Abs.)_{700nm} = 1.50$ Protein conc. (mg/ml) + 0.030	40.00 – 395.30 µg/ml
- with 1 % SDS	$(Abs.)_{700nm} = 1.20$ Protein conc. (mg/ml) + 0.030	47.50 – 394.20 µg/ml

Abs. = Absorbance

conc. = Concentration

Table 3.2: Comparative analysis of protein estimation in maggot (*C. megacephala*) meal extract as determined by different methods under different experimental conditions.

Methods	Treatment 1			Treatment 2			Treatment 3			Treatment 4		
	Protein concentration [mg/ml ± S.E.]	Percentage [(%) ± S.E.]	Arcsine	Protein concentration [mg/ml ± S.E.]	Percentage [(%) ± S.E.]	Arcsine	Protein concentration [mg/ml ± S.E.]	Percentage [(%) ± S.E.]	Arcsine	Protein concentration [mg/ml ± S.E.]	Percentage [(%) ± S.E.]	Arcsine
Biuret (1949)	1.11 ± 0.02	22.2 ± 0.76^{a}	0.49	1.61 ± 0.03	32.2 ± 1.20^{a}	0.60	2.12 ± 0.08	42.4 ± 4.02^{a}	0.71	2.46 ± 0.06	49.2 ± 2.75^{a}	0.78
Bradford (1976)	0.18 ± 0.01	3.6 ± 0.22^{b}	0.19	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Lowry *et al.* (1951)	0.69 ± 0.01	13.8 ± 0.26^{c}	0.38	1.75 ± 0.03	34.9 ± 1.64^{ab}	0.63	1.28 ± 0.02	25.6 ± 2.12^{b}	0.53	2.24 ± 0.04	44.8 ± 1.79^{a}	0.73
Warburg-Christian (1942)	2.30 ± 0.10	46.0 ± 4.96^{d}	0.74	3.48 ± 0.11	69.6 ± 5.24^{bc}	0.99	–	–	–	–	–	–
Semi-micro Kjeldahl (1990)	–	55.5 ± 2.96	0.84^{d}	–	55.5 ± 2.96^{c}	0.84	–	55.5 ± 2.96^{c}	0.84	–	55.5 ± 2.96^{c}	0.84

N.D. = Not determined.

Each value represents the mean of three independent experiments. Mean values shown with superscripts using different letters within each column are statistically significant (5% significance level; Bonferroni adjustment) pairwise multiple comparison when unequal variances in the groups.

This clearly suggested that SDS was either not competent enough to solubilize all membrane-bound proteins or some proteins had undergone aggregation and thus remained in the insoluble fraction. As a whole, comparison of results obtained with biuret method (Gornall *et al*., 1949) and the method of Lowry *et al*. (1951) in presence of 1% (w/v) SDS by adding reagents first (last column of Table 3.2) with that obtained with semi-micro Kjeldhal method (Helrich, 1990) suggested that both biuret method (Gornall *et al*., 1949) and the method of Lowry *et al.* (1951) can be used successfully to determine protein content in biological samples under specified conditions. It seems appropriate as the protein content determined by semi-micro Kjeldhal method (Helrich, 1990) may represent the value towards the higher side due to the presence of other nitrogenous compounds (DNA and RNA) in biological samples.

Characterization of major polypeptides of maggot meal extract was made by SDS-PAGE. Figure 3.4 shows electrophoretic pattern of major proteins of maggot meal extract (MG) along with different marker proteins (M) on 10% (w/v) polyacrylamide gel. As can be seen from the figure, a total of twelve protein bands (MG1–MG12) differing in mobility were visualized on the gel after staining with coomassie brilliant blue R. However, the actual number of protein bands in maggot meal extract may be higher as the resolution and detection of protein bands depend on the sensitivity of the staining dye as well as resolving power of the gel. Since separation of various proteins in SDS-PAGE is based on the size (molecular weight) of a protein, electrophoretrogram of maggot meal extract showed the presence of different sized proteins. Based on the intensity of protein bands it can be said that some proteins were present as major constituents while others had relatively smaller concentrations. Values of relative mobility (R_m) of different protein

bands are given in Table 3.3 which were used to determine their molecular weights. Figure 3.5 shows the standard curve between Log molecular weight and relative mobility (R_m) of various marker proteins. A least squares analysis of the data was found to follow the given straight line equation:

$$\text{Log molecular weight} = -0.9898\ R_m + 5.1979 \quad (1)$$

Substitution of R_m values into the above equation yielded molecular weights of different proteins present in maggot meal extract (Table 3.3). Both high and low molecular weight proteins were present in the maggot meal extract as the molecular weight values of these proteins varied from ~ 17 kDa to ~ 83 kDa. A comparison of data shown in Figure 3.4 and Table 3.3 suggested that maggot meal extract was rich in low molecular weight protein fraction compared to high molecular weight protein fraction. This is more evident from Figure 3.4 where the bottom half of the gel was more densely stained compared to the upper half. This seems advantageous for the selection of maggot meal extract as a protein source in an animal feed since low molecular weight proteins have greater solubility and are easily digested compared to high molecular weight proteins (Duodu *et al.*, 2003).

Table 3.4 shows amino acid composition of maggot sample as obtained after acid hydrolysis. Since acid hydrolysis completely destroys tryptophan and converts amide forms of acidic amino acids (asparagine and glutamine) into their acid forms, quantitation of three amino acids, namely, tryptophan, asparagine and glutamine could not be made. As evident from Table 3.4, majority of essential amino acids were present in maggot sample. Since nutritional quality of the protein in animal feed depends on the presence of essential amino

acids, maggot meal extract offers a better candidate than fishmeal for its selection as the protein source in animal feed (Santiago & Lovell, 1988).

Figure 3.6 shows the fluorescence spectrum of maggot protein sample (obtained by ethanol precipitation) in 0.06 M sodium phosphate buffer upon excitation at 280 nm. As can be seen from the figure, fluorescence spectrum appeared in the wavelength range, 300–400 nm with an emission maximum at 348 nm. Occurrence of an emission maximum ~ 340 nm was suggestive of the presence of tryptophan residues in the sample (Jennifer *et al.*, 1998). Although, this result was qualitative in nature, it confirmed the presence of a significant amount of tryptophan in maggot sample. Tryptophan, being an essential amino acid and its presence in maggot sample adds further to the quality of maggot meal extract and its use as the animal feed.

