LIPID NUTRITION ON GONAD DEVELOPMENT OF MAHSEER, *Tor tambroides* BROODSTOCK

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DOCTOR OF PHILOSOPHY
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MUHAMMAD ABDUH BIN YAZED

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the School of Fisheries and Aquaculture Sciences

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ABSTRACT

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu in fulfilment of the requirements for the degree of Doctor of Philosophy

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Main Supervisor : **Professor Abol Munafi Ambok Bolong, Ph.D.**

Co-supervisor : **Noordiyana binti Mat Noordin, Ph.D.**

School : **School of Fisheries and Aquaculture Sciences**

The present research was conducted to investigate the effects of fish oil (FO) substitutions with corn oil (CO) and lipid levels on the gonad development of *Tor tambroides* broodstocks via three feeding trials. The first feeding trial was conducted to examine the effects of diets containing various FO and CO ratios on female *T. tambroides* broodstocks. Three isonitrogenous (450 g kg^{-1} crude protein) and isolipidic diets (105 g kg^{-1} crude lipid) were formulated to contain FO and CO at ratio of 1:0 , 1:1, and 0:1, respectively and was fed to the female broodstocks for five months. Based on the gonadosomatic index (GSI), hepatosomatic index (HSI), histological observations on the gonad cell, muscle fatty acids (FA) composition, and 17β-estradiol (E2) level, fish fed with diet containing FO to CO ratio of 1:1 demonstrated higher GSI and the farthest development of gonad cell stages for female *T. tambroides* with ratio of n-3 to n-6 of 0.74. To further elucidate the effects of lipid on *T.tambroides* broodstocks, the second and third feeding trials were conducted with isonitrogenous (450 g kg^{-1} crude protein) diets containing 60, 82, 105 and 128 g kg^{-1} of lipid (0, 2.5, 5.0 and 7.5% lipid inclusion respectively) with FO to CO ratio of 1:1 on female and male *T. tambroides* broodstocks respectively, for five months. Based on the GSI, HSI, gonadal histology, FA composition (muscle, liver and gonad) and levels of E2 and testosterone (T), fish fed with diet containing 82 g kg^{-1} of lipid exhibited the best results for both experiments in terms of highest GSI, higher percentage of Stage 4,5 and 6 oocytes, and the ability to express sperm

upon induction. The higher GSI also contributed in higher E2 and T levels for both feeding trials respectively. In contrast with the findings in the first feeding trial, the second and third feeding trial concludes that *T. tambroides* broodstocks preferred diets with ratio of n-3 to n-6 of 1.25 with lower lipid levels. However, arachidonic acid (ARA) levels in male were higher compared to female in all tissue samples which shows different ARA utilization between sexes. These results provide evidence that gonad development of *T.tambroides* broodstocks can be positively affected by diet containing ratio of FO to CO of 1:1 with level of lipid at 82 g kg^{-1}.

ABSTRAK

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

NUTRISI LIPID DALAM PERTUMBUHAN GONAD INDUK MAHSEER, *Tor tambroides*

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Kajian ini dilakukan bertujuan untuk menyiasat kesan penggantian minyak ikan (FO) dengan minyak jagung (CO) dan kandungan lipid terhadap perkembangan gonad induk *Tor tambroides* menggunakan tiga ujian pemakanan. Ujian pemakanan yang pertama dilakukan untuk menguji kesan pemberian diet makanan yang mengandungi nisbah FO terhadap CO yang berbeza terhadap induk betina *T. tambroides*. Tiga diet makanan yang mengandungi kandungan protin dan lipid yang sama (450 g kg^{-1} dan 105 g kg^{-1}) telah di formulasi untuk mengandungi nisbah FO terhadap CO sebanyak 1:0, 1:1 dan 0:1. Makanan ini telah diberikan kepada induk selama lima bulan. Berdasarkan kepada indeks gonadosomatik (GSI) dan indeks hepatosomatic (HSI), pemerhatian histologi atas sel gonad, komposisi asid lemak (FA) dalam isi ikan, dan paras 17β-estradiol (E2), ikan yang diberi makanan mengandungi nisbah FO terhadap CO sebanyak 1:1 menunjukkan nilai GSI yang lebih tinggi dan perkembangan sel gonad terhadapan dengan nisbah n-3 terhadap n-6 sebanyak 0.75. Untuk lebih menjelaskan kesan lipid dalam makanan terhadap kematangan induk *T. tambroides*, ujian pemakan kedua dan ketiga dilakukan menggunakan diet makanan yang mengandungi kandungan protein yang sama (450 g kg^{-1}) yang mengandungi 60, 82, 105 dan 128 g kg^{-1} lipid (0, 2.5, 5.0 dan 7.5% kadar kemasukan lipid) dengan nisbah FO kepada CO sebanyak 1:1 terhadap induk betina dan jantan *T. tambroides* selama lima bulan. Berdasarkan kepada GSI, HSI, pemerhatian histologi terhadap sel gonad, komposisi FA (dalam isi, hati dan gonad ikan), dan kepekatan E2 dan

testosteron (T), ikan yang diberi makanan mengandungi 82 g kg^{-1} lipid untuk kedua-dua eksperimen memberikan nilai GSI tertinggi, jumlah telur pada Peringkat 4,5 dan 6 yang lebih tinggi dan kebolehan mengeluarkan sperma apabila diransang. Nilai GSI yang tinggi juga menyumbang kepada paras E2 dan T yang lebih tinggi bagi kedua-dua ujian pemakanan. Berbanding ujian pemakanan pertama, hasil daripada ujian pemakanan kedua dan ketiga menunjukkan induk *T. tambroides* memilih diet yang mengandungi nisbah n-3 terhadap n-6 sebanyak 1.25 dan paras lipid yang lebih rendah. Walaubagaimanapun, nilai asid arachidonic (ARA) adalah lebih tinggi di dalam ikan jantan berbanding ikan betina dalam semua sampel dan ini menunjukkan perbezaan dari segi penggunaan ARA antara jantina. Berdasarkan keputusan ini, dapat disimpulkan bahawa makan yang mengandungi sumber FO dan CO pada nisbah 1:1 dan tahap lipid sebanyak 82 g kg^{-1} dalam makanan dapat memberikan kesan positif terhadap perkembangan gonad *T. tambroides*.

