

**THE EFFECT OF DIETARY CRUDE PALM OIL AND ALPHA-
TOCOPHERYL ACETATE ON REPRODUCTIVE PERFORMANCE
AND LARVAL QUALITY OF TILAPIA (*Oreochromis* sp.)**

WANG YAN

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REPRODUCTIVE PERFORMANCE AND LARVAL
QUALITY OF TILAPIA (*Oreochromis* sp.)**

by

WANG YAN

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requirements for the degree
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LIST OF SYMBOLS

Symbol	Illustration
α	Alpha
β	Beta
γ	Gama
δ	Delta

ABBREVIATIONS

Abbreviation	Full Name
ARA	Arachidonic acid
CMC	Carboxymethyl cellulose
CPO	Crude palm oil
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FAO	Food and Agricultural Organization
FO	Fish oil
GC	Gas chromatography
GSI	Gonadal somatic index
HPLC	High pressure liquid chromatography
HSI	Hepatosomatic index
IPI	Intraperitoneal fat index
LSO	Linseed oil
LC	Long chain
MT	Metric tonnes
MUFA	Monounsaturated fatty acid
NFE	Nitrogen free extract
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
SGR	Specific growth rate
T	Tocopherol
T3	Tocotrienol
TSI	Testo-somatic index
VSI	Viscera-somatic index

KESAN MINYAK KELAPA SAWIT MENTAH DAN ALFA-TOKOFERIL ASETAT DALAM DIET KE ATAS PRESTASI PEMBIAKAN DAN KUALITI LARVA IKAN TILAPIA (*Oreochromis SP.*)

ABSTRAK

Disertasi ini dijalankan untuk menyiasat kesan-kesan pemakanan lipid dan vitamin E induk kepada prestasi pembiakan tilapia dan kualiti larva. Empat diet yang isonitrogenus (35% protein) dan isolipidik (10%) berasaskan kasein dirumuskan dengan minyak ikan (FO), FO dan minyak kelapa sawit mentah (FO+CPO; 1:1), CPO atau minyak linseed sebagai sumber lipid dalam Eksperimen 1, dan 0 (T0), 60 (T60), 600 (T600) mg α -tokoferil asetat, atau pecahan kaya tokotrienol berasaskan CPO yang ditetapkan untuk menyediakan α -tokoferol pada tahap 60 (PalmE60) mg sekilogram diet kering ditambah dalam Eksperimen 2. Tilapia betina (*Oreochromis sp.*) yang prabertelur dilabel dengan tag warna secara individu, enam betina dan dua jantan distokkan dalam tangki pembiakan yang bersaiz satu tan. Setiap diet diberi kepada dua tangki induk ikan dan prestasi pembiakan individu ikan betina dipantau selama 25 dan 12 minggu, masing-masing. Induk betina diberi makan dua jenis diet berasaskan CPO (Eksperimen 1) dan diet T600 (Eksperimen 2), menunjukkan saiz gonad yang lebih besar secara ketara ($P < 0.05$), mempunyai bilangan tertinggi tilapia yang aktif bertelur dan menghasilkan jumlah telur seekor ikan tertinggi disebabkan oleh selang peneluran yang lebih pendek dan kekerapan menelur yang lebih tinggi. Penetasan telur yang berasal dari rawatan-rawatan ini juga lebih tinggi secara ketara. Penggunaan pelbagai sumber lipid diet mempengaruhi komposisi lemak asid atau kepekatan vitamin E dalam otot, hati, gonad, testis, telur dan larva yang baru ditetas, secara ketara. Dalam Eksperimen 3, larva tanpa karung telur kuning yang diperolehi dari Eksperimen 2

diberi makan diet mengandung kandungan vitamin E yang rendah dengan lipid tidak-teroksida (PV9) atau lipid teroksida (PV160) selama 3 minggu. Keputusan yang diperoleh menunjukkan prestasi pertumbuhan ikan yang diberi diet PV160 adalah lebih rendah daripada ikan yang diberi diet PV9, tetapi larva yang berasal dari tilapia betina yang diberi makan diet T600 kelihatan dapat mengurangkan pengaruh pengoksidaan lipid diet dengan rizab vitamin E yang tinggi. Kesimpulannya, penambahan minyak sawit mentah atau kandungan vitamin E yang tinggi dalam diet induk boleh meningkatkan prestasi pembiakan tilapia dan pemindahan vitamin E induk-anak dapat mengurangkan pengaruh negatif pemakanan pengoksidaan lipid kepada prestasi pertumbuhan larva tilapia apabila diberi makan diet lipid teroksida.

**THE EFFECT OF DIETARY CRUDE PALM OIL AND ALPHA-
TOCOPHERYL ACETATE ON REPRODUCTIVE
PERFORMANCE AND LARVAL QUALITY OF TILAPIA
(*Oreochromis* sp.)**

ABSTRACT

The present dissertation was conducted to investigate effects of the broodstock dietary lipids and vitamin E on the reproductive performance of tilapia and the quality of larvae hatched. Four isonitrogenous (35% protein) and isolipidic (10%) casein-based diets were formulated with added fish oil (FO), FO and crude palm oil (FO+CPO; 1:1), CPO or linseed oil as the lipid source in Experiment 1, and supplemented with 0 (T0), 60 (T60), 600 (T600) mg α -tocopheryl acetate, or a CPO-derived tocotrienols-rich fraction designated to provide α -tocopherol at a level of 60 (PalmE60) mg per kg dry diet in Experiment 2. Pre-spawning female tilapia (*Oreochromis* sp.) was individually color-tagged, and six females and two males were stocked into a one-ton breeding tank. Each diet was fed to two tanks of broodfish and the reproductive performance of 12 individual female fish was monitored over 25 and 12 weeks, respectively. Female broodfish fed the two crude palm oil-based diets and the 600 mg α -tocopheryl acetate/kg added diet, showed significantly ($P < 0.05$) larger ovary sizes, had the highest number of actively spawning tilapia and produced the highest total number of eggs per fish due to the shorter inter spawning interval and higher spawning frequency. The hatchability of eggs originated from these dietary treatments was also significantly higher. The inclusion of various dietary lipid source significantly affected the fatty acid composition or vitamin E concentration of muscle, liver, ovary, testes, egg and newly hatched larvae. In Experiment 3, yolk-sack free tilapia larva hatched

from spawned eggs obtained from the study of Experiment 2 were fed on the low vitamin E diets supplemented with non-oxidized lipid (peroxide value, PV9) or oxidized lipid (PV160) for 3 weeks. Results obtained revealed that the growth performance of fish given the PV160 diet was poorer than larvae fed the PV9 diet but the larvae originating from T600 diet fed parental female could to some extent alleviate the influence of dietary lipid oxidation with the high vitamin E reserve. In conclusion, the addition of crude palm oil or high vitamin E amount into the broodstock diet could enhance the reproductive performance of tilapia and mother-offspring transferred vitamin E thereafter could alleviate the negative influence of dietary lipid oxidation on the growth performance of tilapia larvae when fed oxidized dietary lipid.

