EFFECT OF VARYING DIETARY PROTEIN,LIPID AND HUFA LEVELS ON GROWTH AND REPRODUCTIVE PERFORMANCE OF FEMALE SWORDTAIL X*iphophorus helleri*

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by

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ATP	Adenosine triphosphate
BCP	1-Bromo-3-Chloropropane
bp	base pair
cDNA	Complemetary DNA
C_T	Threshold cycle
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylene diaminetetraacetic acid
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
GSI	Gonadosomatic Index
HSI	Hepatosomatic Index
HUFA	Highlyunsaturated fatty acids
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani
LO	Linseed oil
MOPS	3-[N-Mopholino]propanesulphonic acid
mRNA	Messenger RNA
NCBI	National Centre of Biotechnology Information
OD	Optical density
RT-PCR	Reverse Transcription Polymerase Chain Reaction
PUFA	Polyunsaturated fatty acids
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Reverse transcription
SGR	Specific growth rate
SLO	Squid linseed oil
SO	Squid oil
TBE	Tris-borate-EDTA
UV	Ultra violet

VSI	Viscerosomatic Index
v/v	Volume per volume
w/v	Weight per volume
X-Gal	X5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

KESAN PARAS PEMAKANAN PROTEIN, LIPID DAN HUFA YANG BERLAINAN TERHADAP PERTUMBUHAN DAN PRESTASI PEMBIAKAN IKAN EKOR PEDANG *Xiphophorus helleri* BETINA

ABSTRAK

Ikan air tawar berkeupayaan mensintesis asid lemak sangat tidak tepu (HUFA) daripada asid lemak C_{18} poli tidak tepu (PUFA) yang biasanya didapati dalam diet yang mengandungi minyak sayuran untuk memenuhi keperluan nutrisi pertumbuhan ikan. Namun demikian, pengetahuan tentang keperluan nutrisi HUFA dalam fasa pembiakan aktif ikan air tawar adalah kurang. Dua eksperimen telah dijalankan untuk memeriksa kesan paras pemakanan protein, lipid and HUFA yang berlainan terhadap pertumbuhan and prestasi pembiakan ikan betina ekor pedang. Dua paras pemakanan protein (20% dan 30%) dengan empat paras pemakanan lipid (8%, 12%,16%, 20%) dalam setiap paras pemakanan protein telah diformulasikan sebagai diet ujian. Keputusan menunjukkan paras pemakanan protein mempengaruhi berat akhir, pertambahan berat dan kadar pertumbuhan spesifik (SGR) ikan betina ekor pedang secara signifikan. Pertambahan paras pemakanan lipid dari 8% hingga 12-16% berjaya meninggikan SGR ikan betina ekor pedang pada paras pemakanan 20% dan 30% protein. Interaksi antara pemakanan protein dan lipid berjaya mempengaruhi penyimpanan protein and lipid dalam isi dan ovari ikan betina ekor pedang secara signifikan. Ikan yang diberi diet 30% protein and 12% lipid menunjukkan SGR, indeks gonadosomatik (GSI) dan penyimpanan protein dalam isi yang tertinggi yang justerunya menyebabkan produktiviti anak ikan yang tertinggi. 30% protein dan 12% lipid telah dirujuk sebagai paras pemakanan protein dan lipid yang optimum untuk pertumbuhan dan prestasi pembiakan ikan betina ekor pedang. Berdasarkan paras pemakanan protein dan lipid tersebut, 3 diet yang terdiri daripada 30% protein dan 12% lipid yang mengandungi paras minyak sotong dan minyak linseed yang berlainan (100% minyak sotong, 1:1 minyak sotong:minyak linseed dan 100% minyak linseed) sebagai sumber pemakanan lipid telah diformulasikan. Ikan betina ekor

pedang yang diberi diet mengandungi 1:1 minyak sotong :minyak linseed menghasilkan bilangan anak ikan yang tertinggi. Penyimpanan komposisi asid lemak di hati, isi dan ovari swordtail mencerminkan komposisi asid lemak diet. Kekurangan penyimpanan asid dokosaheksanoik (DHA, C22:6*n*-3), asid eikosapentanoik (EPA, C20:5*n*-3) dan asid arakidonik (AA, C20:4*n*-6) didapati dalam hati, daging dan ovari ikan betina ekor pedang yang diberi diet mangandungi minyak linseed sahaja sebagai sumber pemakanan lipid yang justerunya membawa kepada kekurangan bilangan anak ikan yang dihasilkan. Penyimpanan AA yang tinggi dalam ovari dan anak ikan menunjukkan kepetingan AA dalam proses pembiakan ikan swordtail. Selain itu, ekspresi gene desaturase dan elongase dalam hati dan isi ikan betina ekor pedang didapati bertambah dengan pertambahan paras pemakanan minyak linseed. Ekspresi gene desaturase dalam ovari juga bertambah dengan paras pemakanan minyak linseed tetapi ekspresi gene elongase dalam ovari tidak menunjukkan corak yang spesifik. Secara keseluruhanya, eksperimen ini menunjukkan kesan positif paras pemakanan HUFA terhadap prestasi pembiakan ikan betina ekor pedang walaupun ia mempunyai keupayaan untuk meninggikan transkripsi mRNAs desaturase dan elongase semasa diberi paras pemakanan yang kekurangan HUFA.