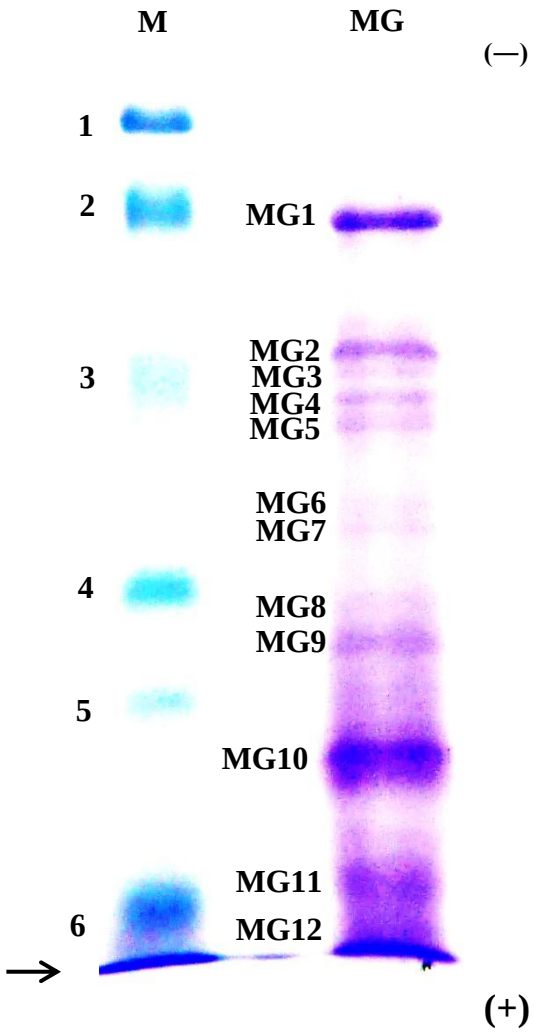

Figure 3.4: SDS-PAGE pattern of maker proteins (M) and maggot (*C. megacephala*) meal extract (MG) on 10% polyacrylamide gel following the method of Laemmli (1970). The arrow shows the position of tracking dye, bromophenol blue. About 10 µl of sample containing 24.6 µg protein was loaded and electrophoresis was performed for about 2 hours. Marker proteins used were: 1. ß-galactosidase, 2. bovine serum albumin, 3. ovalbumin, 4. carbonic anhydrase, 5. soyabean trypsin inhibitor and 6. lysozyme. Major protein bands of maggot meal extract are shown as MG1–MG12.

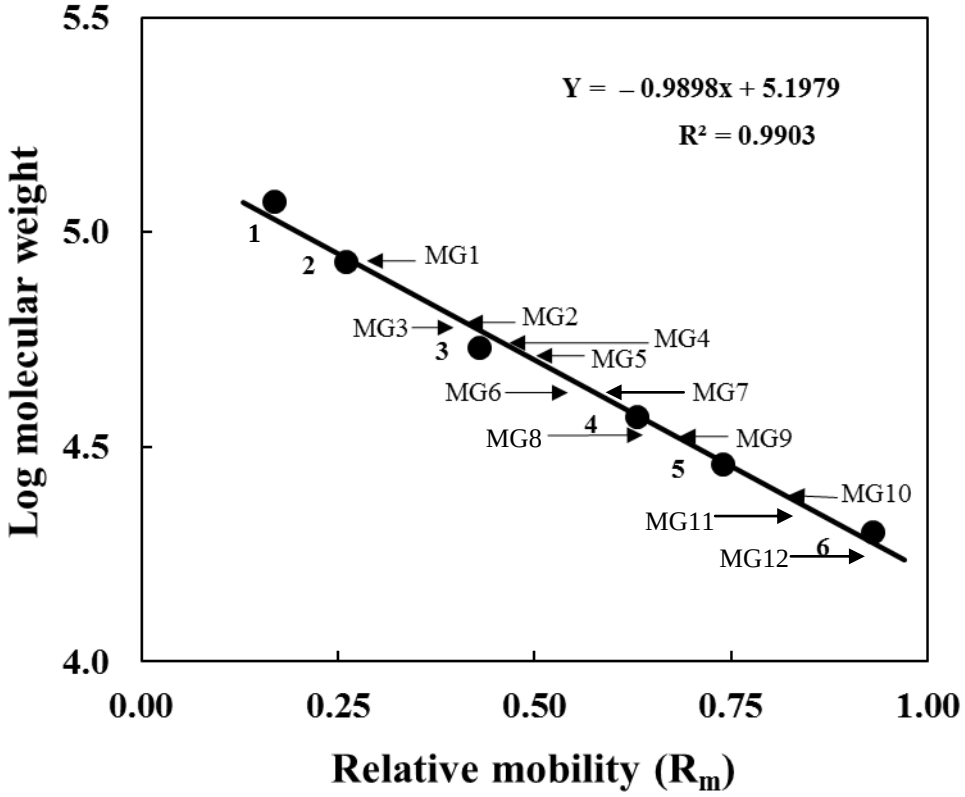

Figure 3.5: Plot of log molecular weight versus relative mobility (R_m) of different marker proteins as obtained from Figure 3.4. Numbers 1–6 refer to different marker proteins, whereas MG1–MG12 represent major protein bands of maggot (*C. megacephala*) meal extract as shown in Figure 3.4. Straight line was drawn using least squares analysis.

Table 3.3: Values of relative mobility and molecular weight of major protein bands present in maggot (*C. megacephala*) meal extract as determined by SDS-PAGE.

Protein band	Relative mobility (R_m)	Molecular weight (Dalton)
MG1	0.28	83,321
MG2	0.41	61,956
MG3	0.43	59,195
MG4	0.44	57,861
MG5	0.48	52,820
MG6	0.56	44,016
MG7	0.57	43,024
MG8	0.65	35,845
MG9	0.69	32,729
MG10	0.81	24,898
MG11	0.91	19,941
MG12	0.98	16,843

Table 3.4: Amino acid composition of (*C. megacephala*) maggot powder.

Amino acids	Relative concentration (%)
Essential amino acids	
Histidine	1.02
Threonine	2.19
Valine	2.20
Methionine	1.02
Isoleucine	4.13
Leucine	3.41
Phenylalanine	1.72
Lysine	3.72
Non-essential amino acids	
Aspartic acid	3.87
Glutamic acid	7.26
Serine	1.94
Glycine	2.27
Arginine	2.43
Alanine	2.73
Proline	2.05
Tyrosine	1.48
Cysteine	0.36

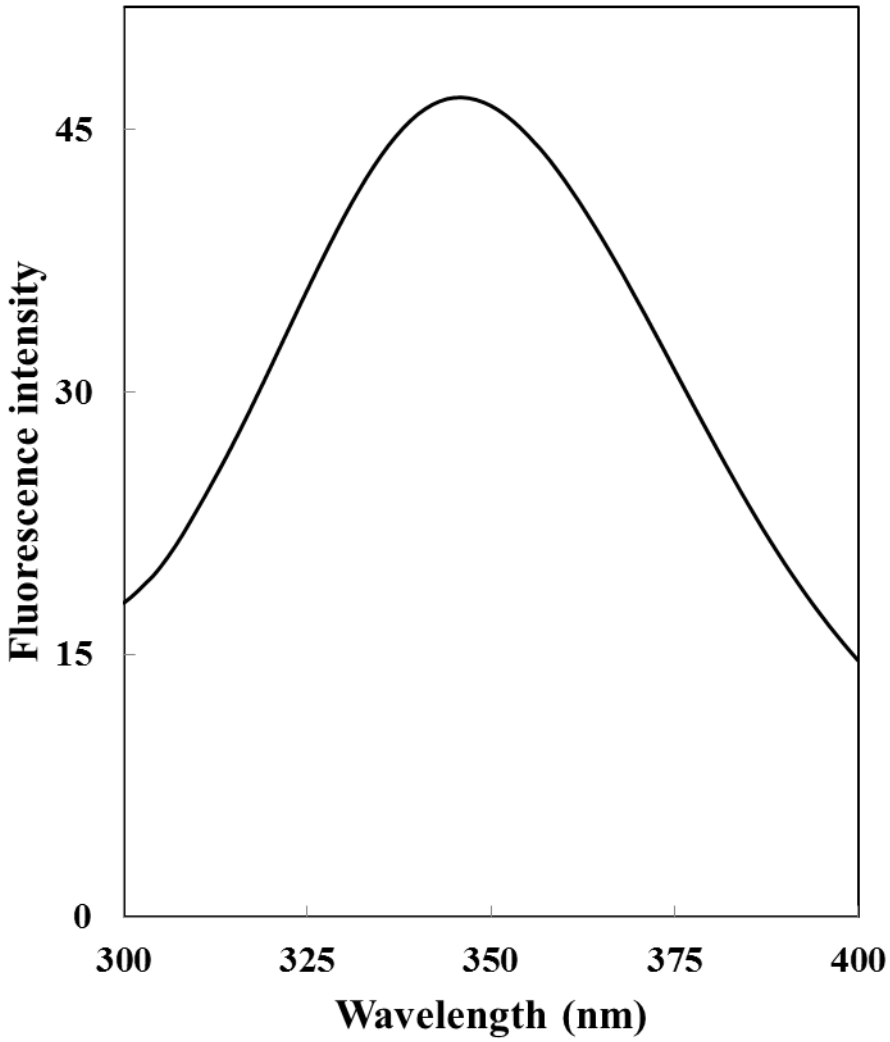

Figure 3.6: Fluorescence spectrum of maggot (*C. megacephala*) protein sample obtained in 0.06 M sodium phosphate buffer, pH 7.0 at 25°C upon excitation at 280 nm.