Chapter 1 Introduction

Tilapia is a collection name for a group of cichlid fish species from the *Cichlidae* family. These fish are mainly living in freshwater and occasionally brackish water in the tropical and subtropical environments. Originally native to the African continent, tilapias have been artificially introduced to be commercially cultured in nearly 100 countries in the world since 1950s because of their rapid growth, hardiness to tolerate harsh conditions, good resistance to diseases and adaptability to a wide range of rearing systems (Gupta & Acosta, 2004). Tilapias have nowadays become the second most widely cultured fish only after the carps. Among them, Nile tilapia, *Oreochromis niloticus*, is the most widely farmed tilapia species. Other species of tilapia also of important commercial importance are Mozambique tilapia (*O. mossambicus*), blue tilapia (*O. aureus*) and their hybrids. Figure 1.1 illustrates the total world production of tilapia and Nile tilapia (FAO, 2012).

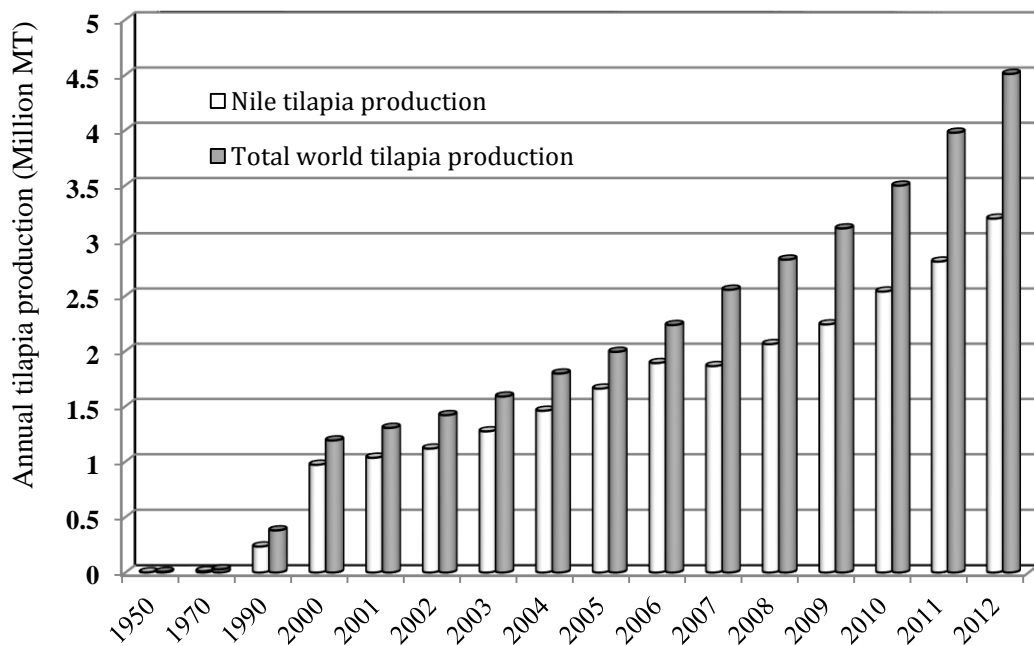


Figure 1.1 Total world tilapia production and Nile tilapia production from 1950 to 2012.

As shown in Figure 1.1, after a relatively slow tilapia production increase from 1950 to 1970, the exponential growth was seen from the subsequent twenty years and thereafter. By 2012, there were totally around 4.5million metric tonnes (Mt) produced, of which was 3.2million Mt Nile tilapia. Together, farmed tilapia contributed around 84% of world tilapia production.

With the ever expanding world population, aquaculture is expected to continue as one of the fastest growing animal food-producing sectors to meet the consumption demand of human for the protein intake as well as the requirement of health-beneficial unsaturated fatty acids like omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In 2011, fish accounted for 16.7 percent of animal protein intake of the global population and 6.5 percent of all proteins consumed (FAO, 2012). FAO (2012) estimated these ratios will grow further and the contribution from aquaculture production in total food supply will rise. Global tilapia production is thus also anticipated to further increase and projected to raise to about 8.9 million tonnes by the year 2020 (Tacon and Metian, 2008).

This rapid rise of tilapia production is due in part to the increasing intensification of farming systems and this has led to a critical need for large quantities of fingerlings for stocking grow-out systems. Furthermore, it is increasingly important to produce high quality tilapia fry due to the low fecundity of broodfish. Tilapias of the *Oreochromis* genus, the major farmed species, are female mouth-brooders and exhibit high parental care with relatively low number of eggs produced in each clutch. The problem in the mass production of tilapia seed is further exacerbated due to the low

degree of female spawning synchrony and reduction in spawning rigor with time (Mires, 1982).

Broodstock nutrition is recognized as a major factor that can influence fish reproduction and subsequent larval quality of many fish species (Izquierdo *et al.*, 2001). Lipids are one of the important nutritional composition of aquafeeds. Their inclusion in the broodstock diets plays a crucial role in maintaining successful spawning performance of broodfish, egg quality and improving hatchability and larval survival and growth (Watanabe *et al.*, 1984; Cerda *et al.*, 1994; Abi-Ayad *et al.*, 1997; Navas *et al.*, 1998; Furuita *et al.*, 2000; Mazoarra *et al.*, 2003). While those studies were primarily conducted on marine species, there is currently a paucity of information concerning the nutrient requirements of tilapia broodfish especially as it relates to lipid nutrition but some information is available on protein and energy requirements (Gunasekera *et al.*, 1996a,b; Siddiqui *et al.*, 1998; El-Sayed and Kawanna, 2008; Lupatsch *et al.*, 2010).