EFFECT OF VARYING DIETARY PROTEIN, LIPID AND HUFA LEVELS ON GROWTH AND REPRODUCTIVE PERFORMANCE OF FEMALE SWORDTAIL *Xiphophorus helleri*

ABSTRACT

Freshwater fish possess higher ability to biosynthesize highly unsaturated fatty acids (HUFA) from C_{18} polyunsaturated fatty acids (PUFA) found in plant-based diets in fulfillment of the nutrient needed for growth and development. However, there is very little knowledge on HUFA requirement during active spawning phase of freshwater broodstock. Two experiments were carried out to determine the effect of dietary protein, lipid and HUFA levels on growth and reproductive performance of female swordtail. Two dietary protein levels (20% and 30%) with 4 dietary lipid levels (8%, 12%, 16%, 20%) within each protein level were formulated as experimental diets. Results showed that dietary protein level significantly influenced final weight, weight gain and specific growth rate (SGR) of female swordtail. In addition, increasing dietary lipid levels from 8% to 12-16% significantly improved SGR of swordtail at both 20% and 30% dietary protein levels. Dietary protein and lipid interaction significantly influenced protein and lipid deposition in muscle and ovary of swordtail. Fish fed diets 30% protein 12% lipid gave the highest SGR, gonadosomatic index (GSI) and muscle protein deposition which evetually led to the highest fry production. 30% protein and 12% lipid were concluded to be the optimum dietary protein and lipid requirements for optimized growth and reproductive performance of female swordtail. Based on these optimum protein and lipid levels, three *iso*-nitrogenous (30% protein) and *iso*-lipidic (12% lipid) experimental diets containing different ratios of squid oil and linseed oil (100% squid oil, 1:1 squid oil:linseed oil and 100% linseed oil) as dietary lipid source were formulated. Swordtail utilizing diet containing mixture of squid and linseed oil produced significantly highest fry production. Fatty acid composition of liver, muscle and ovary of female swordtail reflected dietary fatty acid profiles. Significant lowered levels of docosahexaenoic acid (DHA, C22:6*n*-3), eicosapentaenoic acid (EPA, C20:5*n*-3) and arachidonic acid (AA, C20:4*n*-6) were found in liver, muscle and ovary of swordtail fed diet containing linseed oil as sole dietary lipid source, which probably led to lower fry production. High deposition of AA in oocyte and fry of broodstock also indicated the importance of AA in ovary and larval development. Increased gene expressions of desaturase and elongase were observed in liver and muscle of female swordtail fed diets with increasing levels of linseed oil. In addition, desaturase gene expression in ovary also displayed the same pattern while ovary elongase expression did not show any specific trend. Overall, this study has demonstrated the beneficial effect of dietary HUFA inclusion on the reproductive performance of female swordtail although they possess the ability to increase transcription of desaturase and elongase mRNAs during low dietary HUFA provision.

Chapter 1 INTRODUCTION

1.1 Research background

Ornamental fish culture is an important activity in several Asian countries due to the increasing popularity of aquarium fish. The total production value from Southeast Asian aquarium fish farms is estimated to be US$80-150 million annually (Ng and Tan, 1997). Live bearing ornamental fish species such as guppies (*Poecilia reticulata*), mollies (*Poecilia latipinna, Poecilia sphenops*), swordtails (*Xiphophorus helleri*) and platies (*Xiphophorus maculatus*) are favoured among aquarium hobbyists because of variation in body colours and fin patterns. Swordtails alone accounted for 5.4% of the total number of ornamental fish imported in the United States in 1992 (Chapman *et al.,* 1997).

The culture of swordtail is carried out in earthen ponds and floating net cages, mainly in Singapore, Malaysia, Thailand, Indonesia, India and China. However, feeding practices in farms are poor as they rely mainly on live feed such as bloodworms, *Tubifex* and freshly prepared wet paste containing fish meal and skimmed milk powder (Fernando *et al*., 1991), which lead to problems such as detrimental water effluent, accumulation of harmful pathogens and inability to fulfill overall nutrient requirement of fish. Therefore, development of a proper formulated diet with the correct ratios and levels of different nutrients is important. Formulated diets need to be economical to reduce production cost, practical to ease storage, transportation and supply and also environmental friendly.

In teleosts, nutrition factors such as feed ration, nutrient levels and compositions have great influence in various reproductive parameters such as gonadal development, egg quantity and quality, hatchability, larval quantity and survival (Izquierdo *et al*., 2001;

Watanabe and Vassallo-Agius, 2003). Generally, carbohydrates, proteins and lipids are the major nutrients that provide energy to sustain fish metabolism, growth and reproduction. In addition to this, nutrients such as essential proteins, fatty acids, carotenoids, vitamin C and vitamin E are important in regulating various reproductive processes (Izquierdo *et al.,* 2001; Watanabe and Vassallo-Agius, 2003).

Swordtails are viviparous breeders with females storing transferred sperm within the ovaries for internal egg fertilization, followed by hatching of eggs and a gestation period of 27 days before the release of free-swimming embryos (Siciliano, 1972). The whole process of reproduction from gonad formation and maturation to the production of good quantity and quality offspring is very complicated. Detailed knowledge on the effect of different nutrients influencing different stages of reproduction is greatly needed. Therefore, broodstock nutrition studies are often carried out to evaluate reproductive performances of a particular fish species by determining different levels of nutrients in maternal dietary intake. However, understanding of broodstock nutrition of many fish species is still poor because of difficulties in conducting experiments involving proper feeding and proper measurement of reproductive performances.

Studies have shown that proteins are normally included in broodstock diets for energy and growth. They are present as lipoproteins, hormones and enzymes in teleost eggs, which are crucial in providing nutrient, ensuring proper development and hatching of embryos prior to exogenous feeding (Brooks *et al*., 1997). On the other hand, lipids are known to provide energy and as metabolite storage in fish. Besides this, several studies also denoted the importance of dietary lipids involved in many vital reproductive processes such as oocyte development, egg production and embryo development (Fernandez-Palacios *et al*., 1995; 1997; Bell *et al*., 1997; Mazorra *et al*., 2003). During fish maturation and reproduction, lipids are transported into oocytes from maternal reserves, stored and accumulated in yolk and utilized by developing embryos subsequently (Brooks *et al*., 1997). Studies also showed improved feed conversion ratio (FCR), nitrogen and phosphorus retention when Atlantic salmon was fed diets with higher lipid levels (Hillestad *et al*., 1998; Hemre and Sandnes, 1999). Inclusion of lipid also helps to increase stability of feed in water (Chaiyapechara *et al*. 2003). Moreover, improved growth parameters were found in Atlantic salmon and gilthead seabream fed diets with higher lipid levels (Hemre and Sandnes 1999; Vergara *et al*., 1999). However, excessive inclusion of lipid in diet can cause reduction in feed consumption due to feeling of satiation, which in turn reduce intake of other nutrients. Numerous studies have been carried out to investigate the interactive importance of dietary protein and lipid levels on fish growth performances (Miller *et al*., 2005; Ozorio *et al*., 2006).