CHAPTER 4

EVALUATION OF BLOWFLY (*CHRYSOMYA MEGACEPHALA*) MAGGOT MEAL AS AN EFFECTIVE AND SUSTAINABLE REPLACEMENT FOR FISHMEAL IN THE DIET OF FARMED JUVENILE RED TILAPIA (*OREOCHROMIS* SP.)

4.1 Introduction

Tilapia from the genus *Oreochromis* is in very high demand in the market because it is consumed by people globally. Global tilapia production was predicted up to 3.7 million tons in the end of 2010 and the demand for tilapia still growing especially in United State, the single largest market for tilapia. The high demand in the global tilapia market has thrown the attention from some countries such as Malaysia, Brazil, Thailand and the Philippines to invest in this potential market. Some programs have been implemented to enhance the production of tilapia in those countries, such as selective breeding and genetic improvements technology in tilapia breeding. Nevertheless, the feed still contribute the major production cost in this aquaculture area due to the high price of fishmeal.

Protein is an important dietary nutrient in animal feed as well as in aquaculture diets to enhance the growth of poultry and fish (Marley, 1998). Traditionally, protein is sourced from product such as fish and soybean. Fishmeal contains high nutritional value especially its crude protein (approximately 50%) compared with plant protein. Furthermore, plant proteins lack at least one of the essential amino acids needed by animals. Hence, fish is the best form of protein source (Spinelli, 1978) for formulation of animal diet. However, fish,

which is normally obtained from the wild, has dwindled due to overexploitation, resulting from the ever-increasing human population. Aquaculture has been found necessary as one approach to increase fish production to make sufficient fish/protein available to the population. The high competition for the same foodstuffs between man and domestic animals has increased the price of fishmeal, which is the sole protein source in fish and animal feeds. It is therefore very crucial that an alternative is found to reduce feeding cost, and to make aquaculture a viable and attractive venture (Jauncey, 1982). Various experiments on fishmeal replacement in aquafeed have been conducted to find an alternative effective and sustainable protein source (Cabral *et al*., 2011; Deng *et al*., 2006; Richard *et al*., 2011; Silva *et al*., 2010).

Insect larvae have been found as a very good protein source by many researchers. Housefly (*Musca domestica*) is one of the well reported in literature. However, blowfly *Chrysomya megacephala* from the same Order as the housefly is one of the potential species to be used as protein source in animal feed. *Chrysomya megacephala* maggots are always found on a carcass, are forensically important, and they feed on the decaying organic matter. Hence, the ability of *C. megacephala* to convert the wastes into the better quality of protein needs further investigation. Thus, this study will investigate the potential value of blowflies *C. megacephala* maggots to be used as protein source in red tilapia *Oreochromis* sp.

4.2 Materials and methods

The experimental diets used in this study were formulated and produced at the Aquafeed Laboratory, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, Malaysia. The fish feeding trial was conducted at the Marine and Fresh Water Toxicology Laboratory, Institute of Biological Sciences, University of Malaya, Malaysia.

4.2.1 Maggot meal preparation

Adults of *C. megacephala* were collected from the local wet market and cultured in the Pesticide Toxicology Laboratory, Institute of Biological Sciences, University of Malaya, Malaysia. A slice of fresh beef liver acting as the egg collecting medium was placed in a small container (4 × 2 cm) within a plastic culture container (24 × 28 cm). Once the eggs of *C. megacephala* have been deposited on the beef liver slice, the medium would be transferred to another round plastic container which was then covered with fabric. Maggots hatched from eggs over a period of 4 days were killed by adding hot water. They were sieved and dried in an oven at 100 ˚C for 24 hours before being ground into powder form.

4.2.2 Determination of protein concentration of maggot meal

The protein content of maggot meal was determined using semi-micro Kjeldahl method (Helrich, 1990). A volume of 12 ml of concentrated sulphuric acid was used to digest 0.1 g of maggot powder for 1 hour at 400 ˚C in fume hood using BÜCHI™ Labortechnik digester, model K-435. The digested product was distilled with sodium hydroxide for 5 minutes on an automatic rapid steam distillation machine (C. Gerhardt, UK

Ltd.), model Vapodest20™. Crude protein content was obtained by multiplying the nitrogen percentage value with 6.25.

4.2.3 Determination of amino acid composition of maggot meal

Maggot meal was hydrolyzed with 6 N hydrochloric acid at 100 ˚C for 24 hours. An internal standard amino acid was added into the hydrolyzed sample before filtering through a 0.2 µm cellulose nitrate membrane. Subsequently, a reagent containing methanol, phenylisothiocyanate, triethylamine and water in the ratio 7: 1: 1: 1 was allowed to react with the filtered sample for 20 minutes. This was followed by vacuum drying for 30 minutes. The samples were then dissolved in 0.1 M ammonium acetate (pH 6.5) which was used as the mobile phase for high performance liquid chromatography (HPLC) before filtering with a Millipore membrane. A 20 µl sample was injected into a reversed-phase column in HPLC system (JASCO™ Md 2010) and monitored by UV absorption (PicoTag™ system, Waters).

4.2.4 Experimental diets

Prior to the feed formulation, the approximate nutrient composition of feed ingredients was analyzed. All ingredients were ground into fine powder form with a laboratory grinder. Five experimental diets: a control diet (M0) containing fishmeal as the main animal protein source and four increasing substitution levels of maggot meal to fishmeal at 25% (M25), 50% (M50), 75% (M75) and 100% (M100) were formulated (Table 4.2) to contain approximately 30.0% of crude protein and 20.0 kJg^{-1} of gross energy. This requirement will affect the quantities of the feed components. The diets were

processed using a laboratory scale extruder (Brabender ^{TM} KE19), dried at 50˚C for 12 hours, sealed and stored at room temperature until use.

4.2.5. Feeding trial

Juvenile red tilapia supplied by a local hatchery, were quarantined for a week before being used for the growth trial experiment. All fish were fasted for 24 hours at the beginning of the experiment and body weight of fish was measured individually. Ten juveniles were stocked into each glass tank (60 × 30 × 30 cm) equipped with a closed recirculation water system. Each diet was randomly assigned to a tank in triplicates. Fish were kept in a natural photoperiod regime and the water temperature of 25 ± 1.8 ˚C. Feeding was done twice daily at 0800 and 1600 for 60 days at a daily feeding rate of 5% of total biomass. Throughout the feeding trial period, fish were bulk weighed every two weeks in order to adjust the feeding rate.

At the end of the feeding trial, fish were fasted for 24 hours before the final body weight was recorded. Specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) were calculated as (following Chou and Shia, 1996):

SGR = ((ln (Final body weight) – ln (Initial body weight)) / number of days) X 100

FCR = Total feed intake (g) / (Final body weight – Initial body weight) (g)

PER = (Final body weight – Initial body weight) (g) / Total protein intake (g)

4.2.6. Chemical analysis

At the end of the feeding trial experiment, fish were starved for 24 hours before the body weight was recorded. Subsequently, the fish were sacrificed and dried in an oven at 105 ˚C for 48 hours. The amount of moisture lost was recorded after 48 hours. For proximate composition, the whole fish and dorsal muscle parts (fillets without skin) of 3 – 4 fishes from each replicate were oven dried and analyzed.