Vitamin E is an essential micronutrient found nearly 100 years ago when Evens and Bishop (1922) studied the reproduction of rats. It has now evolved to be a generic name for two groups of potent, structure-related, lipid-soluble, chain-breaking antioxidants, namely tocopherols and tocotrienols. While extensive research had long been carried out for the role of vitamin E on the reproduction of terrestrial animals, it was not until 1970s for the similar research first appearing to conduct on rainbow trout (Kimunaki & Sugii, 1972). During the following years, fish broodstock nutrition studies, fully or partially focusing on vitamin E, were reported on common carp (*Cyprinus carpio*) (Watanabe and Takshima, 1977), ayu (*Plecoglossus altivelis*)

(Takeuchi *et al.*, 1981), red sea bream (*Pagrus major*) (Watanabe *et al.*, 1985), and Atlantic salmon (*Salmo salar*) (Schiedt *et al.*, 1988; Eskelinen, 1989). Vitamin E was eventually recognized for its role on fish reproduction in 1990 (Watanabe, 1990). Since then, this role has been demonstrated in turbot (*Scophthalmus maximus*) (Hemre *et al.*, 1994), seabream (*Sparus auratus*) (Fernández-Palacios *et al.*, 1998), sea bass (*Dicentrarchus labrax*) (Carcia *et al.*, 2000), common dentex (*Dentex dentex*) (Mourente *et al.*, 1999), Japanese flounder (*Paralichthys olivaceus*) (Tokuda *et al.*, 2000), Japanese eel (*Anguilla japonica*) (Furuita *et al.*, 2009a). In contrast, there was little information available for the role of vitamin E on the reproduction on tilapia (El-Gamal *et al.*, 2007; Gammanpila *et al.*, 2007; Areechon and Thaitoungchin, 2003). It is therefore this scarcity guarantees there is a necessity to further study the role of vitamin E on tilapia reproduction.

It is recognised for aquatic animals that the maternal broodstock nutrition has a profound role on the quality of reproduction products such as eggs and/or newly hatched offsprings. Antioxidant like vitamin E is essential for the defence system buildup of developing eggs or newly hatched larvae of fish (Cowey *et al.*, 1985; Mourente *et al.*, 1999). Although a few research noted the association of vitamin E concentrations in eggs and early development stages of larvae of some fish species (Takeuchi *et al.*, 1981; Mourente *et al.*, 1999; Ciarcia *et al.*, 2000), there is no information available for the role of maternal-offspring transferred vitamin E on the protection, if any, for the first-fed larvae when challenged the oxidated diet. Considering tilapia is majorly cultured in the tropical area where the hot weather easily causes the oxidation of lipids in the larva diet, there is an urgency to start the research in this area in order to have a better management of quality tilapia seed production.

Those situation described in the previous paragraphs is not on par with the fact that tilapia is the second most farmed fish. There is a necessity that the related research of broodstock dietary lipids and vitamin E must be conducted to expand our knowledge on the reproductive performance of this economically very important fish.

It is thus the following four objectives have set up for the current dissertation,

1. To evaluate effects of fish oil, crude palm oil, and linseed oil on the spawning performance, egg and larval quality, and the fatty acid composition of muscle, liver, ovaries and testes from broodstock Nile tilapia (*O. niloticus*) (Chapter 4).
2. To determine the distribution of tocopherols and tocotrienols in the muscle, liver, ovaries, testes, egg and larvae from broodstock tilapia (*O. niloticus*) (Chapter 5).
3. To determine if vitamin E supplied as α -tocopheryl acetate or derived from tocotrienols rich fraction of palm oil can enhance the reproductive performance and egg quality of red hybrid tilapia (*O. niloticus* \times *O. mossambicus*) (Chapter 6).
4. To study if the maternal-offspring transferred vitamin E can provide the protective role on the newly-hatched red hybrid tilapia larvae (*O. niloticus* \times *O. mossambicus*) fed on the oxidized dietary lipid (Chapter 7).

The results from these studies will be expected to better help us understand the role of lipids and vitamin E on the reproductive performance of tilapia as well as effects of maternal-offspring transferred vitamin E on the quality of eggs and larval produced by this fish.

Chapter 2 Literature Review

2.1 Lipid and fatty acids requirement of tilapia

Lipid nutrition is one of the most extensively researched topics of many aquatic animals including the tilapia species. Lipid provides the source for energy reserve and essential fatty acids (EFA). It facilitates the absorption of fat-soluble vitamins, serves as precursors of hormones and prostaglandins and maintains proper fluidity and permeability of tissue and cellular membrane structures (Ng & Chong, 2004; Sargent, 1995). Appropriate level of dietary lipid inclusion is important for the growth and well-being of tilapia. Studies on non-brooding tilapias reported optimum dietary lipid level was between 10% and 15% (El-Sayed and Garling, 1988; Chou and Shiau, 1996), whereas a minimum level of 5% must be provided (De Silva *et al.*, 1991; Chou and Shiau, 1996). An even lower level of only 3% was reported on Mozambique tilapia (*O. mozambicus*) if sufficient energy sources contributed from digestible proteins and carbohydrates were guaranteed in the feeds when the fish was intensively cultured in recirculating systems (Fitzsimmons *et al.* 1997). There were numerous studies reported on the protein sparing effect of lipid in tilapia (Anderson *et al.*, 1984; Wee and Ng, 1986), at the same time, under some conditions the amount of dietary lipid requirement of tilapia could be also spared by carbohydrates (El-Sayed and Garling, 1988; Shimeno *et al.*, 1993).

While reviewing the lipid nutrition of tilapia, the fatty acid requirement of this species must also be considered. Tilapia, unlike marine fish, is capable of converting the 18-carbon polyunsaturated fatty acids (PUFA) like linoleic (18:2n-6) and linolenic

(18:3n-3) acids through desaturation and elongation into their respective longer end products arachidonic acid (20:4n-6) and DHA (22:6n-3) (Figure 2.1). Applying the stable isotope tracking technology, both of Olsen *et al.* (1990) and Tocher *et al.* (2002) confirmed this ability of Nile tilapia to synthesize C20 and C22 from C18 n-6 and n-3 PUFA by supplying the fish with ¹⁴C labelled linoleic (18:2n-6) and linolenic (18:3n-3) acids laden diets. Tilapia, like other fish and vertebrates, can not *de novo* synthesize 18:2n-6 and 18:3n-3 (Henderson and Tocher 1987). It was thus these two fatty acids were essential fatty acids for this species and must be provided in the diets (NRC 1993).