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APPROVAL

I certify that an Examination Committee has met on 22nd January 2019 to conduct the final examination of Muhammad Abduh bin Yazed, on his Doctor of Philosophy thesis entitled **"Lipid Nutrition on Gonad Development of Mahseer, *Tor tambroides* Broodstocks"** in accordance with the regulations approved by the Senate of Universiti Malaysia Terengganu. The Committee recommends that the candidate be awarded the relevant degree. The members of the Examination Committee are as follows:

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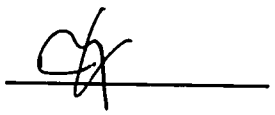
This thesis has been accepted by the Senate of Universiti Malaysia Terengganu as fulfilment of the requirements for the degree of Doctor of Philosophy.



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DECLARATION

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

MUHAMMAD ABDUH BIN YAZED

Date: 29/12/2019

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percent
µm	Micrometer
µg	Microgram
11 KT	11 keto testosterone
AKUATROP	Institute of Tropical Aquaculture
ALA	Alpha linolenic acid
ANOVA	Analysis of variance
ARA	Arachidonic acid
C	Carbon
cm	Centimeter
CO	Corn oil
CPG	Carp pituitary gland
CPO	Crude palm oil
DHA	Docosahexanoic acid
DOF	Department of Fisheries
E2	17-ß estradiol
EFA	Essential fatty acids
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EPA	Eicosapentanoic acid
F1	Filial one generation
FA	Fatty acid
FAMEs	Fatty acids methyl esther
FAO	Food and Agriculture Organization
FAS	Fatty acid synthase
FCE	Fish with confirm eggs
FO	Fish oil
FOM	Final oocyte maturation
FSH	Follicle stimulating hormone
g	Gram
g kg^{-1}	Gram per kilogram
GSI	Gonadosomatic index
Gth	Gonadotropin hormone
h	Hour
HPG	Hypothalamus-pituitary-gonadal
HSI	Hepatosoatic index
HUFA	Highly unsaturated fatty acid
IUPAC	International Union of Pure and Applied Chemistry
kg^{-1}	Per kilogram
LA	Linoleic acid
LCPUFA	Long chained polyunsaturated fatty acid
LH	Luteinizing hormone
m/sec	Meter per second
mg/l	Milligram per liter
ml	mililiter
mm	milimeter
mS/cm	MicroSiemens per centimeter

MUFA	Monounsaturated fatty acids
n-3	Omega 3
n6	Omega 6
NFE	Nitrogen-free extract
ng g^{-1}	Nanogram per gram
OA	Oleic acid
PUFA	Polyunsaturated fatty acid
RAS	Recirculating aquaculture system
RBDPO	Refined, bleached, deodorised palm oil
RBDPOo	Refined, bleached, deodorised palm olein
RBDPOs	Refined, bleached, deodorised palm stearin
RMK	Rancangan Malaysia ke
S	Stage
SFA	Saturated fatty acid
SC	Spermatocyte
SG	Spermatogonia
SE	Standard error
SEM	Standard error of the mean
sGNRH	Salmon gonadotropic releasing hormone
sp.	Species
ST	Spermatid
SZ	Spermatozoa
T	Testosterone
UMT	Universiti Malaysia Terengganu

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Malaysian Mahseer, *Tor tambroides* is known as one of the highest valued fresh water fish in Malaysia (Asaduzzaman et al., 2016), where the price started rising from RM500 kg^{-1} (Ambak *et al.*, 2007) to RM800-RM1,200 (UPM, 2013) in recent years. Locally known as "Kelah" in Peninsular Malaysia (Mohsin and Ambak, 1983) this species is considered as one of the high valued game fish and ornamental fish (Ng, 2004). Kelah production in fresh water pond had increased from 18.06 tonnes in 2014 to 31.14 tonnes in 2015 with an increase of value from RM672,410 to RM1,645,820 respectively. Its production and value showed an increase of 45.01% and 59.14% respectively (Department of Fisheries, 2015).

The development of Kelah culture is the main focus for the Department of Fisheries Malaysia (DOF) in the Eleventh Malaysia Plan (RMK-11). The Ministry of Agriculture & Agro-Based Industry Malaysia has launched a programme: "Pembangunan Sumber Baru Akuakultur Kelah" with the objectives to produced and develops Kelah broodstock as a high potential aquaculture species and also to replenish and increase the population in its wild habitat. The program had started in 2016 with a total funding of RM 194,000.00 (Deparment of Fisheries, 2016).

In Malaysia, efforts are being done to increase the production of Kelah hatchling and fry by the Malaysian Government hatcheries. In 2014, the production of Kelah fry is 63,286 tails, where 15,455 tails were released into public water bodies to replenish the wild population. This values increases in 2015, where 1,400 hatchling and 220,167 Kelah fry were produced and 37,750 fry were released to the wild (Department of Fisheries, 2015).