The crude protein, crude lipid, ash, fiber and gross energy of the experimental diets, the whole fish and fillets were determined according to the AOAC method (1990). An adiabatic bomb calorimeter was used to estimate the gross energy of the dry sample.

4.2.7 Statistical analysis

All the data were recorded as mean ± standard deviation and were subjected to one-way analysis of variance (ANOVA). All percentage data were arcsine transformed prior to analysis. The differences among treatment means were analyzed using Duncan's multiple range test using R 2.13.1.

4.3 Results and Discussion

In this study, the potential of *C. megacephala* as an alternative protein source in red tilapia feed was investigated and the best performance was observed when fishmeal was substituted with maggot meal at 100%.

The crude protein and amino acid profile of *C. megacephala* maggots (different days after hatching from eggs) are summarized in Table 4.1. Crude protein content of maggot meal powder derived from maggots hatched from eggs over a period of 4 days varied between 52% and 56% while the amino acids composition were slightly different (Table 4.1). These values are similar to the housefly maggot meal that was reported by Awoniyi *et al*. (2003), but are slightly higher when compared to the data published by Gado *et al*. (1982) and Atteh and Ologbenla (1993). However, Calvert *et al*. (1971) found that the crude protein of housefly maggot meal is 65% which is much higher than the others. Such variations in protein content for the same maggot meal can be attributed to the processing, drying, storage and protein estimation method employed or the media for the production of housefly maggots (Awoniyi *et al*., 2003; Ogunji *et al*., 2008).

Maggot meal derived from maggots that hatched after 1 day have the highest concentration of amino acids as compared to the others. However, the difference in the concentration of the essential amino acids among the maggot meal of the various days are very small (< 2%) except Cystine from the non-essential amino acid group. Since, acid hydrolysis completely destroys tryptophan, asparagine and glutamine, quantification of these three amino acids could not be performed (Tsugita & Scheffler, 1982). As evident from Table 4.1, the majority of essential amino acids are present in the maggot samples.

Since nutritional quality of the protein in animal feed depends on the presence of essential amino acids, maggot meal extract is a better candidate than fishmeal for selection as the alternative protein source in animal feed (Santiago & Lovell, 1988).

All the experimental diets had similar crude protein content at 292.0 – 311.0 g kg^{-1} and gross energy 19.9 – 21.8 kJ g^{-1}, while crude lipid ranged from 468.0 – 562.0 g kg^{-1}. The ash content decreased from 104.0 – 43.0 g kg^{-1} as the level of substitution maggot meal to fishmeal increased. The fiber content decreased from 60.0 – 22.0 g kg^{-1} in the M0 to M50 diets and gradually increased to 31.0 g kg^{-1} when the fishmeal was totally replaced by maggot meal (M100). All the experimental diets contain 60 – 70% of moisture except the diet with 25% substitution of maggot meal to fishmeal (48%).

The initial body weight of juvenile red tilapia was similar (~ 3.00 g) for all the treatments before the feeding trial experiment. At the end of the experiment, the final body weight of juvenile red tilapia increased when the percentage of maggot meal substituting fishmeal in the diet increased. This is shown in Figure 4.1. The highest final body weight (10.63 g) was recorded from the group of fish fed with the diet that contained only maggot meal without fishmeal. Therefore, this group of fish has the highest percentage of weight gain at the end of the experiment (~ 250%) and it is significantly different from the other groups of fish (Figure 4.2). Other studies also revealed that maggot meal with partial substitution levels for fishmeal is accepted by omnivorous fish species such as catfish and Nile tilapia but not at the total substitution level (Brinker & Reiter, 2010; Cabral *et al*., 2011; Ogunji *et al*., 2008; Oyelese, 2007). In addition, other researchers reported that the level of replacement fishmeal with housefly maggot at below 50% appeared to be the

optimum substitution level for broiler feed (Adenji, 2007; Awoniyi *et al*., 2003). This is because of the high level of replacement fishmeal by housefly maggot meal which is associated with low body weight gain in both fish and broiler.

Table 4.3 summarizes the initial and final body weight of fish, survival, special growth rate, food conversion ratio and protein efficiency ratio of juvenile red tilapia after feeding with the experimental diets for 60 days. At the end of the experiment, the highest survival rate of juvenile red tilapia was observed in those fed with 100% substitution (80.0%) maggot meal. Even though a lower survival rate was recorded in the lower levels of replacement maggot meal to fishmeal, there are no significant differences ($P > 0.05$) in survival rate among the various experimental diets. Special growth rate (SGR) increased when the level of substitution increased. The maximum SGR value (2.02% day^{-1}) was recorded when fishmeal was totally replaced by maggot meal and is significantly different ($P < 0.05$) from other the diets. When fed on a fishmeal-free diet (M100), the juvenile red tilapia had the best feed conversion ratio (1.34) and protein efficiency ratio (0.30). There is no significant difference ($P > 0.05$) among the experimental diets in feed conversion and protein efficiency ratio.

Table 4.1: Analyzed crude protein (%) and amino acid composition (%) of blowfly maggot (different day after it hatched from egg).

	Day			
	1	2	3	4
Protein concentration	55.0	56.2	54.0	52.4
Essential amino acid				
Histidine	2.5	1.4	1.5	1.5
Threonine	4.7	2.7	2.3	2.3
Valine	3.5	2.3	2.4	2.6
Methionine	2.1	1.2	1.3	1.5
Isoleucine	3.7	1.9	1.9	2.1
Leucine	4.9	3.4	3.5	3.7
Phenylalanine	2.9	2.5	3.2	4.0
Lysine	5.3	4.1	4.4	4.6
Non-essential amino acid				
Aspartic acid	12.1	10.0	10.7	12.1
Glutamic acid	15.7	13.2	13.8	14.4
Serine	2.9	2.0	2.0	2.2
Glycine	3.2	2.4	2.4	2.3
Arginine	3.4	2.7	2.2	2.6
Alanine	3.9	3.1	2.8	2.8
Proline	3.7	2.0	2.0	2.2
Tyrosine	3.2	1.8	2.9	4.2
Cystine	2.9	0.2	0.1	0.1

Table 4.2: Feed composition and proximate analysis of the experimental diets.

	Experimental Diets				
	M0	M25	M50	M75	M100
Ingredients (g kg^{-1})					
Fishmeal	300.0	225.0	150.0	75.0	0.0
Maggot meal	0.0	75.0	150.0	225.0	300.0
Soybean meal	210.0	213.0	248.0	254.0	259.0
Tapioca starch	200.0	200.0	282.0	276.0	271.0
Rice bran	222.0	236.0	50.0	50.0	50.0
Fish oil	15.0	15.0	50.0	50.0	50.0
Corn meal	0.0	0.0	50.0	50.0	50.0
Sunflower oil	34.0	16.0	15.0	5.0	2.0
Mineral premix[a]	10.0	10.0	10.0	10.0	10.0
Vitamin premix[b]	10.0	10.0	10.0	10.0	10.0
Proximate composition (g kg^{-1} dry matter)					
Crude protein	295.0	310.0	292.0	295.0	311.0
Crude carbohydrate	468.0	497.0	562.0	555.0	546.0
Ash	104.0	93.0	58.0	52.0	43.0
Fiber	60.0	52.0	22.0	25.0	31.0
Moisture	73.0	48.0	62.0	72.0	69.0
Gross energy (kJ g^{-1})	21.8	20.8	19.7	19.9	20.0

[a] Mineral premix (g kg^{-1}): potassium chloride, 9; potassium iodide, 0.004; dicalcium phosphate dihydrate, 50; sodium chloride, 4; copper sulfate, 0.3; zinc sulfate, 0.4; cobalt (II) sulfate, 0.002; ferrous sulphate, 2; manganese (II) sulfate, 0.3; calcium carbonate, 21.5; magnesium hydroxide, 12.4; sodium selenite, 0.003; sodium fluoride, 0.1.