Dietary lipid might contain different oil sources and thus, containing different fatty acid composition. Arachidonic acid (ARA, 20:4*n*-6), eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) are three highly unsaturated fatty acids (HUFA) widely studied in order to determine optimal dietary levels required for fish growth, reproduction and development. In general, these HUFA are responsible for maintaining cell membrane integrity. More specifically, they act as precursors for eicosanoids synthesis, an important group of paracrine hormones involved in the regulation of a wide range of physiological processes. Eicosanoids are an active range of chemical molecules that control vital reproduction pathways such as ovulation and steroidogenesis (Sorbera *et al*., 2001). HUFA is also identified as a vital nutrient in broodstock diet to ensure reproduction success and offspring survival. Many studies in the past two decades have identified lipid, and in particular HUFA, as important nutrients influencing reproductive performances in numerous fish species (Fernandez-palacious *et al*., 1995; Furuita *et al*., 2000; Izquierdo *et al*., 2001; Bell and Sargent, 2003; Mazorra *et al*., 2003; Watanabe and Vassallo-Agius, 2003; El-sayed *et al*., 2005). However, essential fatty acids requirement varies among different fish species (Sargent *et al*., 1999a).

HUFA are provided in broodstock diet by adding fish oil, a traditional dietary lipid source in aquaculture containing high level of AA, EPA and DHA. It was predicted that global production of fish oil, 1.2-1.4 million tons/year, might not be able to meet the need of animal feed in the next few years (Izquierdo *et al*., 2004). Various studies were conducted to seek suitable replacement for fish oil as its cost is increasing and the supply is diminishing (Barlow, 2000; Chamberlin and Barlow, 2000). These studies usually aimed to substitute fish oil fully or partially with vegetable oil as it is a cheap and sustainable resource (Bell *et al*., 2003; Fonseca-Madrigal *et al*., 2005; Montero *et al*., 2003; 2005; Regost *et al*., 2003; Izquierdo *et al*., 2004; Rennie *et al*., 2005; Ruyter *et al*., 2006; Francis *et al*., 2006). Overall studies showed that soybean oil and linseed oil can be good alternative oil sources for salmonids, sea bream, turbot and some freshwater species without compromising growth rates and feed conversion. However, vegetable oils are rich in linolenic acid (LNA, 18:3*n*-3) and linoleic acid (LA, 18:2*n*-6) but devoid of HUFA. Fish fed on vegetable oils usually reflect low HUFA profiles in their body fatty acid composition. This low HUFA composition might affect fish reproductive performance and bring negative value for human consumption. Therefore, knowledge on dietary HUFA requirement of cultured species is important in order to incorporate appropriate level of plant oil as dietary lipid source in aquafeeds. Besides that, improper inclusion of AA, EPA and DHA in diet will cause an imbalance in HUFA biosynthesis pathway as they compete with each other in some metabolic pathways (Sargent *et al*., 1999b).

Most of the freshwater fish are known with their ability to synthesize HUFA from LNA and LA. LNA and LA are considered essential fatty acids (EFA) for freshwater fish as EPA and DHA are synthesized from LNA of the *n*-3 series fatty acids, while AA is synthesized from LA of the *n*-6 series fatty acids. In HUFA biosynthesis pathway, desaturase and elongase are major enzymes responsible for fatty acid desaturation and elongation from shorter chain of fatty acid to HUFA mediated by microsomal system (Tocher *et al*., 2002a; 2002b; Seliez *et al*., 2003). However, several studies have shown the benefits of dietary inclusion of HUFA in freshwater species even though they possess the ability to synthesize these fatty acids endogenously. Mukhopadhyay and Rout (1996) reported highest growth rate was obtained from Catla carp with the inclusion of fish oil and sunflower oil as dietary lipid source. Higher growth performance was not achievable over 50% replacement of fish oil by linseed oil in freshwater Murray cod (Francis *et al*., 2006). Smith *et al*. (2004) also reported inclusion of dietary HUFA benefited weight gain in silver perch. However, very little is known about HUFA dietary requirements and utilization in freshwater fish especially during reproduction phase.

However, marine fish require substantial levels of dietary HUFA because they do not possess the ability to synthesize these HUFA de novo nor from shorter chain precursors such as LNA and LA. Therefore, EPA, DHA and AA are considered EFA for marine fish. Even though many studies have shown the essentiality and beneficial effects of dietary HUFA to marine fish, it remains to be seen if a similar practice will improve reproductive performance of freshwater broodstock.

1.2 Objectives

In light of the above discussions, the present study is aimed to:

a. investigate the effect of different dietary protein and lipid levels on the growth and reproductive performances of female swordtail

b. investigate the effect of different dietary HUFA levels on the reproductive performances, tissue fatty acid profile and desaturase and elongase mRNAs of female swordtail

Chapter 2 LITERATURE REVIEW

2.1 Swordtail Biology

The swordtail is a freshwater fish grouped within the family of Poeciliidae. The poeciliid family contains few of the most popular ornamental species in the aquarium trade known as the "big four." The genus *Xiphophorus* includes species of the swordtail and the platy while the genus *Poecilia* includes species of the molly and the guppy. These fish are hardy, relatively peaceful, colourful, and among the easiest fish to breed

The natural geographical origin of the common swordtail, *Xiphophorus helleri*, extends from northern Mexico to the central and western parts of Guatemala and Honduras in Central America. The species was introduced and has become popular in southern Florida, California, the Lake Mead area of Arizona and Nevada, Hawaii, Canada, Puerto Rico, Africa, Sri Lanka, Australia, Guam, Fiji and United Kingdom (Dawes, 1991; Jacobs, 1969).

The male swordtail grows to a maximum overall length of 14cm and the female to 16 cm. Swordtails are sexually dimorphic and the males and females are easily identified. It is reported that swordtail attains sexual maturity at the length of 2.5-3.0cm or at 10-12 weeks of age (Milton and Arthington, 1983; Dawes, 1991). Swordtails are completely polygamous and do not form mating pairs after they reach sexual maturity. The male fish will not only court females of their own species, but also they will mate with females of other poeciliids that resulted in hybridization.