1.2 Statement of Problems

High market demands of *T. tambroides* at various stages of their life-span has seriously threat their wild-stock population and is now considered as an endangered species in Malaysia (Jalal et al., 2005; Ng, 2004). Besides overfishing (Haryono, 2006), the sharp decline of wild *T. tambroides* were contributed by agricultural and industrial development including deforestation and poor irrigation practices that lead to water pollution (Ingram et al., 2005; Esa et al., 2006; Ismail et al., 2011). Without scientific knowledge on the biology and management of *T. tambroides*, it is feared that they will face extinction. Therefore, it is crucial for scientific research on *T. tambroides* be carried out to replenish the wild-stock of *T. tambroides* and at the same time satisfy high market demands of this species with production from aquaculture industry.

Research on breeding program of *T. tambroides* has long been carried out since 2001 by government agency such as Fisheries Department and private industries (De Silva et al., 2004b). The fish has been successfully reproduced through natural and induce spawning methods under captive conditions (Ingram et al., 2005) and they are capable to spawn several times per year in small batches (Ingram *et al.*, 2007a). However, the aquaculture of *T. tambroides* is still yet to develop due to difficulties in obtaining high quality mature broodstocks, inconsistent number of eggs produces and production of viable larvae.

Fish raised in captivity often experience one or more forms of reproductive dysfunction that can be attributed to some incongruence between their wild and captive environment (Zohar and Mylonas, 2001; Mylonas *et al.*, 2010). In recent years, the use of exogenous hormones has been touted as a method of overcoming reproductive dysfunction in captive populations. Hormonal injections are widely used in induced breeding of fish broodstock in captivity for cyprinid fish (Glasser *et al.*, 2004; Mousavi and Yousefian, 2012; Podhorec *et al.*, 2016; Xu *et al.*, 2016). It was also used in the induction of mahseer like Golden Mahseer, *T. putitora* (Ayub *et al.*, 2006), Deccan mahseer, *T. Khudree* (Sangma & Basavaraja, 2010) and Malaysian mahseer, *T. tambroides* and *T. douronensis* (Ingram *et al.*, 2007b; Azuadi

et al., 2013a, 2013b). Besides its effect on induce breeding, the effects of hormonal injection on the reproduction hormones were also investigated. In these study, gonadal steroid profile [testosterone (T) and 17β estradiol (E2)] can be used to depict cycles of activity of pituitary gonadotrophs that are directly related to development of gonads and reproduction of fish (Ismail *et al.*, 2011).

An improvement in broodstocks nutrition and feeding has been shown to greatly improve the eggs and sperm quality of fish, resulting in strong and healthy offspring (Izquierdo *et al.*, 2001; Ling *et al.*, 2006; and Muchlisin *et al.*, 2006). In a study conducted for three years, where the breeding performance of *T. tambroides* was monitored, it was found that the spawning of *T. tambroides* was significantly affected by diets (Ingram *et al.*, 2007c).

Among nutritional components that are important for this cyprinids group, lipid has been highlighted as the crucial component of their diet, as a source of digestible energy and fatty acids (Muchlisin, 2005 and Misieng *et al.*, 2011). In female fish broodstocks, the lipid reserves in fish eggs are important for larval development, in term of metabolism and as a vital component in membrane fluidity (Sargent, 1995; Bell *et al.*, 1997; Henrotte *et al.*, 2010). These lipid requirements by the oocytes, followed by lipid accumulation in yolk (during vitellogenesis) and utilization of lipid by developing embryos and larvae are essential processes in reproduction and larval development (Brooks *et al.*, 1997).

Meanwhile, in male broodstocks, fatty acids are important as it has been demonstrated that it influenced sperm quality parameters such as sperm count (Asturiano *et al.*, 2001 and Nandi *et al.*, 2007), sperm motility (Vassalo-Agius *et al.*, 2001), sperm volume (Duangjai *et al.*, 2017). It has also been observed that certain fatty acids delayed maturation in male broodstocks (Wassef *et al.*, 2012 and Bogevik *et al.*, 2014). Although several studies had been conducted on lipid requirements of *T. tambroides* juveniles (Ng and Andin 2011; Ramezani *et al.*, 2012a), there is still no research conducted in determining the suitable lipid dietary levels for *Tor sp.* broodstocks.

Due to over dependent toward fish oil, many plant products (essentially grain sources), by-products, microbial production of terrestrial animal and microalgae are currently being tested to identify possible alternative lipid sources to replace fish oil (Glencross, 2009). The dietary substitution of part of fish oil or totally by various vegetable oils has been extensively studied in various species. The inclusion of dietary vegetable oils such as corn, soybean, flaxseed, palm, and rapeseed oil at the expense of fish oils which is rich in highly unsaturated fatty acids such as eicosapentanoic acid (EPA) and docosahexanoic acid (DHA), is reflected in tissue fatty acid composition (Watanabe *et al.*, 1985, Maina *et al.*, 2003, Bell et al., 2013, Izquierdo et al., 2015 and Ghaedi et al., 2016). Corn oil has high level of linoleic acid (LA) with less or none highly unsaturated fatty acids (HUFA) in it (Watanabe *et al.*, 1991 and Maina *et al.*, 2003). It had been used as substitute of fish oil in several fish broodstock such as red sea bream, *Pagrus major* (Watanabe *et al.*, 1985), sea bass, *Dicentrarchus labrax* L. (Navas *et al.*, 1997), Nile tilapia, *Orechromis niloticus* (Santiago and Reyes, 1993), white bass, *Morone chrysops* (Lane and Kohler, 2006) and Japanese eel *Anguilla japonica* (Furuita *et al.*, 2007). While most of the study found deteriorating effects of more than 50% of fish oil substitution with corn oil, Santiago and Reyes (1993) found that *O. niloticus* fed with fish oil to corn oil ratio of 1:1 gives better reproductive performances. Meanwhile, buoyant egg and fertilization percentages were higher in fish fed with corn oil as primary lipid sources for *A. japonica* (Furuita *et al.*, 2007).