[b] Vitamin premix (g kg^{-1}): ascobic acid, 45; myo-insitol, 5; choline chloride, 75; niacin, 4.5; riboflavin, 1; pyridoxine, 1; thiamin mononitrate, 0.9; ca-pantothenate, 3; retinyl acetate, 0.6; cholecalciferol, 0.08; vitamin K menadione, 1.7; α-tocopheryl acetate (500 IU g^{-1}), 8; biotin, 0.02; folic acid, 0.1; vitamin B_{12}, 0.001; cellulose, 845.1.

Superior growth performance in total replacement with *C. megacephala* maggot meal was observed in this study whereas high inclusion replacement levels with other animal or plant proteins have led to growth reduction of fish (Begum *et al*., 1994; Millamena, 2002; Ogunji *et al*., 2007). The possible reasons for the reduced growth of fish in this instance may be due to deficiencies in essential nutrients such as essential amino acids in animal or plant proteins. Deficiency of essential amino acids in diet is manifested as a reduction in weight gain. Moreover, insufficient amount of certain indispensable amino acids in any given diet can cause fish to suffer from cataracts (methionine and tryptophan) and scoliosis (tryptophan) (Cowey, 1994). Present study showed that *C. meagacephala* maggot meal contains all the indispensable amino acids that are needed by juvenile tilapia (Santiago & Lovell, 1988) and in sufficient amounts and thus is a better candidate for selection than the other diets as the alternative protein source in tilapia feed.

Proximate analysis ash content in experimental diets shows that diets with higher inclusion levels of maggot meal contain lower ash content. Diet digestibility is affected by the ash content in any given feed. High ash content in diet may contribute to low digestibility of the diet. In this study, the apparent digestibility of maggot meal has not been measured for tilapia but the higher values of feed conversion ratio (FCR) for control (fishmeal) and low levels replacement of fishmeal with *C. megacephala* maggot meal diets suggested that high levels of ash will lower growth rates of juvenile tilapia.

In conclusion, *C. megacephala* maggot meal could be an alternative protein source to replace fishmeal in tilapia diet. The total replacement of fishmeal with cheaper maggot meal in tilapia diet directly reduces the production costs of tilapia as well as dependency on

trash fish that is comprehensively being used as feed. Further studies under on-farm conditions should be carried out to determine the growth performance of fish fed with fish replacement diets and its long-term effects.

Table 4.3: Initial body weight (g), final body weight (g), special growth rate (SGR), food conversion ratio (FCR) and protein efficiency ratio (PER) of red tilapia *Oreochromis* sp. after feeding with the experimental diets for 60 days.

	Experimental diets				
	M0	M25	M50	M75	M100
Initial body weight (g)	2.75 ± 0.57	3.00 ± 0.27	3.21 ± 0.22	3.29 ± 0.21	3.11 ± 0.14
Final body weight (g)	5.64 ± 0.93	6.34 ± 1.71	7.47 ± 1.44	8.53 ± 2.22	10.63 ± 2.65
Survival rate (%)	63.33 ± 11.55^{a}	60.00 ± 10.00^{a}	76.67 ± 15.28^{a}	73.33 ± 25.17^{a}	80.00 ± 26.46^{a}
SGR (% day^{-1})	1.21 ± 0.34^{a}	1.21 ± 0.37^{a}	1.39 ± 0.21^{a}	1.55 ± 0.34^{ab}	2.02 ± 0.27^{b}
FCR	2.89± 0.99^{a}	2.63 ± 1.10^{a}	2.08 ± 0.35^{a}	1.97 ± 0.50^{a}	1.34 ± 0.14^{a}
PER	0.18 ± 0.06^{a}	0.17 ± 0.06^{a}	0.20 ± 0.05^{a}	0.21 ± 0.07^{a}	0.30 ± 0.12^{a}

Value (mean ± standard deviation) of three replications in same row followed by different letters are statistically significant using, Duncan's multiple range test, $P < 0.05$.

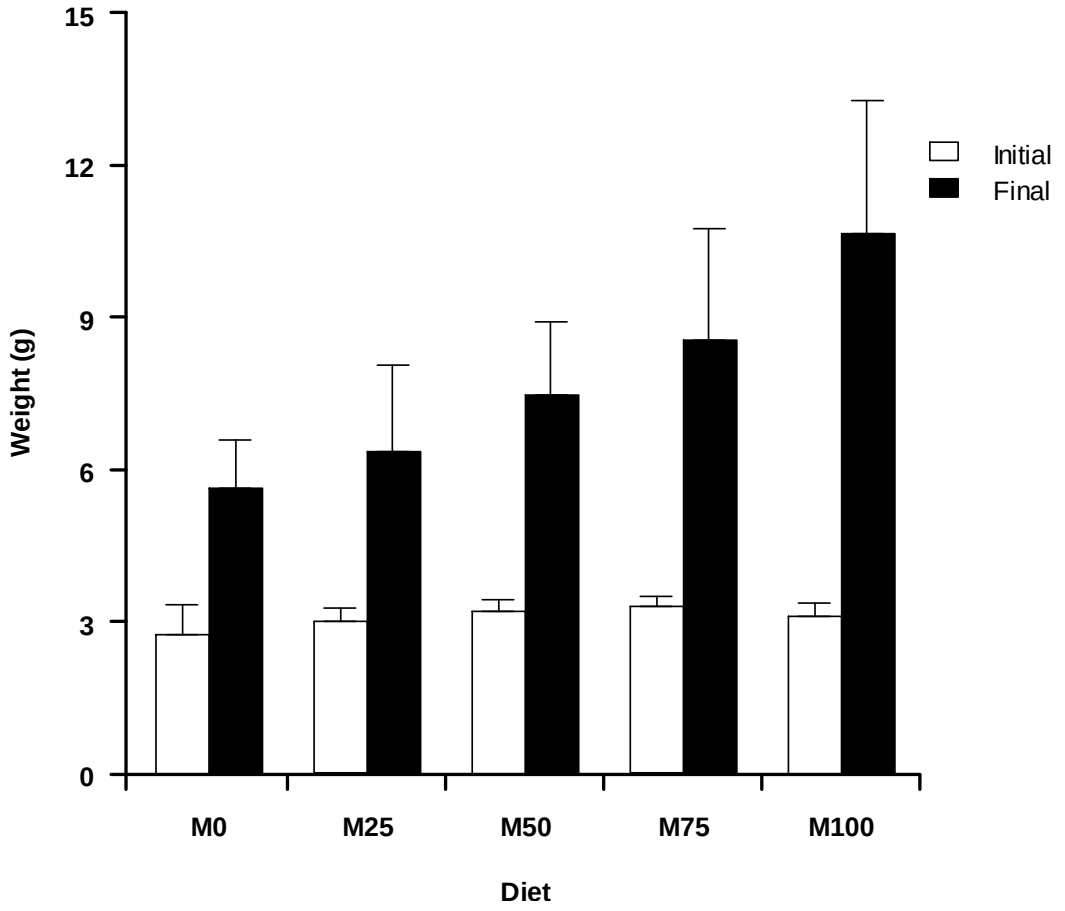

Figure 4.1: The body weight of red tilapia *Oreochromis* sp. at the beginning and end of the experimental period (60 days).