All poeciliid males possess a modified anal fin called a gonopodium. It is used to insert sperm into the female in order to fertilize the eggs during the mating process. Matured male swordtails have elongated lower caudal fin that looks like a sword.

Meanwhile, Poeciliid females pocess a distinguishing feature called the "gravid spot", a dark area just slightly above and forward of the anal fin which changes in size and darkness with the development of the embryos. Female swordtails are livebearing fish in which fertilization, hatching of eggs and development of the embryos will take place within the females until live young are released from her body. Female swordtails can store viable sperm in the folds of their oviducts to fertilize matured eggs when needed. Therefore, subsequent clutches of eggs in the females can be fertilized even after long contact with a male fish. A single copulation can provide viable sperm for fertilization up to two years and a female can give birth to five to nine consecutive broods from a single mating event (Axelrod and Wischnath, 1991).

Optimal water temperature was reported to be between 22^{o}C to 26^{o}C for reproduction in all livebearers and the gestation period was estimated to range from 26 to 30 days (Dawes, 1991). Studies of the reproductive activities of wild swordtails in Australia have revealed that temperature can influnce the number of fry produced per female per month (Milton and Arthington, 1983). Data showed that with the rise of every degree in water temperature from 22^{o}C, the number of fry per spawn will correspondingly increase by three, with the highest average number of fry found in water with a temperature of 29^{o}C. It also reported decrease in fry production when water temperatures drop below 18^{o}C or rise above 29^{o}C. The fecundity of swordtail has been reported to be as high as 242 fry/female (Breder and Rosen, 1966). However, an average female swordtail spawns about 30 fry/female.

Commercial production of livebearer fish are normally carried out in earthen ponds. Broodstock are kept in the ponds and allowed to spawn freely for 6-7 months. After that, the fish are harvested and graded for market and new batch of broodstock are introduced into the ponds. However, this type of large-scale production produces higher number of off-color and deformed fish. In order to maximize the production of a desired colour or fin variety, the broodstock should be kept in traps or small cages within aquaria, tanks, or ponds. Further more, this can avoid cannibalism of the fry by the broodstock and increase the survival of fry from one particular strain. Simple plastic net cage, clear plastic box or any container with holes that allows fry to escape from the broodstock can be used for swordtail culture.

2.2 Protein

Nutritional status of fish often affects their reproductive performances including time to first maturation, fecundity, egg size, egg and larval quality. The nutrient requirements vary between species and within species, at different stages of life. Energy is present in the chemical bonds of molecules that hold the nutrients together. Energy is partitioned by fish between various physiological processes including maintenance, growth and reproduction. It will be used for metabolism first and the excess will be divided between growth and reproduction.

Protein is the largest molecule in cell made up from amino acids. There are 20 naturally occurring amino acids that will be incorporated into proteins. Proteins are linked by peptide bonds between the carboxyl group of one amino acid to the amino group of another amino acid in chains of different orders and lengths. Amino acids have a general structure of $–NH_2CHRCOOH-$, where R is the organic side chains made up from some or all of carbon, hydrogen, oxygen, nitrogen and sulphur atoms. The structure of protein is determined by the sequence of amino acids it is composed from. This sequence is determined by the gene coding for the production of that particular protein.

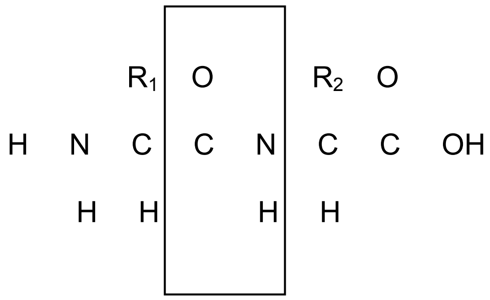

Figure 2.1 Two amino acids linked by a peptide bond

Proteins are divided into essential and non-essential amino acids. The non-essential amino acids can be synthesized by the animal from other chemical compounds. Essential amino acids are the amino acids that cannot be synthesized by fish. Therefore, adequate level of protein and correct ratios of all essential amino acids and most non-essential amino acids must be provided in fish diet for protein synthesis and growth. Some of the amino acids will also be deaminated and degraded for energy. Synthesis and degradation of protein will occur continuously in tissues. Fish is able to grow when the rate of protein synthesis is greater than the rate of protein degradation. If there are insufficient essential amino acids, then protein degradation will be greater than protein synthesis and the fish is unable to grow. Fish meal is used as a traditional protein source in fish diet to provide most of the non-essential and essential amino acids.

2.2.1 Dietary protein requirement of fish

Optimum protein level in diet promotes fish growth, earlier puberty and oocyte maturation. Larger size broodstock has been reported to display higher spawning rates (Gunasekera *et al*., 1996a;1996b; El-Sayed *et al*., 2003), higher fecundity (Milton and Arthington, 1983) and egg size (Seghal and Toor, 1991; Bromage *et al*., 1992;). Research has shown that optimum dietary protein requirement for fish ranges from 30% to 50% of the diet (De Silva and Anderson, 1995).

Carnivorous fish species such as salmonids, percids and marine flatfish require 40-55% of dietary protein. Other species such as cyprinids and tilapias are capable to grow when fed with lower protein level around 30-40% (Joblings, 1994). Watanabe *et al*. (1984) found that 45% of dietary protein level resulted in highest number of eggs produced, number of viable eggs and larval hatchability in red sea bream. Chong *et al*. (2004) suggested that a minimum of 30% protein should be included in the diet of female swordtail broodstock for optimum reproductive performance. It is concluded that dietary protein is crucial for both the somatic growth and the reproduction process of female swordtail. Khan *et al*. (2004) suggested protein level of 30% for optimum growth and reproductive performances in grass carp, *Ctnenopharyngodon idella*.