Some studies show that lipid also affected hormone concentration. Navas *et al.* (1998) shows that in seabass, *Dicentrarchus labrax,* fish fed with higher lipid and n-3 PUFA had higher E2 and luteinizing hormone (LH) levels compared to control fish. Da Silva *et al.* (2016), founds that Europen eel, *Anguila anguila*, fed with higher PUFA, shows better usage of hormone manipulation in inducing gonad development. Arachodonic acid (ARA) and EPA are precursor of eicosanoids, which later produced series II and III prostaglandins respectively. Prostaglandin produced from EPA is biologically less active than those produced by ARA (Sargent *et al.*, 2002). Eicosanoids modulates the steroid synthesis and regulation, and also spermiation during sexual maturation in males (Asturiano *et al.*, 2000; Norambuena *et al.*, 2013a; Norberg *et al.*, 2017). These studies suggested that lipid affected the hormonal level of fish broodstocks. However, information is still lacking in this area.

It is important for this aspect to be studied further, so that its implication on breeding fish in captivity can be understood.

1.3 Objectives of the Study

The aquaculture of *Tor tambroides* is restricted due to limited information that affects production such as maturation and nutritional requirement. Through nutritional approach, it is hoped that the nutritional requirements data gathered for *T. tambroides* will enable further development for mass production of healthy and viable *T. tambroides* fry for long-term culture. Thus, the objectives of this research are as follow:

a. To study the effects of fish oil substitution with corn oil on gonad development of female *T. tambroides* broodstocks,
b. To determine the effects of varying lipid levels on gonad development of female *T. tambroides* broodstocks,
c. To determine the effects of varying lipid levels on gonad development of male *T. tambroides* broodstocks.

CHAPTER 2

LITERATURE REVIEW

2.1 Malaysian Mahseer, *Tor tambroides*

The *Tor* fishes are a group of cyprinids commonly referred to as Mahseer. Family Cyprinidae are among of the most popular food and ornamental fishes cultured in Asia (Ng, 2004). Mahseer or locally known as Kelah, Empurau and Belian in the Borneo, are renowned as the king of the mountain river as well as a sport fish currently fetching high market price as well as in other South-East Asian countries (Ingram et al., 2005; Esa et al., 2008). Nowadays, over exploitation of the natural stocks due to high demand and the environmental pollution effected in declining populations of Mahseer in the wild (Ogale, 2002).

2.1.1 Taxonomy

According to Desai (2003), there are 10 species and three subspecies of *Tor* (Table 2.1) documented worldwide. Four of them can be found here in Malaysia namely the *T. tambroides* (Bleeker), *T. soro* (Cuvier and Valenciennes), *T. tambra* (Cuvier and Valenciennes), and *T. douronensis* (Valenciennes) (Mohsin and Ambak, 1992; Ambak *et al.*, 2007). Among these, *T. tambroides* is the most popular and mostly studied species in the Tor sp. in Malaysia.

Table 2.1: Valid species and sub species of Mahseer in the world.

Scientific Name
Species
Tor putitora (Hamilton)
Tor tor (Hamilton)
Tor mosal (Sykes)
Tor khudree (Sykes)
Tor mussullah (Sykes)
Tor progenensis (McClelland)
Tor douronensis (Valenciennes)*
Tor sinensis (Wu)
Tor tambroides (Bleeker)*
Tor zhobensis (Mirza)
Subspecies
Tor khudree longispinis (Guther).
Tor khudree malabaricus (Jerdon)
Tor mosal mahamadicus (David)

* Tor species found in Malaysia

According to Mohsin and Ambak (1992), the taxonomy for *T. tambroides* is as below:

Kingdom:	Animalia
Phylum:	Pisces
Class:	Teleostomii
Order:	Cypriniformes
Family:	Cyprinidae
Genus:	*Tor*
Species:	*Tor tambroides, Bleeker 1854*

Tor tambroides sub-terminal mouth has a protruding and sectorial feature (Ambak *et al.*, 2007). Long median lobe was present at the lower lip reaching a line connecting corners of mouth and tubercles were absent on the snout and cheek (Kottelat, 1998; Desai, 2003). It has powerful rows of pharyngeal teeth, with large scales. *Tor tambroides* can morphologically be identified based on the presence of strong osseous dorsal rays, smooth, and its stiff portion as long as head (Siraj *et al.*, 2007). There are two series of scale between the lateral line and the root of the ventral fin. Lips of this species very thick, both with well-developed lobes; mouth

type is inferior. The body is compressed, with the profile of the back curves. Snouts are moderate length and its caudal fin deeply forked (Ambak *et al.*, 2007).

The lack of information on *T. tambroides* sexual dimorphism has also been a major bottleneck for the development of its broodstocks in captivity. There are no clear differences between both male and female (Figure 2.1). Breeders found it difficult to identify the sex of this fish to be paired for breeding which make sex identification critical for its breeding success. However, there are some research been conducted on Himalayan mahseer, *Tor tor*. Islam (2005), stated that *T. tor* do shows sexual dimorphism though there is no further explanations on that aspect. Tubercles can only be found in male Mahseer (Pathani, 1978), however it only occurs during spawning seasons (Desai, 1973). Sex differentiation can only be determined if the female fish is fully ripe where when slight pressure were applied to the abdomen, it will release eggs (Desai, 2003).