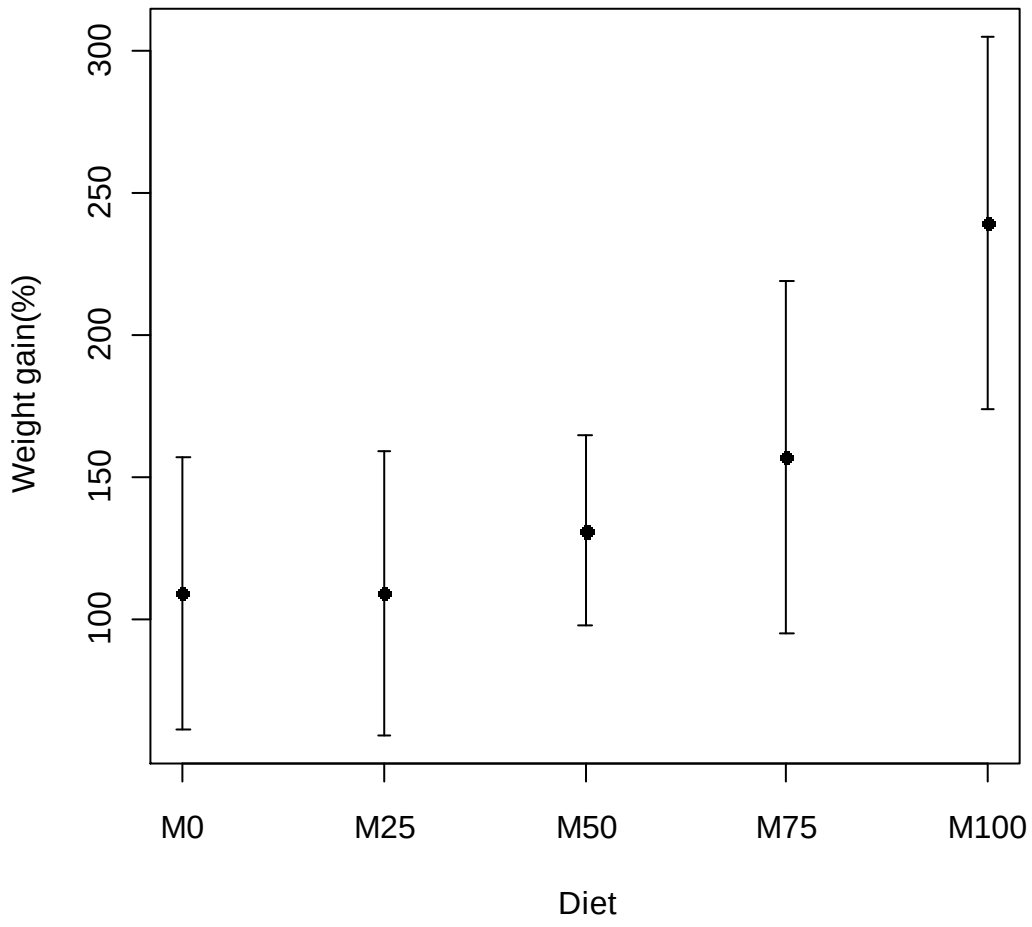

Figure 4.2: 95% confidence interval of mean percentage weight gain (%) according to feed types.

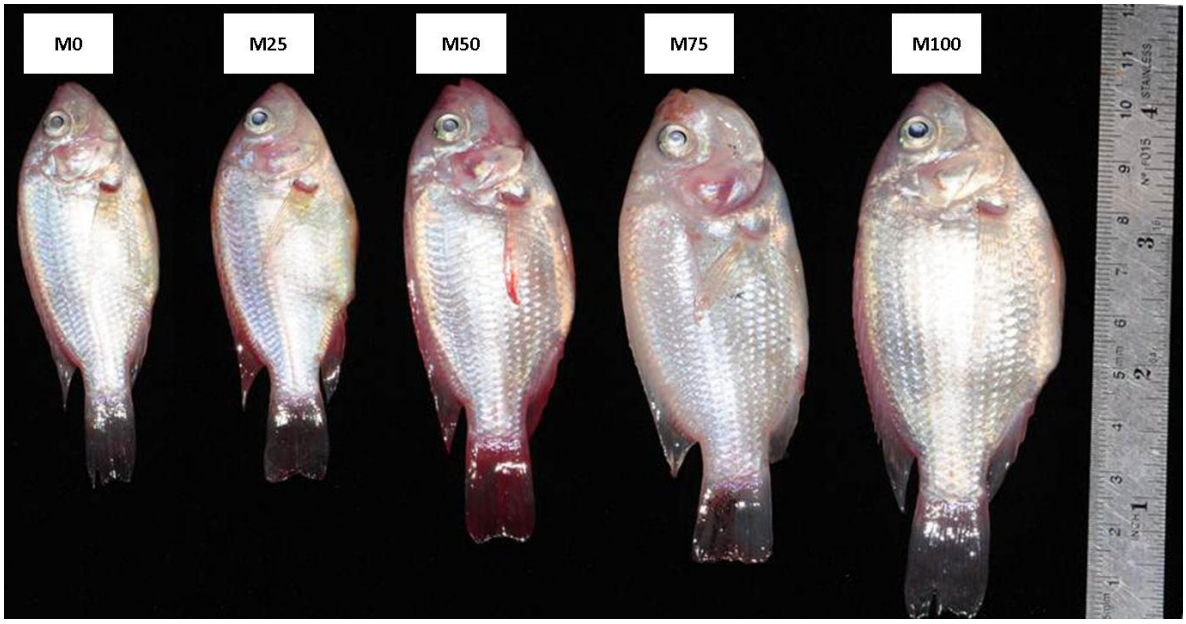

Figure 4.3: Body sizes of red tilapia *Oreochromis* sp. after feeding with the experimental diets for 60 days.

CHAPTER 5

PRELIMINARY STUDY ON MAGGOT STRAIN IMPROVEMENT USING ARTIFICIAL SELECTION

5.1 Introduction

Artificial selection, also known as selective breeding, refers to the process of enhancing certain traits of an original 'wild' type through a controlled breeding program. It is in contrast with natural selection and has been applied domestically for centuries in animals and plants in order to gain desirable traits such as high yield strain crops that are preferred by farmers (Snustad & Simmons, 2010).

Selection experiments are able to magnify the differences of phenotypic characteristics between selected lines and control lines; this can contribute to an understanding of physiological mechanisms involved in the response to selection (Harshman & Hoffmann, 2000). Moreover, resultant improved strains from using the selection method are relatively stable in terms of genetic dynamic because only the homozygous will remain at the end of selective breeding program (Gjedrem, 2005; Moen & Zhuikov, 2007).

The size and body weight of an organism can be influenced by genes, environment, ecology, life history and physiology (Hillesheim & Stearns, 1992). Generally, body weight is considered to have moderate heritability (Dunnington & Siegel, 1985). However, under highly control conditions body weight responds significantly to artificial selection.

In 1989, Eisen reported that body weight and protein weight are highly correlated. Thus, the objective of this study is to produce a robust and high protein strain of *Chrysomya megacephala* maggots to be used as animal feed by artificial selection.

5.2 Materials and methods

5.2.1 Sampling and colonization of flies

The *C. megacephala* fly population used in this study was sampled by using a sweep net from a single wild population at the Sungai Way Wet Market in April 2010. Collected wild flies were brought to the laboratory and used to generate the next generation. They were reared in plastic containers (24 x 28 cm) supplied with granulated sugar, water and small pieces of fresh beef liver (protein source) in separated petri dishes. These flies were kept at 27˚C on a 12:12 light: dark photoperiod.