Protein is the most abundant nutrient found in fish egg (Watanabe and Kiron, 1994) besides being structural, functional and energetic constituent in fish tissues. It is also important for fish fertilization and normal development of embryo as amino acids act as a reservoir of materials used for biosynthetic activities during embryogenesis. Relationship between dietary protein and few reproductive parameters varies between different species, dietary compositions and culture conditions. Some studies showed increased dietary protein levels increased the total number of eggs produced per female in tilapia, *O. niloticus* (Siddiqui *et al*., 1998), spawning frequency and the number of eggs per spawn in nile tilapia (El-Sayed *et al*. 2003) and number of eggs per body weight in bighead carp, *Aristichthys nobilis* (Santiago *et al*., 1991). Increasing protein levels in diet also improved fertilization and hatchability of fish eggs in *O. niloticus* (Gunasekera *et al*., 1996a) and *C. carpio* (Manissery *et al*. 2001) as high protein diets most probably produce more oocytes and hatch into larvae. Increased dietary protein levels up to 35% caused an increase in Gonadosomatic index (GSI) in grass carp (Khan *et al*., 2004), while no significant effect was found in tilapia, *O. niloticus* (Gunasakera and Lam, 1997; Gunasakera *et al*., 1997). Various contradictory results also showed that relative fecundity and egg diameter were not affected by dietary protein in carp (Khan *et al*., 2004), tilapia (Gunasekera *et al*., 1996a, 1997), and European sea bass (Cerda *et al*., 1994).

Few studies also indicated that further increase in protein intake did not benefit fish reproductive performances once the dietary protein requirement has been met (Gunasekera *et al*., 1996a; Emata and Borlongan, 2003). Excessive protein level over 35% in diet caused a marked decline in weight gain and fish growth of grass carp (Khan *et al*., 2004). This might have resulted from extra energy being used to deaminate and excrete excess amino acids and caused a reduction in dietary energy available for growth.

Table 2.1 Estimated protein requirements of some juvenile fish (adapted from De Silva and Anderson, 1995)

Species	Protein source	Optimum protein level in diet (%)
Channel catfish	Whole egg protein	32-36
Common carp	Casein	38
Gilthead bream	Casein, Fish protein concentrate	40
Grass carp	Casein	41-43
Red sea bream	Casein	55
Chinook salmon	Casein, gelatin and amino acids	40
Rainbow trout	Fish meal	40
Snake head	Fish meal	52
Tilapia	Casein, egg albumin and fish meal	30-56

2.3 Lipid

Lipids are organic molecules which contain hydrogen, oxygen and many carbon atoms in variation of chain or ring conformations. They are biological substances, soluble in organic solvent such as chloroform, hydrocarbons or alcohols but insoluble in water. Lipids occur in all living cells and contribute to its cellular structures. They also provide stored fuel and participate in many biological processes such as transcription of genetic code, regulation of genetic pathways, physiological responses and so on. Fatty acids, triglycerides, phospholipids, sterols and sphingolipids are the five major classes of lipids.

Fatty acids usually exist in esterified form in nature called triacylglycerols or triglycerides, which mean triesters of glycerol. They are formed by esterification of fatty acids with glycerol and represent the major storage lipids of both plants and animals. Triacylglycerols are an efficient form to store metabolic energy as they are less oxidized than carbohydrates and proteins. Hence, they yield more energy on oxidation.

$$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6COOH$$

Figure 2.2 Chemical structure of a fatty acid - Linoleic acid, C18:2(*n*-6).

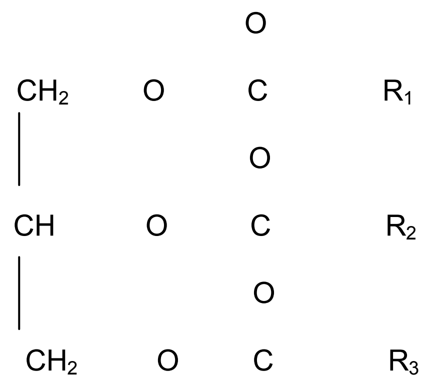

Figure 2.3 Structure of triacylglycerol, where R_1, R_2 and R_3 are fatty acids.

Phospholipids are also called glycerophospholipids. They are the major lipid component of biological membranes. Majority of phospholipids contain nitrogen and phosphorus in addition to carbon, hydrogen and oxygen. Cholesterol is the most notable sterol lipid. It is a major component of animal cell membrane, a precursor of steroid hormones and bile acids. Corticosteroid hormones of interrenal tissues are involved in the regulation of salt and water balance and the metabolism of carbohydrates and proteins. Gonadal steroids such as androgens and oestrogens are vital hormones in reproduction as they induce the production of vitellogenin in the liver and initiate some behavioral changes in spawning activity. Vitellogenin is the main yolk precursor protein in teleosts. Sphingolipids are also major membrane component and are commonly found in the membranes of nerve cells.

2.3.1 Dietary lipid requirement of fish

Lipids are important source of energy and essential fatty acids (Watanabe, 1982). In addition to being highly digestible and well metabolized by fish, lipids also increase diet palatability and stabilizes feed pellet during manufacture, transportation and storage. Increasing dietary lipid will be an effective strategy to improve feed efficiency and reduce protein utilization as it is able to spare the use of protein as energy source.

Maximum growth of white seabass fingerlings was obtained from diets inclusion of 15.5% and 18% lipids with 61% protein (Lopez *et al*., 2006). Other marine species such as red drum, black rockfish, red sea bream and Asian Seabass have showed improved growth fed with diets containing around 17% lipid and 30%-50% protein (Craigh *et al*., 1999; Lee *et al*., 2002; Williams *et al*., 2003). Surubim, *Pseudoplatystoma coruscans*, fed experimental diet with the highest lipid level, 18% lipid and 46% protein showed the best nutritional performance in weight gain, protein retention and energy retention (Martino *et al*., 2002). They also concluded that dietary 10-20% lipid gave the optimal growth rates in fish. In salmonids, dietary lipids up to 30% improved feed and protein utilization and reduce nitrogen excretion (Beamish and Medland, 1986; Hillestad and Johnsen, 1994; Helland and Grisdale-Helland, 1998).