Figure 2.1: *Tor tambroides* broodstock. ((a): Female; (b): Male)

2.1.2 Distribution of *Tor tambroides*

Tor tambroides are found around Java, Borneo and Sumatra in recent years this species has been found in Thailand (Ambak *et al.*, 2007). In Malaysia, wild populations of *T. tambroides* can be found in rural such as Tembat River, Terengganu River, and Kiang River in Terengganu (Sopha, 1999), Royal Belum Forest, Perak, Pahang National Park, Pahang (Ismail *et al.*, 2011) and Andang River in Sarawak (Razak *et al.*, 2017). The habitats of *Tor* species ranged from mountain streams and rivers to fast flowing rivers in the plains, often preferring clear, swift-flowing waters with stony, pebbly or rocky bottoms (Desai, 2003). According to Sopha (1999), *T. tambroides* is a territorial species. Populations of adults mostly dominates the deeper parts or pools in the upstream reaches of large streams whereas the juvenile of this species are distributed in all parts of streams and lakes.

2.1.3 Natural Spawning and Breeding of *Tor tambroides*

In the wild, *T. tambroides* is a slow-growing and can take up to a year just to reach 500 to 600 g (Ng, 2004) and pilot trials in ponds using captive-bred fish showed that an average weight of 790 g (range 390 g to 1200 g) could be reached by three years of age (Ingram *et al.*, 2007a,c), although some reported a growth of only 200 g in the first year. Since fish of one to two kg are usually the ideal serving sizes in restaurants, the *T. tambroides* may require a prolonged grow-out period. Reproduction maturity is based on the size and weight of the mahseer, where mature males and females usually weight 2.5 kg and 3.9 kg, and measuring 60 cm and 70 cm respectively (total length) (Kunlapapuk and Kulabtong, 2011). Ambak *et al.* (2007) stated that *T. tambroides* matures at the age of 3 years, at 2.5 kg (female), while male reaches maturity in 20 months, weighing about 0.75 kg. A research in Sarawak shows the smallest mature *T. tambroides* female was 2.5 kg (De Silva *et al.*, 2004b; Ingram *et al.*, 2005) while later study suggested 1.25 kg (Ingram *et al.*, 2007b). Meanwhile, research carried out by Ismail *et al.* (2011) shows that female as small as 0.95 ± 0.36 kg can exert eggs by gentle pressure in the abdomen. Currently

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

Shrestha, (1997) has provided details of the natural life cycle of golden mahseer, *T. putitora* (Figure 2.2). It shows that the breeding starts from August, followed with development into juveniles for nine months. This species then need one to three years until maturity. According to Tan (1980), breeding season for *T. tambroides* is between the month of November and January, while most recently Ambak *et al.* (2007) stated it started from July to September. During these months, the river environment and water quality are in their optimal state for breeding purposes (Kunlapapuk and Kulabtong, 2011) (Table 2.2). Breeding takes place in the clearer upper reaches of the river stones and sandy beds.