(n-6) Series



(n-3) Series

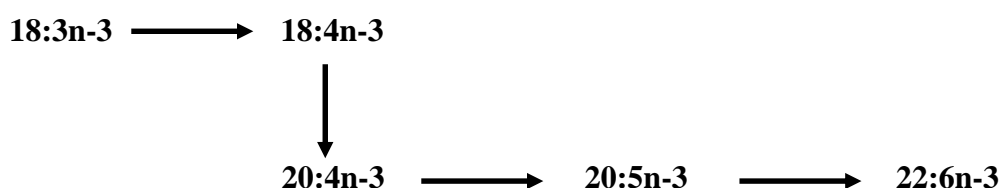


Figure 2.1 Pathways of conversion of C18 (n-6) and (n-3) polyunsaturated fatty acids to their higher C20 and C22 derivatives. (Adopted from Sargent, J.R., 1995)

However, similar to other freshwater fish, tilapia tends to require greater amounts of n-6 fatty acids in contrast to n-3 fatty acids for the maximal growth performance (NRC, 1993) though this was not accompanied with uncontradictory reports. Takeuchi *et al.* (1983) reported Nile tilapia required 0.5% 18:2n-6 albeit not 20:4n-6 for the optimal growth when fed the purified diets. Kanazawa *et al.* (1980) found red-belly tilapia, *Tilapia zillii*, had the fatty acid requirement for 18:2n-6 or 20:4n-6 at a level of about 1% of the better growth performance. These authors also showed that dietary 18:3n-3 or n-3 HUFA either had no growth improvement or even

to the worse reduced growth performance or feed utilization of the afore-mentioned tilapias. Similar findings were reported in blue tilapia (*O. aureaus*) (Stickney and McGeachin, 1983) and hybrid tilapia (*O. niloticus* × *O. aureaus*) (Huang *et al.*, 1998; Ng *et al.*, 2001). Contrary to the notion, Chou *et al.* (2001) reported that hybrid tilapia fed diets containing up to 5% n-3 PUFA rich cod liver oil (CLO) had better growth performance while those fed a diet added no CLO had poorer growth and showed a typical n-3 deficiency, suggesting that n-3 PUFA may be required for maximal growth. The qualitative requirement of broodstock tilapia for EFA has to date not been determined yet.

2.2 Alternative lipid source for tilapia

Fish oil is a traditional key important ingredient for the aqua-feeds as a source for energy and n-3 PUFA. Due to the over-exploitation for pelagic fisheries, the global annual production of fish oil has stagnated around one million MT (Figure 2.2) for many years after peaking at slightly less than two million MT during the late 1980s. The International Fishmeal and Fish Oil Organisation estimated 0.8 million MT of global fish oil was used for the aquaculture and 3% of this amount for tilapia in 2009. Although the total amount of fish oil for the aquaculture use was to some extent reduced in recent years but the fish oil use in the tilapia feeds was increased to 5.3% according to The World State of Fisheries and Aquaculture by FAO in 2012. To reduce the reliance on this expensive and finite oil source, quite some work have been carried out to research the economical alternatives for tilapia (Gaber, 1996; Huang *et al.*, 1998; Ng *et al.* 2001, 2006; Bahurmiz and Ng, 2007; Trushenski *et al.*, 2009; Szabo *et al.*, 2011).

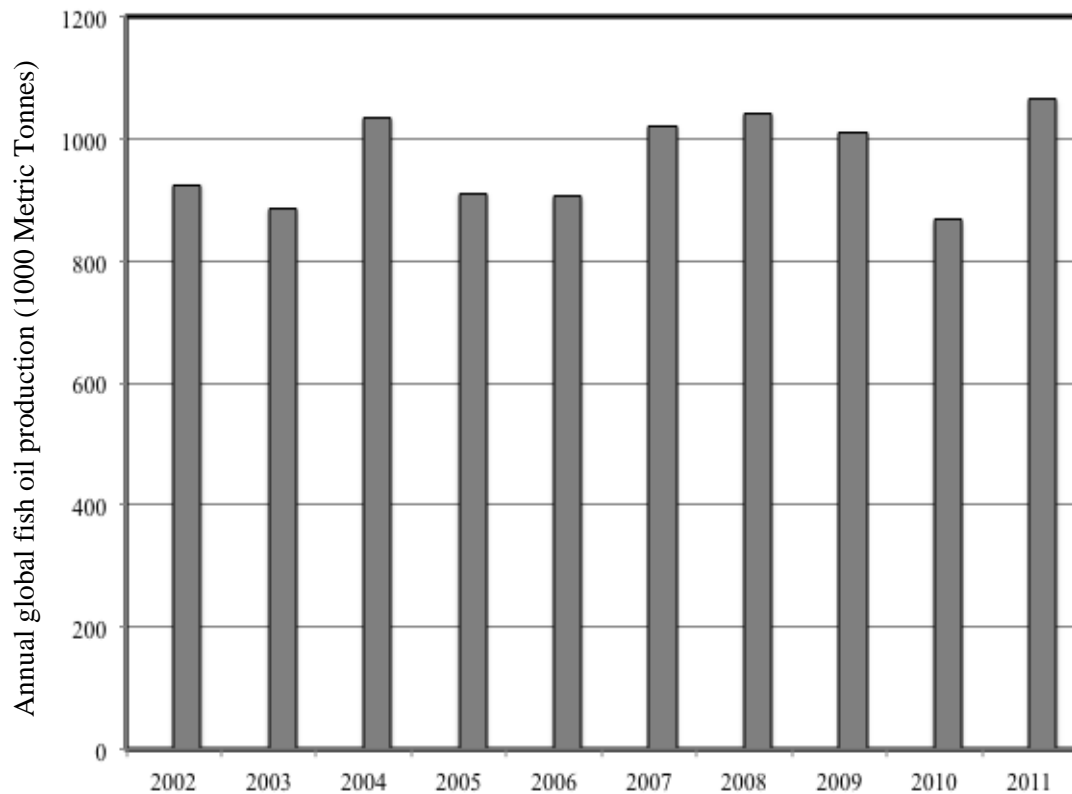


Figure 2.2 Global fish oil production between 2002 and 2011. (FishStat Plus: Universal software for fishery statistical time series, www.fao.org/fishery/statistics/software/fishstat/en).