Excess dietary lipid will cause a decrease in feed consumption and therefore, reduce the intake of other dietary nutrients and fish growth (Watanabe, 1982; Ellis and Reigh, 1991; Lopez *et al*., 2006). The growth reduction effect from excessive dietary lipid level was probably due to the limited ability of fish to digest and absorb high amount of lipid, excess lipid accumulation in liver and visceral imbalance in metabolic activities. Lopez *et al*. (2006) demonstrated that increasing dietary lipid to more than 18% with 61% protein did not improve weight gain and SGR of white sea bass fingerlings. Wang *et al*. (2005) showed that dietary lipid level over 15% reduced feed intake and had a negative influence on growth of Cobia. Few studies (Bromley, 1980; Lie *et al*., 1988; Hellestad and Johnsen, 1994; Perez and Oliva-Teles, 1999) have shown that excessive level of dietary lipid promoted undesirable increase of lipid deposition in fish, especially in carcass, visceral and tissue cavity. The localization of excess lipid deposits strongly reduces the nutritional value, organoleptic properties, transformation yields and storage time of fish carcass.

Fish proximate and fatty acid composition normally reflect dietary lipid and fatty acids levels. As dietary lipid level increased from 5% to 25% in Cobia, energy retention, daily lipid gain, viscerosomatic index (VSI), hepatosomatic index (HSI), body lipid content and muscle lipid content also increased dramatically (Wang et al., 2005). Similar result was found in carcass lipid content in Japanese sea bass (Ai *et al*., 2004).

2.3.2 Lipid metabolism

Nutrients in feed are digested in fish gut and absorbed into bloodstreams through gut lining. These nutrient molecules circulate around the body and are taken up by various tissues. They are then subjected to metabolism reactions including catabolism to liberate energy and anabolism to produce new tissues.

Carbohydrate will be broken down to glucose, and protein to amino acids. Lipids are broken down to fatty acids and are resynthesized into lipids after absorption through gut lining to form droplets and lipoproteins that circulate in fish blood system. Lipoproteins help the insoluble lipid components to be maintained in aqueous environment. Lipoproteins and droplets are hydrolyzed to their constituent fatty acids and glycerol by lipoprotein lipase enzyme in target tissues outside the cell. They are then transported across cell membrane and form triacylglycerols or oxidized for energy. Triacyglycerols stored in adipose tissues and required for metabolism will be hydrolyzed by triacylglycerol lipase before passing out from the cell.

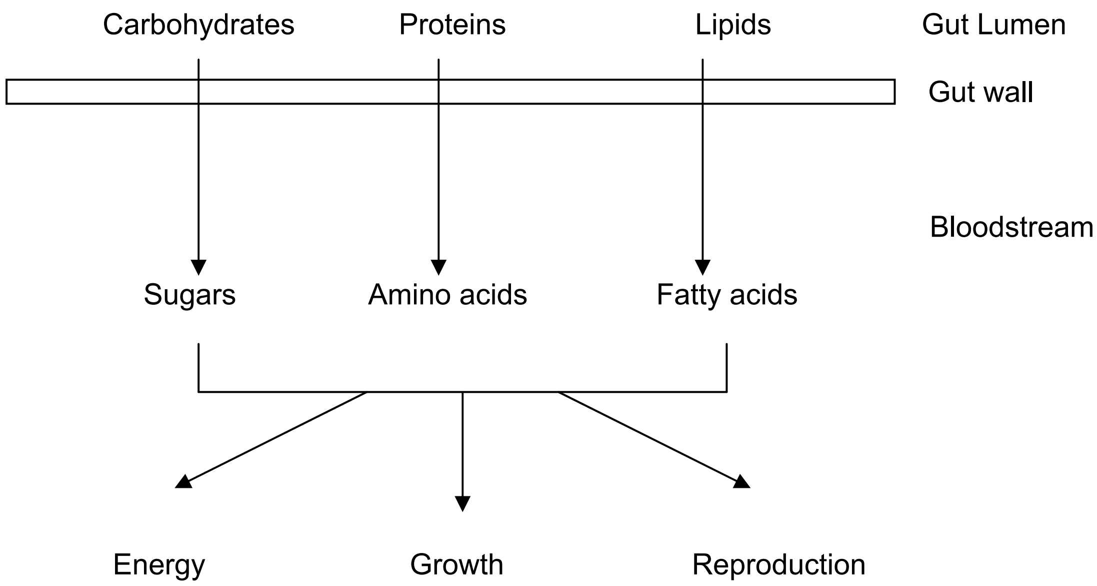

Figure 2.4 The fate of gross nutrients (adapted from De Silva and Anderson, 1995).

The degradation of glucose, amino acids and fatty acids will reach a common intermediate compound called acetyl Co-A. Acetyl Co-A will enter citric acid cycle, which is then connected to the process of oxidative phosphorylation. Oxidative phosphorylation will consume oxygen and release carbon dioxide and energy in the form of ATP (Adenosine triphosphate).

2.3.3 Protein sparing effect of lipid

Growth of fish will often be limited as it needs to direct some amino acids into energy liberating pathway to provide energy for basal metabolism despite protein synthesis. Lipid and carbohydrate will normally be included in fish diet by formulator to replace protein as alternative energy sources so that most of the amino acids will be used for protein synthesis. This is referred as protein-sparing effect. Sparing dietary protein from use as energy also reduces ammonia and nitrogen waste production in aquatic environment from metabolism of amino acids (Bromley, 1980; Shyong *et al*., 1998; Vergara *et al*., 1999; Lopez *et al*., 2006).

Lipid is an effective source of non-protein energy compared to other energy sources as it releases more energy per unit weight. It is easily digested and metabolized compared to carbohydrate and therefore, serves as a better source of energy for protein sparing. Besides that, inclusion of lipid can produce cost-effective diet compared to high cost protein sources.