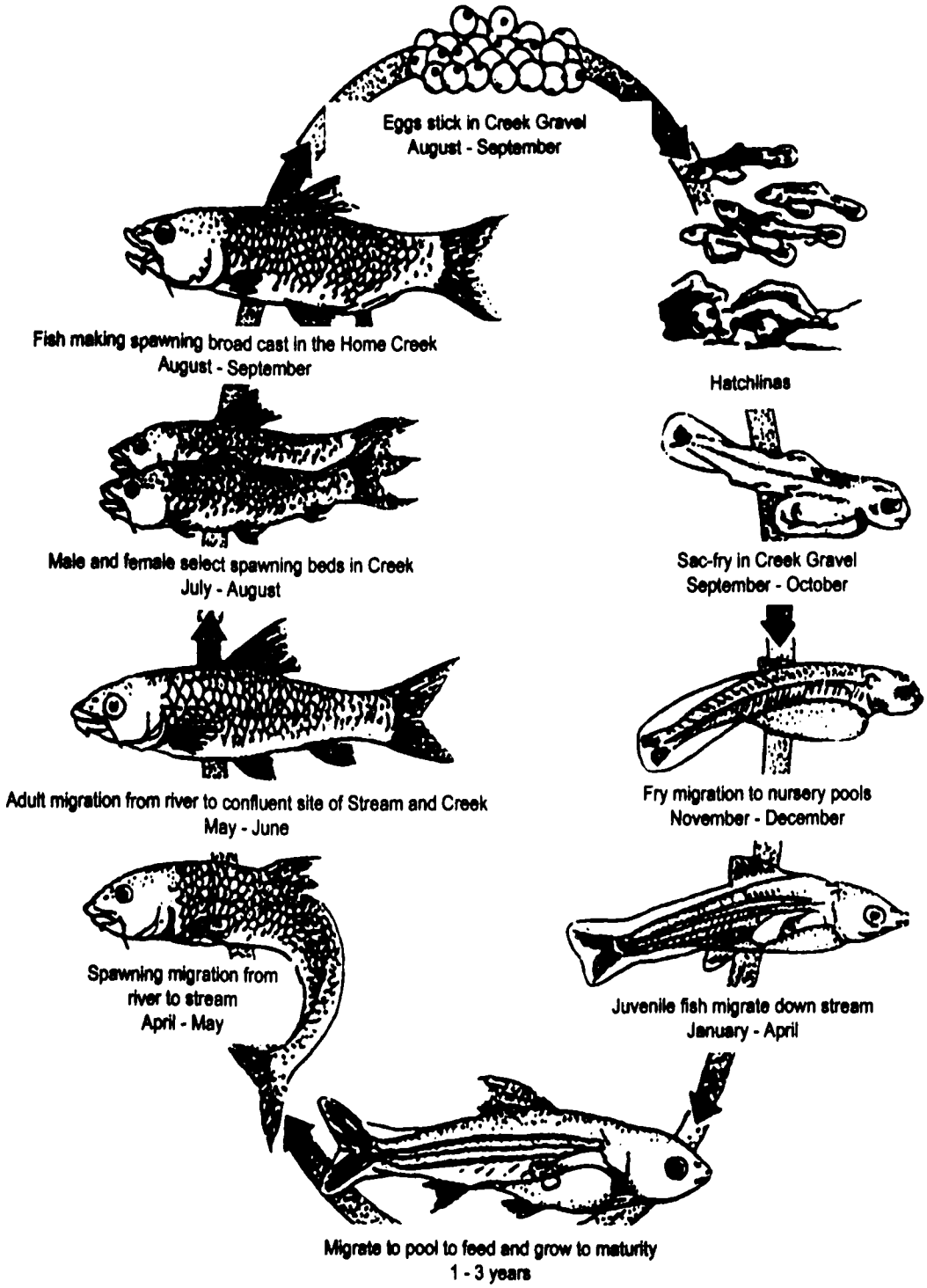


Figure 2.2 Life cycle of Golden Mahseer, *Tor putiotora* in (Shrestha, 1997).

Table 2.2: Optimal water quality requirement of *T. tambroides* during its breeding season (Kunlapapuk and Kulabtong, 2011)

Water quality parameters	Average range
Dissolved oxygen (mg/L)	6.30-8.34
Conductivity (2mS/cm)	0.051-0.118
Velocity (m/sec)	0.27-0.86
Water color	Clear to turbid
Temperature (°C)	25.26-27.30
pH	6.81-7.09

2.1.4 Dietary Requirement of *Tor tambroides*

Many studies on the dietary requirements of *T. tambroides* are mainly focused on larvae and juvenile stages. Ng *et al.*, (2008) and Misieng *et al.*, (2011) both suggested 48% and 40% of dietary protein is recommended for maximum growth of *T. tambroides* juveniles, respectively. In previous study on *T. putitora*, the optimum dietary protein level for maximum growth lies between 45% and 50% (Islam & Tanaka, 2004).

For maximum growth in *T. tambroides* juveniles, it was concluded that 5% of lipid was recommended (Ng and Andin, 2011, Ramezani-Fard *et al.*, 2012a). They also found that *T. tambroides* juveniles preferred lipid sources with high n-6 PUFA, high monounsaturated fatty acids and low n-3 PUFA content. Recently, Asaduzzaman *et al.* (2016) documented that artificial formulated feed (crude protein 50% and crude lipid 15%) gave better results than feeding live feeds to *T. tambroides* larvae. Meanwhile Chowdhury *et al.*, (2016), found out that incorporation of phototrophic purple bacteria with artificial feed (crude protein 42% and crude lipid 8%) helps improve growth performance of *T. tambroides* fingerlings. Crude palm oil (CPO) was the most cost effective palm oil type and giving higher PUFA ratio in *T. tambroides* muscle compared to refined, bleached, deodorised palm oil (RBDPO), RBD palm olein (RBDPOo) and RBD palm stearin (RBDPOs) (Bami *et al.*, 2017a).

Although this fish is also known to consume on ripe fruits falling onto the water surface from several species of trees near the river banks, study conducted by

Bami, *et al.*, (2017b) shows that canarium fruit oil (*Canarium odontophyllum*) was not suitable to be considered as dietary lipid source, even as low as 1.25% as feed basis for *T. tambroides* juveniles. However, crude illipe oil (*Shorea macrophylla*) shows no negatives impact on growth performance of *T. tambroides* juveniles although its inclusion resulting in slightly inferior retention of dietary lipid and muscle n-3 and n-6 PUFA in treated fish (Kamarudin *et al.*, 2018).

De Silva *et al.*, (2004b) reported that sub-optimal diet and husbandry nutrition with insufficient amounts of highly unsaturated fatty acids, in particular, eicosapentanoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (ARA) in the diets leads to the failure of artificial induction. They found out that diet with proximate composition of protein and lipid at 45 and 16% respectively improved the breeding performance of *T. tambroides* and *T. douronensis* in captivity. Research done by Azuadi *et al.* (2013a) used two types of pellet with different composition of protein and lipid (62.6% protein with 17.6% lipid and 16% protein with 4% lipid, respectively), while others used diet containing 40% of protein for maintaining *T. tambroides* broodstocks in captivity (Ismail *et al.*, 2011). It is anticipated that with proper nutrition, the commercial farming of this species can be a viable enterprise. As until now, there is currently no published information on the nutrient requirements of *T. tambroides* broodstocks. The summary of studies conducted on nutritional requirement of *Tor sp.* is shown in Table 2.3.