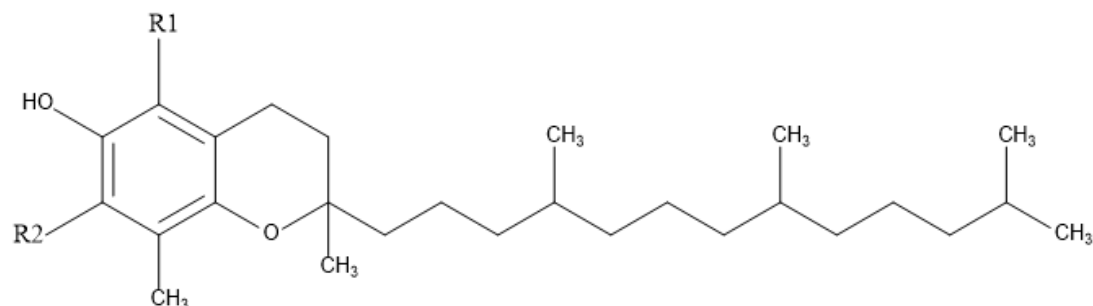
Results from these studies showed vegetable oils like soybean oil (Gaber, 1996; Huang *et al.*, 1998) and crude palm oil (Ng *et al.* 2001, 2006; Bahurmiz and Ng, 2007) appeared to be suitable candidates. Especially for crude palm oil, it is cheap and readily available as this oil was projected to take over soybean oil as the most abundant plant oil a decade ago (Gunstone, 2001). Its richness in natural antioxidants such carotenoids and vitamin E, high concentration of saturated 16:0 and monounsaturated 18:1n-9 fatty acids leads not hard to believe it is not only reduce the incidence of rancidity of feeds but also lend the benefits to the health of tilapia consuming this oil. Furthermore, Shiranee & Natarajan (1996) reported the incorporation of crude palm oil into the diet enhanced the reproductive potential of pearlspot *Etroplus suratensis*. Hajizadeh *et al.* (2008) first reported the addition of crude palm oil into the broodstock diet had no

negative effect on egg and larval quality in *O. niloticus*. Further research should be conducted to investigate effects of crude palm oil, the foremost potential fish oil substitute for tilapia, on the reproductive performance of this fish (Chapter 4).

2.3 Vitamin E nutrition of tilapia

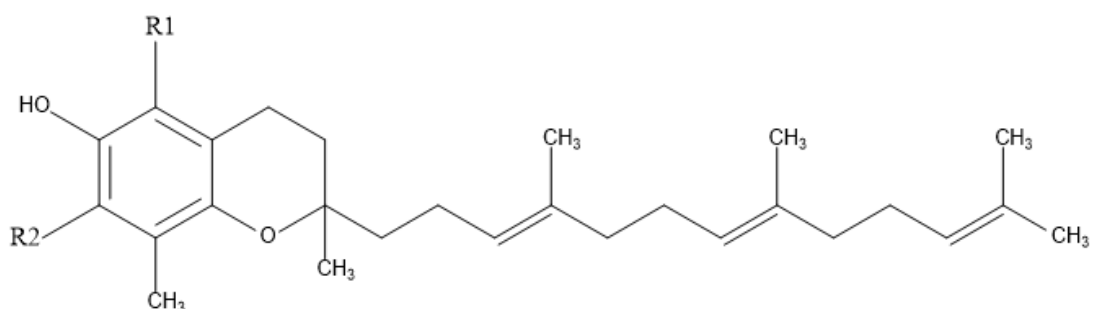
Vitamin E found by Evans and Bishop (1922) is nowadays used to describe for a group of structure-related, lipid-soluble compounds. Its eight naturally occurring isoforms consists of a common chromanol head and a hydrocarbon side chain (Figure 2.3). Those with the saturated tail are called tocopherols whereas those bearing the unsaturated one named tocotrienols. The vitamin E homologue, α , β , γ or δ -tocopherol or tocotrienol, is designated according to the number and the position of the methyl groups (CH_3 -) attached to the aromatic ring. Various homologues of vitamin E have the different biological activity with the following order was reported, α -tocopherol > β -tocopherol > α -tocotrienol > γ -tocopherol > β -tocotrienol > δ -tocopherol > γ -tocotrienol, corresponding to a respective biopotency factor to 1, 0.5, 0.3, 0.1, 0.05, 0.03 and 0.01 based on the amount of vitamin E necessary to prevent foetal resorption in pregnant and vitamin E-deficient rats (Sheppard and Pennington, 1993; Drotleff and Ternuies, 1999). Nevertheless, the biological activity of these homologues is not necessarily associated with their antioxidant activity. For example, Cooney *et al.* (1993) reported that γ -tocopherol was superior to α -tocopherol to detoxify nitrogen dioxide (NO_2), suggesting that the former had the more potent antioxidant activity than the latter. Serbinova *et al.* (1991) reported α -tocotrienol possessed 40-60 times higher antioxidant activity against Fe^{2+} -induced lipid peroxidation in rat liver microsomal membranes than α -tocotrienol. Similarly, Kamat *et al.* (1995 & 1997) reported

tocotrienols had more potent antioxidant capabilities than α -tocopherol in rat brain mitochondria and liver microsomes, respectively.



Tocopherol	R1	R2
α	CH ₃	CH ₃
β	H	CH ₃
γ	CH ₃	H
δ	H	H

(a)



Tocotrienols	R1	R2
α	CH ₃	CH ₃
β	H	CH ₃
γ	CH ₃	H
δ	H	H

(b)

Figure 2.3 Structure of naturally occurring vitamin E isoforms: tocopherols (a) and tocotrienols (b).

Like terrestrial animals, fish can not synthesize vitamin E in the body and thus must obtain it from the diet (NRC, 1993). DL- α -tocoperhyl acetate, chemically synthesized from substituting the hydrogen atom of hydroxyl group on phenolic ring on the chromanol nucleus of tocopherol by the acetate group (CH₃COO-) (Figure 2.4),

is the most commonly used vitamin E compound due to its stability. The deficiency of vitamin E in fish can cause poor growth, muscular dystrophy, anemia, impaired erythropoiesis, depigmentation, and among other symptoms (NRC, 1993).

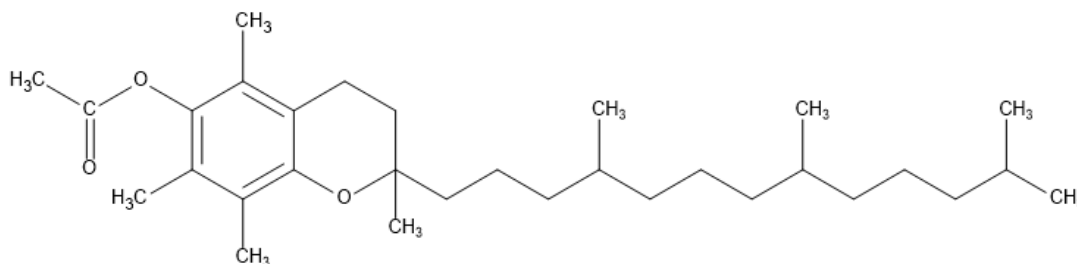


Figure 2.4 Structure of chemically synthesized vitamin E: α -tocopheryl acetate.