The protein sparing effect of lipid varies between species. Overall, inclusion of 15-18% lipid will be optimal to replace protein energy in diet (Lie *et al*., 1988). Ai *et al*. (2004) concluded 41% protein and 12% lipid was the optimal dietary level of protein and lipid for Japanese sea bass. At three protein levels of 36%, 41% and 46%, increasing dietary lipids from 8% to 16% improved specific growth rate (SGR), protein efficiency ratio (PER), protein productive value (PPV) and energy retention in Japanese sea bass, showing protein sparing effect of lipid. Bromley (1980) showed that protein sparing action of lipid was most significant in turbot fed to three-quarter of satiation as 42% of dietary protein was converted into fish protein in diet containing 6% lipid. Meanwhile, only 32% conversion of protein was demonstrated in diet containing 0.5% lipid. Lee *et al*. (2002) demonstrated that increasing dietary lipid levels significantly improved hepatosomatic index (HSI), viscerosomatic index (VSI), protein efficiency ratio and protein retention in juvenile rockfish, *Sebastes schlegeli*, indicating protein sparing effect of lipid. It was concluded that 42% protein and 14% lipid was optimal dietary level for the growth and protein utilization of juvenile rockfish. Lee and Sang (2005) showed that increase of dietary lipid from 10% to 19% with 42% dietary protein caused improved growth and protein utilization in bagrid catfish, *Pseudobagrus fulvidraco*. They suggested a diet containing 42% protein and 19% lipid would be suitable for bagrid catfish.

Boujard *et al*. (2004) concluded sea bass could be fed diets containing up to 30% lipid and 54% protein. These high energy diets increased nitrogen retention, reduced phosphorus excretion and the use of fish meal as protein energy intake. Carmona-Osalde *et al*. (2005) found that increased dietary lipid levels improved maturation index and GSI in crayfish, *Procambarus llamasi.* Diet containing 20% protein 12% lipid was recommended for growth in crayfish while diet containing 30% protein 12% lipid for maturation. Protein sparing effect also has been reported in several fish species fed diets containing lipid as main energy source (De Silva *et al*., 1991; Vergara *et al*., 1999).

On the other hand, Ozorio *et al*. (2006) indicated that increasing dietary lipid from 12% to 16% with dietary protein of 15% and 28% did not induce protein sparing effect in white seabream, *Diplodus sargus* due to a low protein requirement in this species. Studies elsewhere also showed no protein sparing effect by dietary lipid in sharpsnout seabream (Hernandez *et al*., 2001) and dentex (Espinos *et al*., 2003). High energy diet and high inclusion of carbohydrate could also inhibit lipid absorption and reduced protein sparing effect by lipid (Ozorio *et al*., 2006).

Protein sources for fish farming depend mostly on fish meal, which is produced from demersal fish as raw material from northern water. The population of fish will eventually decrease in the near future. Therefore, it is necessary to lower the content of fish meal in fish diet and substitute it with other energy sources. Dietary protein utilization needs to be improved for protein synthesis rather than for energy. Besides that, adequate levels of protein and lipid need to be considered carefully to produce optimum dietary protein and lipid ratio in feed. An imbalance in the diet will have adverse influences on fish growth, nutrient utilization, body lipid deposition, increased production cost and deterioration in water quality.

2.4 Fatty acids

Fatty acyl groups form the hydrophobic interior of all cell membranes. This condition provides an impermeable barrier to water and polar molecules and separates the cell from the surrounding environment. Fatty acids are carboxylic acids with long-chain hydrocarbons as their side groups. The nomenclature of fatty acids is as follow:

C x:y(*n*-z) where,

x = the number of carbon atoms

y = the number of double bonds in the hydrocarbon chain

z = the position of carbon at which the first double bond appears numbering from the non-carboxyl (COOH) end

In addition to that, C represents carbon and *n* represents omega.

Saturated fatty acids are straight chain or branched-chain structures with an even number of carbon atoms without any double bond. For example, palmitic acids (C16:0) with a chemical structure of $CH_3(CH_2)_{14}COOH$. The most common saturated fatty acids lie in the range of 12 to 22 carbons. However, acids from 2 carbons to longer than 30 carbons have been reported. Acids such as ethanoic, propanoic, butanoic and hexanoic are in this category. Branched-chain fatty acids are usually found in bacteria, butter fats and skin lipids. They occur at low concentration in animal fats and some marine oils.

Monounsaturated fatty acids (MUFA) are fatty acids with a chain length of 16-22 carbon atoms and contain one double bond between the carbon atoms. For example, Oleic acid (C18:1n-9) with chemical structure of $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$. Saturated and monounsaturated fatty acids serve as major energy yielding nutrients in fish. Polyunsaturated fatty acids (PUFA) contain 2 to 4 double bonds between its carbon atoms while highly unsaturated fatty acids (HUFA) contain more than 4 double bonds. Chain length and the degree of unsaturation (number of double bonds) will determine the physical and chemical properties of the fatty acids. Saturated fatty acids are normally solid at room temperature while unsaturated fatty acids have low melting points and are fluid oils at room temperature.

Fatty acids existing in terrestrial plants and animals have a relatively low degree of unsaturation (less number of double bonds) with 14-18 of carbon chain lengths while fatty acids in aquatic organisms have high degree of unsaturation and longer chain lengths. Unicellular algae are the primary producers of essential fatty acids in marine ecosystem. They contain 20% of their dry weight as lipid, with approximately 50% of it present as *n*-3 PUFA. Besides that, red algae are rich in AA as well as *n*-3 PUFA. Crustacean zooplanktons are the major consumers of phytoplankton, and they are subsequently consumed by planktivorous fish. Throughout this food chain *n*-3 PUFA are generally elongated. Fatty acids are found mostly in algae as C_{18} PUFA while C_{20} and C_{22} HUFA are found in zooplankton and fish. In fish, the amount of C_{22} HUFA is greater than C_{20} HUFA.

Freshwater microalgae and insects normally contain higher amount of *n*-6 PUFA compared to marine algae (De Silva and Anderson, 1995). This eventually causes fatty acid composition of freshwater fish to contain higher proportions of *n*-6 PUFA compared to marine fish which contain higher composition of *n*-3 PUFA. Nevertheless, both are rich in EPA and DHA. Therefore, fatty acid composition of marine fish and freshwater fish will be different depending much on the fatty acid composition of the prey present in the two environments.