5.2.2 Artificial selection based on body weight

The above wild flies were separated into two different lines. These are the control (non-selected) line and the selected line. Each line consisted of three independent replicates and was kept in different plastic containers at 27˚C on a 12:12 light: dark photoperiod. C1, C2 and C3 represented the three replicates for the control line whereby S1, S2 and S3 represented the three replicates for the selected line. All maggots were weighed individually by using a precision balance and their body weight was recorded. The non-selected line will be random breeding without introducing any selection force until the tenth generation. However, the artificial selection criteria will be based on the 20% of the highest maggot weight in the distribution curve and this will be carried out until the tenth generation.

5.2.3 Determination of protein content

Half of the selected maggots from the previous section (5.2.2) were killed by using hot water. They were dried in an oven at 100˚C for 24 hours and then grounded into powder form. Subsequently, the powder was used to prepare maggot meal extract following Treatment 4. Method of Lowry *et al*. was used to estimate the protein content of maggot meal for all generations.

5.2.4 Flies breeding

The other half of the selected maggots were kept in a new plastic container for breeding purposes. The above procedures were repeated up to 10 generations for both selected and non-selected lines under laboratory condition.

5.3 Results and Discussion

The maggot body weight of each generation, mean and standard deviation for selected and non-selected lines of maggots (10 generations) were summarized in Table 5.1. The means were variable in the non-selected line and fluctuated from 0.049 g (lowest) to 0.0713 g (highest) throughout the 10 generations. Moreover, the range of body weight in the non-selected line within the 95% confidence interval is found to be overlapping after observing all the 10 generations. On the other hand, a slight increase in body weight was observed in the selected line when selection pressure was continuously introduced to the population. The lowest and upper boundary (0.0500 — 0.0842 g) of the 95% confidence interval body weight for the selected line increased at the tenth generation compared to parental (0.0387 — 0.0686 g).

Many complex traits such as body size, weight and height are influenced by genes and do not show simple patterns of inheritance. Genetic variation in a population's gene pool determines the evolutionary potential of the population as well as the response to selection. Nonetheless, the populations are phenotypically variable and are strongly related with the genetic variation. Hence, the majority of quantitative traits in a population could be permanently altered after selection force was continuously introduced to the population (Fristrom & Clegg, 1987). Thus, in this study the maggot body weight in the selected line showed a slight increase through the selection breeding experiment.

During the selection experiment, the positive assortative mating with a single extreme homozygous genotype will reduce the range of variation if the assorting is cued on heritable traits. This is because the frequency of a single phenotypically homozygous genotype will be increased in the population and uniformity of genetics might occur

(Fristrom & Clegg, 1987). Therefore, the range of body weight from the selected line was reduced as compared to the parental.

The protein content of maggot meal from this experiment showed a weak relationship with body weight because the maggots from the selected line have higher body weight but lower protein content as compared to parental. Hence, selection for body weight could be accompanied by raising fatness as there is a high correlation between fatness and weight (Bunger *et al.*, 1998).

Table 5.1: Number of maggots (N), mean body weight (g) and standard deviations (S.D), 95% confident interval (CI) and coefficient of varience (C.V) in each generation for selected and non-selected lines of *C. megacephala* maggots.

Generation	Non-Selected				Selected			
	Mean ± S.D (g)	**Range (g)**	**95% CI (g)**	**C.V**	**Mean ± S.D (g)**	**Range (g)**	**95% CI (g)**	**C.V**
Parental	0.0512 ± 0.0089 (N = 1341)	0.0350 — 0.0799	0.0387 — 0.0686	0.1738	0.0512 ± 0.0089 (N = 1341)	0.0350 — 0.0799	0.0387 — 0.0686	0.1738
F1	0.05611 ± 0.0085 (N = 537)	0.0368 — 0.0861	0.0395 — 0.0728	0.1515	0.0525 ± 0.0086 (N = 1380)	0.0335 — 0.0861	0.0356 — 0.0694	0.1638
F2	0.05197 ± 0.0063 (N = 1257)	0.0341 — 0.0705	0.0473 — 0.0623	0.1212	0.0598 ± 0.0082 (N = 1056)	0.0375 — 0.0846	0.0437 — 0.0759	0.1371
F3	0.0490 ± 0.0063 (N = 1277)	0.0337 — 0.0721	0.0367 — 0.0613	0.1286	0.0651 ± 0.0101 (N = 360)	0.0416 — 0.0912	0.0453 — 0.0849	0.1551
F4	0.0618 ± 0.0064 (N = 820)	0.0426 — 0.0844	0.0499 — 0.0743	0.0337	0.0596 ± 0.0053 (N = 118)	0.0462 — 0.0710	0.0492 — 0.0700	0.0889
F5	0.0576 ± 0.0073 (N = 763)	0.0374 — 0.0805	0.0433 — 0.0719	0.1267	0.0598 ± 0.0065 (N = 129)	0.0428 — 0.0730	0.0471 — 0.0725	0.1087
F6	0.0563 ± 0.0079 (N = 701)	0.0348 — 0.0802	0.0408 — 0.0718	0.1403	0.0608 ± 0.0104 (N = 126)	0.0428 — 0.0867	0.0404 — 0.0812	0.1711
F7	0.0713 ± 0.0077 (N = 196)	0.0454 — 0.0878	0.0562 — 0.0864	0.1080	0.0685 ± 0.0107 (N = 384)	0.0500 — 0.0955	0.0475 — 0.0895	0.1562
F8	0.0612 ± 0.0073 (N = 754)	0.0400 — 0.0885	0.0469 — 0.0755	0.3548	0.0698 ± 0.0110 (N = 378)	0.0406 — 0.0957	0.0482 — 0.0914	0.1576
F9	0.0573 ± 0.0079 (N = 763)	0.0381 — 0.0759	0.0418 — 0.0728	0.2958	0.0671 ± 0.0084 (N = 123)	0.0406 — 0.0860	0.0506 — 0.0836	0.1252
F10	0.0529 ± 0.0057 (N = 275)	0.0405 — 0.0701	0.0417 — 0.0641	0.1078	0.0671 ± 0.0087 (N = 123)	0.0410 — 0.0860	0.0500 — 0.0842	0.1297

Table 5.2: Mean protein content (mg/ml) and standard deviations (S.D), 95% confident interval (CI) in each generation for selected and non-selected lines of *C. megacephala* maggots.