Table 2.3: Studies conducted on nutritional requirement of *Tor sp.*

Species	Study	Results	Reference
Tor khudree (juvenile)	Different levels of sardine oil supplementation	6% of sardine oil supplementation had superior feeding efficiency	Bazaz and Keshavananth, 1993
Tor putitora (juvenile)	Different protein level	Optimum level between 45-50%	Islam and Tanaka, 2004
Tor tambroides (juvenile)	Different protein level	Optimum level of 48%	Ng *et al.*, 2008
Tor tambroides (juvenile)	Different level of lipid and n3 to n6 ratios	Optimum level of 5% and n3 to n6 ratio of 0.3	Ng and Andin, 2011
Tor tambroides (juvenile)	Different protein level	Optimum level of 40%	Misieng *et al.*, 2011
Tor tambroides (juvenile)	Different lipid level	Optimum level of 5%	Ramezani-Fard *et al.*, 2012a
Tor tambroides (juvenile)	Fish meal replacement with poultry offal meal	Up to 100% replacement of poultry offal meal	Ismail *et al.*, 2012
Tor tambroides (juvenile)	Fish oil replacement with vegetable oil	50-100% replacement with vegetable oil	Kamarudin *et al.*, 2012
Tor tambroides (juvenile)	Different carbohydrate level	Optimum level of 20-25%	Ishak *et al.*, 2016
Tor tambroides (juvenile)	Different type of palm oil	Crude palm oil gives higher PUFA ratio and more cost effective	Bami *et al.*, 2017a
Tor tambroides (juvenile)	Different level of Canarium fruit oil	Better results in control diet (0% canarium oil)	Bami *et al.*, 2017b
Tor putitora (juvenile)	Effects of Nano selenium and vitamin C	Better growth with supplementation at Vit C 300mg kg^{-1}+ Nano Se 0.68 mg kg^{-1}	Khan *et al.*, 2017
Tor tambroides (juvenile)	Different level of Illipe fruit oil	Added Illipe oil didn't give negative impact on growth and whole body proximate.	Kamarudin *et al.*, 2018

2.2 Broodstock Nutrition on Fish Reproduction

The quality of fish gametes can be highly variable due to a significant number of external factors or broodstock management practices in aquaculture practice. This is because all of the diet is generally provided by artificial feed. Broodstock may need to be reared for many years and if their diet is not optimal then levels of some essential nutrients may become depleted over time (Izquierdo *et al.*, 2001). Hence, research focusing on the improvement of broodstock management and its effects on egg and larval quality is considered a priority from both the scientific and technical perspectives (Mylonas and Robles, 2014). The nutritional status of broodstock has been reported to have effect on the quality of reproduction of farmed fish including the chemical composition of eggs, fertilization, hatching rates and larval survival rates (Bell *et al.*, 1997; Izquierdo *et al.*, 2001). Several studies have demonstrated that reproductive performance and egg quality are influenced by nutrients like protein, lipid, minerals and vitamins in fish such as gilthead seabream, *Sparus aurata* (Fernandez- Palacios *et al.*, 1995), sea bass, *Dicentrarchus labrax* (Cerda' *et al.*, 1994), tilapia, *Oreochromis niloticus* (Santiago and Reyes, 1993; Siddiqui *et al.*, 1998) and common carp, *Cyprinus carpio* (Manissery *et al.*, 2001).

Reproductive performance in fish such as, egg size, chemical composition of egg and also embryonic developments is highly affected by the nutritional status of fish (Shepherd and Bromage, 1988; Carillo *et al.*, 2000). Various reports have emphasized the importance of broodstock nutrition in enhancing reproductive performance of fish in captivity (Cerda *et al.*, 1994; Coward and Bromage, 2000). Among dietary nutrients, lipids have been reported to influence reproductive performance in both freshwater and marine fish broodstock (El-Sayed *et al.*, 2005; Izquierdo *et al.*, 2001). These studies mainly showed how dietary lipid affects the broodstock breeding performances.

2.2.1 Lipid and Fatty Acid

Lipids are a group of compounds generally insoluble in water which are widely involved in the functioning of all organisms by playing an integral part in the structure of bio membranes, energy storage and metabolic control (Sargent *et al.*, 2002). Animal lipids, including fish lipids, can be divided into two broad classes; neutral and polar lipids. Neutral lipids, that are completely soluble in non-polar solvents, are composed principally of triacylglycerols (TAG). Wax esters constitute another class of neutral lipid, consisting of a single molecule of fatty acid esterified to a single molecule of fatty alcohol, which can be present in considerable amounts in body tissues and the egg of some species (Tocher, 2003). Polar lipids are composed principally of phospholipids. The most important simple lipid (e.g. a lipid that does not contain fatty acids) in all animals, including fish, is cholesterol. This is the most common form of the tetracyclic hydrocarbon compounds, collectively called sterols. It may exist in an unesterified form as an essential component of cell membranes, or in a neutral lipid storage form esterified to a fatty acid (Tocher, 2003). TAG are a major class of the neutral lipids and consist of three fatty acids esterified in the *sn*-1, *sn*-2 and *sn*-3 positions of L-glycerol, usually saturated or monounsaturated FA located in the *sn*-1 and *sn*-3 and PUFA in *sn*-2 (Sargent *et al.*, 2002). TAG is the main form in which lipids are stored in fish tissues. Specifically, in most fish species, the primary storage sites are the mesenteric adipose tissue, the adipose tissue within the white muscle and to a smaller extent the liver, although the latter can be the major lipid storage site for many marine fish species (Nanton *et al.*, 2007).