2.4.1 Dietary Fatty acids requirement of Fish

Inadequate provision of essential fatty acids will cause low growth rate, poor food conversion rate and affect reproductive performances of broodstocks. Research on the dietary fatty acids requirement has changed from considerations of optimal dietary levels of *n*-3 HUFA to optimal dietary ratios of the two important *n*-3 HUFA, EPA and DHA. Recent research aims to find the optimal dietary levels and ratios of the three important dietary HUFA, which are EPA, DHA and AA.

The EFA requirement of fish differs considerably between different species. Rainbow trout require *n*-3 fatty acids, while carp, eel, tilapia and salmon require both of the *n*-3 and *n*-6 fatty acids for good growth. HUFA such as EPA and DHA are important EFA for red sea bream, plaice and yellowtail, LNA and LA are considered as non-essential fatty acids (Watanabe, 1982). El-Sayed *et al*. (2005) found that Nile tilapia broodstock reared in brackish water required dietary *n*-3 HUFA for optimum spawning performance. Meanwhile, soybean oil which is rich in *n*-6 HUFA may meet the fatty acids requirement of broodstock reared in freshwater.

Studies also showed that HUFA especially EPA and DHA have a higher EFA value and growth enhancing effect than LNA of the *n*-3 fatty acids. Similarly, AA has a higher EFA value than LA of the *n*-6 fatty acids (Watanabe, 1982). Watanabe & Takeuchi (1976) found that replacing pollock liver oil which is high in HUFA with a comparable amount of methyl LNA did not improve the growth of trout. Other studies by Takeuchi and Watanabe (1977, 1980) showed that 0.5% of dietary supplementation of EPA and 0.5% of dietary *n*-3 HUFA mixture of EPA and DHA produced higher growth rate than dietary supplementation of 1% LNA in carp, eel and salmon.

Seed oils in terrestrial plants are rich in *n*-6 PUFA but devoid in *n*-3 PUFA. Lipids found in aquatic food chain have high proportion of *n*-3 PUFA as well as *n*-6 PUFA that satisfy the fatty acids requirement of fish. Therefore, farming fish relies closely on fish oil and fish meal for provision of dietary *n*-3, *n*-6 fatty acids and HUFA. Lee *et al*. (1967) obtained reduced growth in trout fed corn oil as sole lipid source but the growth increased when trout were fed with Salmon oil which is rich in C_{20} and C_{22} *n*-3 fatty acids.

Fish tissues generally have higher concentrations of DHA and EPA than AA showing a dietary requirement of *n*-3 HUFA. However, this should not obscure the fact that AA is also an important minor component in fish cell. DHA is an important dietary HUFA as it is present in a very high concentration in visual and neural cell membrane in both fish and mammals, especially in rod cell outer segment membrane and synaptosomal membrane. Juvenile herring deprived of DHA were not able to capture prey at low light intensities efficiently (Bell *et al*., 1995a; 1995b). AA is the major precursor of eicosanoids. Eicosanoids are a range of highly active C_{20} compounds formed in trace amounts in every tissue in the body. Nevertheless, they are involved in variety of physiological functions such as cardiovascular, inflammatory response and reproduction. They are also produced in response to stressful situations in both the cellular and body level. Eicosanoids formed by AA are more biologically active than those eicosanoids formed by EPA. Watanabe (1982) concluded that most of the commercial diets for rainbow trout, carp, eel, salmon and red sea bream contain 4-6% lipid and 0.4-0.6% of EPA and DHA to satisfy the EFA requirement of these fish.

Yu & Sinnhuber (1976) concluded that addition of 1% LA stimulated growth of rainbow trout, but the growth was depressed when the dietary LA level increased to 2.5% and 5%. Extremely high levels of *n*-3 fatty acids and more than 1% of *n*-6 fatty acids in diet depressed the growth of Coho Salmon (Yu *et al*., 1979). Excess addition of LNA or HUFA mixture of EPA and DHA of 4 times higher than the dietary requirements produced poor growth and low feed conversion in rainbow trout (Takeuchi & Watanabe, 1979). Sargent *et al*., (1999b) concluded that excess supplementation of EPA in the diet of turbot larvae was not deleterious but an excess of AA was. Essentially normal survival, growth, pigmentation and metamorphosis were shown by turbot larvae fed on rotifers and brine shrimp nauplii supplemented with tuna orbital oil with a ratio EPA:AA of 4.2. At ratios of EPA:AA of 1.5 and 0.4, achieved by elevating dietary AA levels, pigmentation and metamorphosis of the larvae were found impaired. Excess dietary AA was found to alter brain eicosanoid production and cause a biochemical-induced stress. For sea bass larvae, optimal dietary ratio of DHA:EPA was found to be circa 2:1 while optimal dietary ratio of EPA:AA was circa 1:1. For turbot and halibut, optimal dietary ratio of DHA:EPA was 2:1 while optimal dietary ratio for EPA:AA was 10:1 (Sargent *et al*., 1999a). We should consider a balance proportion of fatty acids for fish growth compared to the amount of fatty acids present in the diet by taking into account the ratio of n-3:n-6 fatty acids, ratios of DHA:EPA:AA.

Sargent *et al*. (1999b) stated that imbalanced levels and ratios of dietary EFA will cause competitive interactions between different series of fatty acids, for example *n*-3 and *n*-6 series, and also between fatty acids of different chain lengths and degrees of unsaturation within the same series such as C20:5*n*-3 (EPA) and C22:6*n*-3 (DHA). Competitions will occur between C18:2*n*-6 (LA) and C18:3*n*-3 (LNA) for delta-6 fatty acid desaturase and between C18:3*n*-6 and C18:4*n*-3 for fatty acid elongase. C20:4*n*-6 (AA) and C20:5*n*-3 (EPA) will compete for cyclo-oxygenases that synthesize 2-series prostanoids, 4-series leukotrienes and lipoxygenasis that synthesize 3-series prostanoids and 5-series leukotrienes. These eicosanoids produced from AA are biologically more active than those produced from EPA and they compete for the same cell membrane receptors. High level of EPA competitively inhibits the formation of eicosanoids formed from AA. Therefore, high tissue ratio of AA:EPA results in enhanced