The dietary requirement for vitamin E has been established for many fish species including tilapia with the levels ranging from 25 – 119 mg/kg (NRC, 1993). The dietary vitamin E requirement of tilapia may be species specific and also affected by the dietary lipid level or dietary HUFA. For example, Satoh *et al.* (1987) reported the dietary vitamin E requirement of Nile tilapia was 50–100 mg DL- α -tocopheryl acetate/kg dry diet for a diet added with 5% lipid but significantly increased to 500mg/kg when the dietary lipid level was increased to 10-15%. In the meanwhile, Roem *et al.* (1990) reported the requirement of blue tilapia *O. aureas* was estimated to 10mg and 25mg/kg in diets supplemented with 3% and 6% dietary lipid, respectively. Futhurmore, Shiau & Shiau (2001) re-evaluted the vitamin E dietary of tilapia to be 42-44 mg/kg and 60-66 mg/kg when fed diets containg 5% and 12% lipid, respectively, based on the findings from their laboratory that both n-3 HUFA and n-6 fatty acids were required by tilapia for maximal growth (Chou and Shiau, 1999; Chou *et al.*, 2001). To date, the qualitative requirement for vitamin E of broodstock tilapia is not determined.

In fish nutrition, the vitamin E studies were primarily focused on α -tocopherol/ α -tocopheryl acetate, only a few research were conducted on other tocopherols like β , γ or δ -tocopherol in salmonids (Sigurgisladottir *et al.*, 1994; Hamre and Lie, 1997; Parazo *et al.*, 1998) and tocotrienols in African catfish (Ng *et al.* 2004) and red hybrid tilapia (Wang *et al.*, 2006). The results obtained from these studies showed α -tocopherol was always the predominant isoform accumulated in all tissues of fish examined, indicating there was no conversion between the most biological active α -tocopherol and its β , γ or δ -counterpart or four tocotrienols. A study by Hamre *et al.* (1998) indicated there might be present of a hepatic tocopherol binding protein called α -tocopherol transfer protein (α -TTP) existing in mammals (Blatt *et al.*, 2001) to differentiate α -tocopherol in liver from other tocopherols during the absorption, also in Atlantic salmon (*Salmo salar* L.). Ng *et al.* (2004) assumed α -TTP was also present in African catfish and went on to propose this was the cause leading to the very low levels of α - and γ -tocotrienols deposited into the tissues of this fish detected despite that one of groups of animals were fed on a diet containing 10% palm fatty acid distillate rich in tocotrienols (3889.2mg total vitamin E/g oil, with 79.5% consisting of tocotrienols and 20.4% tocopherols). The further work from this laboratory led Wang *et al.* (2006) first to report the deposition of tocotrienols in the tissues of tilapia fed diets added palm oil-originating tocotrienols fraction. Results obtained there further added the credence of existence of assumed α -TTP in fish although the exact mechanism of this fat-soluble vitamin remains to be uncovered. There is also no report available on the deposition and distribution of various vitamin E homologues in the broodstock tilapia, which warrants the necessity of doing the relevant studies in this regard (Chapters 5 & 6).

2.4 Broodstock nutrition of tilapia

Tilapia farming is developing at the fast step as it is the second most farmed fish only after the carps in the world. However, to have the sustainable tilapia production, it is not difficult to understand the demand for fry supply must be met. The *Oreochromis* tilapia and its hybrids are mouthbrooders and infamous for their asynchronous spawning and low fecundity breeding traits which hinders to expand commercial tilapia aquaculture (Bhujel, 2000). Broodstock nutrition is recognized to play the important role on gonadal maturation, egg and larval quality of fish (Izquierdo *et al.*, 2001). To date, protein is the foremost researched nutrient on the reproduction of tilapia. Results available showed a dietary requirement for protein of tilapia should be ranging from 20 to 40%. Santiago *et al.* (1985) found Nile tilapia fed on the 40% protein diet consistently had the higher larvae production but only a portion of those on the 20% protein one had the comparable results with the remaining having significantly inferior reproductive performance. Gunasekara *et al.* (1996a) reported Nile tilapia maintained on the diets with 20 or 35% protein level had higher numbers of eggs per spawn and significantly shorter spawning intervals compared to those on a diet of 10% protein level though relative fecundity and egg size was relatively similar. A following work by the same authors observed Nile tilapia had the similar numbers of eggs per spawn and percent fertilizability when also fed the 20 and 35% protein level diets but those on the higher protein diet had significantly higher hatchability of eggs and percentage of normal larvae (Gunasekara *et al.*, 1996b). Similarly, Bhujel *et al.* (2001) observed no difference for the reproduction performance between the two groups of broodstock Nile tilapia fed on two commercial catfish diets with the protein content at 25% and 30%, respectively. El-sayed *et al.* (2003) reported that 40% dietary protein was required for optimum spawning performance of Nile tilapia reared at

different salinities. Nevertheless, the excess of protein inclusion in diets might have a negative effects on the tilapia reproduction. Both Wee and Tuan (1988) and De Silva and Radampola (1990) found tilapia fed on the diets containing more than 40% crude protein produced less larvae or total number of spawnings per female. When high dietary protein levels were used for broodstock tilapia, an optimal protein to energy (P/E) ratios from 20.5 to 23.6 mg CP KJ/g was recommended (El-Sayed and Kawanna 2008; Lupatsch *et al.*, 2010). In the meanwhile, deficiency of protein in the broodstock diets of tilapia was reported to delay sexual maturity, slow oocyte maturation, and reduce the fertility of spawned eggs and postpone the spawning intervals (Gunasekara *et al.*, 1995, 1996a, b).