Generation	**Non-selected**		**Selected**	
	Mean ± S.D (mg/ml)	**95% CI (mg/ml)**	**Mean ± S.D (mg/ml)**	**95% CI (mg/ml)**
Parental	2.10	2.03 — 2.57	2.10	2.03 — 2.57
F1	2.17	1.38 — 2.95	2.11	1.18 — 3.04
F2	2.37	1.59 — 3.16	2.48	2.27 — 2.69
F3	2.36	2.24 — 2.49	2.42	2.21 — 2.63
F4	2.49	2.32 — 2.66	1.90	1.76 — 2.04
F5	2.35	2.20 — 2.50	2.51	2.32 — 2.70
F6	2.78	2.68 — 2.87	2.38	2.20 — 2.56
F7	2.32	2.25 — 2.40	3.02	2.92 — 3.12
F8	2.98	2.26 — 3.11	2.54	2.37 — 2.71
F9	2.88	2.46 — 3.30	1.93	1.70 — 2.17
F10	2.90	2.65 — 3.15	2.00	1.41 — 2.60

CHAPTER 6

GENERAL DISCUSSION

Protein is an important component in animal feed to enhance the growth of poultry (SRAC, 1998). Thus, protein concentration in a feed supplement needs to be determined before using it for animal feed formulation. A comparative study was made to determine the protein content in *C. megacephala* maggot meal by semi-micro Kjeldahl method, biuret method (Gornall *et al*., 1949), dye-binding method (Bradford, 1976), Warburg-Christian method (1941) and the method of Lowry *et al*. (1951). These methods (except semi-micro Kjeldahl) were equivalent being commonly used and easy to perform. Furthermore, the comparative study may be helpful in detecting potential interference in protein estimation by other substances present in the feed. Different methods produced different estimates of the total protein amount present in the *C. megacephala* maggot meal. However, Warburg-Christian method (1941) yielded the similar results to that obtained with semi-micro Kjeldahl method for total protein amount. In view of the presence of other nitrogenous compounds (nucleic acids and free amino acids) in maggot meal extract, results obtained from these two methods seem to be questionable. Presence of detergent, sodium dodecyl sulfate (SDS) in the solubilization buffer also resulted in a higher value of protein content in each of the colourimetric methods compared to that obtained in the absence of SDS. This is because SDS solubilized the membrane-bound proteins in maggot meal which would otherwise remain insoluble in the residue upon centrifugation. A comparison of results obtained with biuret method (Gornall *et al*., 1949) and the method of Lowry *et al*. (1951) using prior addition of reagents in the presence of 1% (w/v) SDS before centrifugation with that obtained with semi-micro Kjeldhal method (Helrich, 1990), suggested that these two

methods can be used successfully to determine protein content in maggot meal and other multicellular biological samples.

Protein analysis of *C. megacephala* maggot meal based on SDS-polyacrylamide gel electrophoresis, amino acid analysis and fluorescence spectra suggested it is a good protein source in tilapia feed due to high protein content (55.5%), abundance of low molecular weight proteins and good amount of essential amino acids. Presence of low molecular weight proteins in the maggot meal also increases the feasibility due to their greater solubility and digestibility as compared to high molecular weight proteins (Duodu *et al*., 2003). In addition, presence of essential amino acids in maggot meal further adds to its quality as the protein source in animal feed (Santiago & Lovell, 1988).

After determining the suitability of *C. megacephala* maggot meal as a protein source in animal feed, a feeding trial was conducted to evaluate the growth performance and feed utilization of juvenile red tilapia (*Oreochromis* sp.) with diets containing increasing substitution levels of dietary fishmeal by a mixture of maggot meal sources. Results showed that the juvenile red tilapia diet which constituted a 100% replacement with maggot meal protein had the highest weight gain, survival rate, special growth rate (SGR), protein efficiency ratio (PER) and lowest food conversion ratio (FCR). This superior growth performance at the highest inclusion replacement level of maggot meal to fishmeal clearly indicated that maggot meal contains sufficient nutrients such as essential amino acids and fatty acids which are important for growth and development of tilapia.

There are other advantages in using maggot meal as an alternative protein source rather than plant protein. Different groups of scientists have been studying the potential practicability of plant protein to replace fishmeal in aquafeed. Most of the results showed

that plant protein can only partially replace fishmeal because the inclusion of plant protein in substantial amounts has adverse effects on fish growth. A common problem of plant protein is their relatively low sulfuric amino acid content (cystine and methionine) which are essential amino acids for fish and also, the presence of anti-nutritional factors. Sudaryono *et al*. (1999) and Espe *et al*. (2007) reported low feed intake and low digestibility in shrimps and fishes when given feed containing anti-nutritional factors. Additionally, the high occurrence of plant cell walls present in feed are resistant to digestion by fish and other animals (Calvert, 1979).

In earlier studies, high survival rates of fish when fed with diets containing different levels of housefly maggot meal have been recorded by different groups. Ogunji *et al*. (2007) found that the inclusion of maggot meal in nile tilapia fingerling diet did not cause oxidative stress generation in tilapia liver. Thus, no high reactive oxygen species (ROS) were observed. Furthermore, in the wild, insect larvae are the natural food sources for animals including fish. From the sustainable standpoint, maggot larvae is a suitable protein source because it can be mass produced in a short period of time and the maggots are ready to be harvested within a brief period (less than 1 week). Besides, fish feed protein should be sourced from other forms of organisms because using aquaculture wastes such as discarded fish heads and other unwanted fish parts in fishmeal (especially from the same species), could spread diseases and/or cause environmental contamination. However, before maggot meal can be used commercially in red tilapia feed, a larger trial over a longer period on-farm condition should be conducted to confirm its performance and long-term effects.

The artificial selection experiment was designed to produce a more robust strain of *C. megacephala* with increased protein content. However, results (Table 5.1) showed that there is a reduction of variances in body weight rather than a change, with the mean trait

leaning towards higher body weight. This might due to the stabilizing of selection. Earlier studies suggested stabilizing selection caused genetic variance reduction which is in contrast with the disruptive selection experiment (Bulmer, 1971; Kaufman *et al.*, 1977).

Table 5.2 showed the dispersion pattern of protein content after the selection experiment up to 10 generations. The lack of information on the distribution of gene effects as well as trait frequencies of protein content in maggots and changes in variance cannot be fully explained by the change in gene frequency. Furthermore, it is difficult to detect changes in rates of response to selection in the short term; therefore, long term experiments are needed to ascertain the inheritance of quantitative traits.

CHAPTER 7

SUMMARY

Chrysomya megacephala maggot meal was found to be suitable as a protein source in animal feed because of its high protein content, essential amino acids and abundance of low molecular weight proteins.

Moreover, *C. megacephala* maggot meal has very good potential as a protein source for red tilapia diet. The total substitution of fishmeal by maggot meal in the replacement level diets showed the best growth performance and feed utilization in juvenile tilapia.

The artificial selection experiment failed to meet the third objective of this study but has succeeded in narrowing the range of body weight of maggot (a slight increase in the lowest boundary). However, this will not negate the effectiveness of *C. megacephala* maggot meal as a protein source in animal feed.

In conclusion my study has demonstrated that there is a great potential for the use of *C. megacephala* maggots as a protein source in tilapia feed.

This could make a significant impact on the cost of producing farmed tilapia contributing to the sustainable development of the aquaculture industry a crucial concern in light of rising human and global food demands.

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LIST OF PUBLICATION AND PRESENTATION

1. **Sing, K. W**., Sofian-Azirun., M. and S. Tayyab. (2012). Protein analysis of *Chrysomya megacephala* maggot meal. *Animal Nutrition and Feed Technology 12*: 35-46.

2. **Sing, K. W**., Salleh, K. M., Tayyab, S. and Sofian-Azirun. M. (2012). *Chrysomya megacephala (Fabricus) maggot meal as an alternative protein in red tilapia (Oreochromis sp.) feed*. Presented orally at the 48th Annual Scientific Conference of The Malaysia Society of Parasitology and Tropical Medicine.

3. **Sing, K. W**., M. Sofian-Azirun and S. Tayyab. (2011). *Chrysomya megacephala (Calliphoridae) maggot meal as an alternative protein in feed industry*. Paper presented at the 16th Biological Sciences Graduate Congress.

4. **Sing, K. W**. and Sofian-Azirun, M. (2011). The potential of fly maggot-derived meal as an alternative protein in feed industry. Paper presented at the Universiti Malaysia Terengganu 10th International Annual Symposium.

5. **Sing, K. W**., Nor Shariza, Sofian-Azirun, M., and Tayyab, S. (2010). *Protein Content in Chrysomya megacephala Maggot Meal: Methods Revisited*. Paper presented at the 15th Biological Sciences Graduate Congress.