Aside from protein, lipid also plays a crucial role on the reproductive performance and larval quality of fish (Bell *et al.*, 1997; Mazorra *et al.*, 2003). Although the lipid nutrition of tilapia attracted many interests of fish nutritionists in the past decades (see Section 2.1), fewer studies have by so far been performed on the broodstock tilapia. Santiago and Reyes (1993) studied effects of different dietary lipid sources (cod liver oil, soybean oil, corn oil, coconut oil or a cod liver oil and corn oil mixture), on the reproductive performance of Nile tilapia. They found that fish fed the diet added cod liver oil rich in n-3 HUFA as the sole lipid source had the poorest spawning performance while fish fed on the diet supplemented with soybean oil abundant with n-6 18:2-6 had the most breeders and the highest larvae production. El-sayed *et al.* (2005) also researched the reproductive performance of Nile tilapia fed on different dietary lipid sources (fish oil, soybean oil, or the mixture of the two oils) added at a level of 10% when rearing in the different salinities (0 - 17‰). These authors found Nile tilapia fed all dietary lipid sources had similar fecundity and

number of eggs per spawn at the 0 ‰ salinity but fish fed fish oil-based diets had shorter egg hatching and yolk-sac absorption time at higher salinities at 7 and 14‰ in contrast to those soybean oil fed. They went on to suggest the broodfish of Nile tilapia reared in the salty water require a source of dietary n-3 HUFA for optimum spawning performance. In another study comparing broodstock dietary lipid source, Hajizaheh *et al.* (2008) fed diets added 10% of cod liver oil, palm oil, or a blend of palm oil and cod liver oil with a commercial trout diet as a control to Nile tilapia from the starting of exogenous feeding. It turned out fish on the diet with cod liver oil as the sole source suffered high mortalities and did not spawn while fish fed diet containing palm oil or mix of palm oil with cod liver liver had higher fecundity and shorter spawning intervals than fish fed the control diet. Other than those information summarized above, no optimal dietary lipid and n-3 or n-6 fatty acids levels were reported for the broodstock tilapia. Further studies should be considered in this area to expand our current knowledge on the lipid broodstock nutrition of the second most farmed fish.

Vitamin E has been recognized for its importance in the reproduction of fish (Watanabe *et al.*, 1991; Izquierdo *et al.*, 2001). However, there were few reports available for this fat-soluble antioxidant on the reproductive performance of tilapia. Both El-Gamal *et al.* (2007) and Gammanpila *et al.* (2007) confirmed the synergistic effects of this vitamin together with vitamin C, selenium or zinc on the spawning performance and egg and larval quality. For example, El-Gamal *et al.* (2007) observed Nile tilapia fed α -tocopheryl acetate at a dietary level of 34mg/kg combined with vitamin C 120 mg/kg and/or selenium 0.2 mg/kg had larger egg diameter, higher fecundity, fertilization percentage, hatching rate and survival percentage of hatched fry compared to the fish fed on the control diet supplemented with none of them. At the same time,

Gammanpila *et al.* (2007) found the supplementation of vitamin E at 600 mg α -tocopheryl actate/kg diet (or vitamin C at 1250 mg/kg) alone in the diet did not affect the spawning efficiency, total fry production and number of eggs per breeder of Nile tilapia but the broodfish fed diets supplemented with the combination of vitamin E and vitamin C with or without zinc (added as 120 mg/kg zinc sulphate) had significantly higher numbers of eggs per spawning. An early report by Areechon and Thaitoungchin (2003) also did not observe the enhanced spawning of this species though the broodfish fed with the diet added a high level of vitamin E (600 mg/kg) slightly increased number of spawning fish. It is therefore further studies are worth of being done to examine the requirement of vitamin E for broodstock tilapia in order to improve the reproductive performance (Chapter 6). And thereafter, what extent and how the maternal-offspring transferred vitamin E (as antioxidant) can provide to the first-feeding larvae when reared on the lipid oxidative stressed diets? The question can not be answered until the related research (Chapter 7) is done since there is no any ready information available for fish.

There is by so far almost no reports of other nutrients aside from protein, lipid (including fatty acids) and vitamin E available on the tilapia reproduction. Abdelhamid *et al.* (2010) reported both commercial feed additives Therigon® (Adwia Co, El Oubor, Egypt) consisting of GnRH stimulant α -amino-p-hydroxyhydrocinnamic acid and Nuvisol Hatch P® (Newtrix Co, Lille, Belgium) making up of various B vitamins, progesterone, biotin and L-carnitine, added at the level of 1 g/kg of diet, fed to female broodstock Nile tilapia significantly increased the total number of eggs produced. Besides, there were some reports available for the influence of non-nutrition factors on the spawning activities and fry production. For example, Siddiqui and Al-Harbi

(1997) found a stocking density of 2 fish/m² in concrete tanks under a sex ratio of 1:2 and 1:3 (male : female) showed the better spawning performance of hybrid tilapia (*O. niloticus* × *O. aureas*) in terms of number of eggs/female/day and number of eggs/kg female/day. While using the same sex ratio as Siddiqui and Al-Harbi (1997), Ridha and Cruz (1999) obtained the best results with respect to fecundity, spawning synchrony and number of swim-up fries for the breeders of Nile tilapia stocked at a density of 4 fish/m² than at 8 and 12 fish/m². Environmental factors like temperature and light condition was also reported to affecting the spawning performance of tilapia (Ridha *et al.*, 1998; Ridha and Cruze, 2000; El-sayed and Kawanna, 2007). It was suggested that an ambient water temperature of 29 ± 2 was optimal for the spawning performance of the tilapia species *O. spilurus* (Ridha *et al.*, 1998) and a light intensity of 2500lux combined a light/night photoperiod of 18h/day improve the seed production and spawning synchrony of Nile tilapia broodfish reared in concrete tanks (Ridha and Cruze, 2000) but a 12h/day photoperiod regime was recommended in order to obtain maximum fecundity, egg production and spawning efficiency of Nile tilapia broodstock cultured in intensive, recirculating systems (El-sayed and Kawanna, 2007).

Chapter 3. Materials and Methods

3.1 Introduction

Based on the research objectives, three separate but related feeding trials were carried out. This chapter describes the materials and general methods used in the present dissertation.

In Experiment 1 (Chapter 4), the prime intention was to examine how the replacement of fish oil with 0, 50 and 100% of crude palm oil as the dietary lipid in broodstock diets while including linseed oil as a sole lipid source in a fourth diet for comparison purposes affect the reproduction of Nile tilapia, *Oreochromis niloticus*. Because of the high endogenous vitamin E occurrence in these diets originating from the dietary oils, the second part (Chapter 5) of this experiment studied the deposition and distribution of tocopherols and tocotrienols in various tissues of female and male broodfish, and reproduction products like eggs and yolk-sac free larva.

Experiment 2 (Chapter 6) was designed to examine if the supplementation of various levels of vitamin E in the α -tocopheryl acetate form or from a palm oil-based tocotrienols rich fraction into broodstock diets could boost the reproductive performance of brooding tilapia. It was expected that high broodstock dietary α -tocopheryl acetate could lead to the high α -tocopherol reserve in the body of larvae having originated from the parental female fed on the afore-mentioned diets.

Experiment 3 (Chapter 7) was thus conducted to investigate whether the resultant high α -tocopherol level in the body of yolk-sac free larva had positive effects

on the growth performance, body vitamin E status and fatty acids profile when given no vitamin E added diet supplemented with or without highly oxidized oil.

3.2 Proximate analysis

Proximate analysis were performed to check the composition of dietary ingredients before formulating diets as well as to verify the composition of all experimental diets after pelleted. Dietary protein sources were made up of Danish fish meal, soybean meal, gelatin and casein. These ingredients were either locally sourced or purchased from multinational cooperates. Danish fish meal was supplied by Tan Trading Sdn. Bhd. (Alor Setar, Kedah, Malaysia), soybean meal from Sun Sun Feedstuffs Co. (Butterworth, Penang, Malaysia), gelatin of bovine origin from Liang Traco Trading (George Town, Penang, Malaysia) and purified casein was bought from Sigma-Aldrich Chemical Co. (St. Loius, MO, USA).

Fish meal, casein and soybean meal were analysed for moisture, crude protein, crude lipid, fiber and nitrogen extract (NFE). NFE was not determined directly, but by the amount remaining after subtracting the total amounts of other proximate constituents from 100%, that is $\%NFE = 100 - (\%Ash + \%Protein + \%Lipid + \%Fiber)$. The procedures to perform these analyses were done according to AOAC standard methods (Association of Official Analytic Chemists, 1997).

Table 3.1 shows protein concentration of dietary protein sources used in three experiments of the present study.

Table 3.1 Protein concentration of dietary protein source (dry weight basis, %) in Experiment 1, 2 & 3¹.

Ingredients	Dry Matter	Protein	Lipid	Ash	NFE ²
<i>Experiment 1</i>					
Fish Meal	92.42 ±0.15	77.75 ±0.21	12.33 ±0.04	13.10 ±0.13	TR ³
Casein	89.51 ±0.06	91.21 ±0.13	TR	2.11 ±0.39	6.68 ±0.23
Gelatin	91.10 ±0.13	100	TR	TR	TR
SBM ⁴	93.28 ±0.05	48.86 ±0.16	3.57 ±0.08	6.87 ±0.07	35.99 ±0.10
<i>Experiment 2 & 3</i>					
Fish Meal	91.68 ±0.10	72.33 ±0.76	12.33 ±0.04	13.10 ±0.13	TR ³
Casein	92.86 ±0.10	89.01 ±0.20	TR	1.14 ±0.41	9.84 ±0.26
Gelatin	87.57 ±0.04	100	TR	TR	TR
SBM	93.69 ±0.20	48.08 ±0.44	5.17 ±0.08	5.93 ±0.01	37.93 ±0.48

¹ Results were expressed as mean ± S.D. after triplicate samples were analyzed.

² NFE, nitrogen-free extract.

³ TR = trace.

⁴ SBM = soybean meal.

3.3 Fatty acid analysis

Fatty acid analysis was done using a Shimadzu GC-2010 GC (Shimadzu, Japan). It is equipped with a flame ionization detector and Shimadzu GC solution integrator.

All fatty acid methyl ester were resolved and analyzed by this Shimadzu system. The esters were separated on a SPTM-2560 fused silica capillary column (100 m x 0.25 mm ID, 0.2µm film thickness, Supelco, Bellefonte, PA, USA). Column temperature was set at 140 °C for the first 2 min, then increased to 225 °C at 2 °C/min and held at this temperature for 5 min, then increased again to 240 °C at 2 °C/min and held for 3 min. A

SPL-2010 injector (split ratio 1:100) was used. Injector port and detector temperatures were 250 °C. Nitrogen was used as the carrier gas.

Fatty acids were identified by comparing retention time with those of known standards (Supelco 37 Component FAME Mix, PUFA No.2 and PUFA No.3; Supelco, Bellefonte, PA). The resulting peak areas were then quantified relative to the internal standard. An internal standard (23:0) was added for quantitative measurement of individual fatty acid present in the samples.

Solvents and chemicals used during the sample preparation, such as methanol, chloroform, sodium chloride, anhydrous sodium sulphate (Na_2SO_4) and heptane, all of analytical-grade, were purchased from Thermo Fisher Scientific (Hampton, NH, USA), while methanolic boron trifluoride (BF_3) were bought from Merck (Darmstadt, Germany). Oils samples could be directly processed to be methylated and transesterified with methanolic BF_3 (AOAC, 1997) before fatty acid methyl esters were extracted into a volume of heptane and finally a portion of it was analyzed on GC.

3.4 Vitamin E analysis

Vitamin E (tocopherols and tocotrienols) was analysed by high performance liquid chromatography (HPLC). Solvents and chemicals used during the sample preparation, such as butylated hydroxytoluene (BHT), sodium chloride (NaOH, ACS-graded), absolute ethanol (>99.6%), hexane (HPLC-grade), tetrahydrofuran (THF), ethyl acetate were all purchased from the Malaysian branch of Fisher Scientific Ltd., except for potassium hydroxide (KOH, ACS-graded) that was supplied by Merck (Darmstadt, Germany).

The HPLC system consisted of a LiChroCART Purospher STAR Si (5 μm) 250 \times 4.6 mm column from Merck (Darmstadt, Germany) equipped with a LiChroCART 4-4 guard column, a Rheodyne 7125i sample injector fitted with a 20- μl sample loop, a Shimadzu LC-20AT pump, and a Shimadzu RF-10Ax1 Fluorescence Detector (Shimadzu, Japan). Results were recorded and analyzed using Shimadzu LC solution software. The excitation and emission wavelengths of detector was set at 196 nm and 330nm, respectively. The isocratic mobile phase used was hexane – THF (100:4), v/v) at a flow rate of 2 ml per min.

α -tocopherol, and tocotrienols (α -, γ - and δ -) were identified and quantified with the help of an in-house reference standard complimented by Carotech (Ipoh, Malaysia). γ - tocopherol standard was purchased from Sigma-Aldrich (MO, USA).

As tocopherol and tocotrienols are light and heat sensitive, it is important to perform this bio-assay in a dim and low temperature environment. Oil samples were weighed accurately about 0.01g or experimental diets, fish tissues (muscle, liver, ovary, testes) into a 10ml centrifuge tube, followed by adding 2 ml absolute ethanol containing 0.2% BHT, 0.2 ml of 2% NaCl and 0.4 ml of 60% KOH. The tube was flushed nitrogen gas, capped and thoroughly mixed on a votex machine before saponified in a 70°C water bath for 20 minutes. After cooling on ice, the tube was added 2 ml of 2% NaCl. The tocopherols and tocotrienols were extracted with 5 ml of hexane containing ethyl acetate (v:v = 9:1). 100 μl of aliquots of the hexane layer was injected into the HPLC.