Fatty acids are a family of lipids, which are generally aliphatic monocarboxylic acids that have the ability to be liberated via hydrolysis from naturally occurring fats and oils. Fatty acids can be broadly classed into three structurally and functionally diverse groups based on the presence or absence of carbon to carbon double bonds within the hydrocarbon chain of the molecule. A fatty acid is referred to as saturated fatty acids (SFA) when it contains no carbon to carbon double bonds. Conversely, monounsaturated fatty acids (MUFA) contain one double bond whilst fatty acids with two or more double bonds are termed polyunsaturated

fatty acids (PUFA) (Sargent *et al.*, 2002; Tocher, 2003). Some authors are also referring fatty acids with carbon chain length more than 20 as highly unsaturated fatty acids (Furuita *et al.*, 2002; Tocher *et al.*, 2003; Ling *et al.*, 2006) or long-chained PUFA (Sargent *et al.*, 1999; Tocher, 2015).

2.2.2 Fatty Acid Biosynthesis, Elongation and Desaturation

Fish are able to endogenously synthesize SFA 16:0 and 18:0 and desaturate them to 16:1-n7 and 18:1 –n9, by removal of 2 carbon acetyl units (Sargent *et al.*, 1993). Like other vertebrates, fish have the absolute requirement for –n3 and –n6 PUFAs in their diets such as linoleic acid (LA) and linolenic acid (ALA). They have the ability of desaturation and chain elongation but are incapable of de novo synthesis of C20 and C22 PUFAs. Sargent *et al.* (2002) demonstrated that the cytosolic enzyme system "fatty acid synthase (FAS) multienzyme complex" in fish is responsible for the de novo biosynthesis of fatty acids of up to C16 and, its synthesis occurs mainly in the liver.

Fatty acid desaturation is an aerobic reaction catalyzed by terminal oxygenase ("desaturase"), introducing a double bond (or unsaturation) into fatty acyl chains, whereas fatty acid elongation is a reaction that occurs in four steps, each catalyzed by a specific enzyme ("elongase") and as their name indicates, elongate a preexisting fatty acid chain by two carbon atoms (Tocher, 2003). DHA rather than EPA is the main end product of desaturation and elongation of ALA, whereas ARA is the end product of desaturation and elongation of LA. The degree of HUFA synthesis from C18 PUFA is dependent on enzymatic activities from desaturases and elongases (Bell & Tocher, 2009).

Desaturation and elongation of C20 and C22 metabolites to their end products ARA, EPA and DHA depend on two crucial enzymes (Δ5 and Δ6). These fatty acids have essential physiological roles. ARA and EPA are precursors of eicosanoids, biologically active compounds that regulate and modulate several

physiological processes. DHA is an essential component of cell membrane lipids. The synthesis of long chain polyunsaturated fatty acids (LCPUFA) occurs in the microsomal fraction of the liver except for the chain shortening from 24:6-n3 to 22:6-n3 and from 24:5-n6 to 22:5-n6, which occurs in the peroxisomes by β-oxidation (Figure 2.3) (Castro *et al.*, 2016)

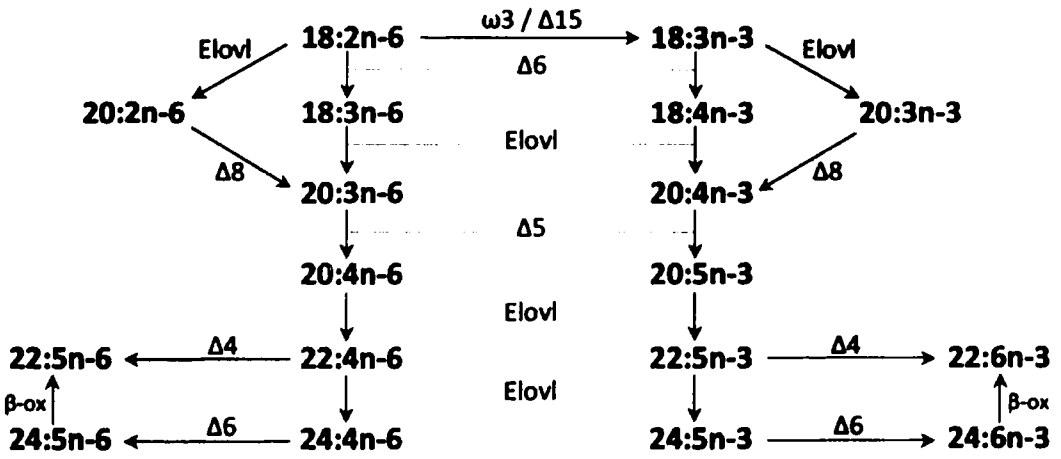


Figure 2.3: Potential pathways in fish for the biosynthesis of long-chain polyunsaturated fatty acids from the C18 precursors, 18:2n-6 and 18:3n-3. (Castro *et al.*, 2016).

Recently, an alternative more direct way for DHA biosynthesis has been shown by the presence of fatty acyl desaturase with Δ4 activity, demonstrating that an alternative pathway via direct Δ4-desaturation of 22:5-n3 was possible for the production of DHA from EPA in two freshwater fish, Pike silverside, *Chirostoma estor* (Fonseca-Madrigal *et al.*, 2014) and striped snakehead, *Channa striata* (Kuah *et al.*, 2015).

2.2.3 Importance of Lipids in Reproduction

Lipids are organic constituents of the fish body that play an important role as source of energy and essential fatty acids (EFA). The requirement of EFA as membrane components is to maintain both structure and function of the cell membrane (Sargent *et al*, 1995; Bell *et al.*, 1997; Sargent *et al.*, 2002). The major lipid storage sites of fish are liver, mesenteric fat, and muscles. Diets high in lipid content lead to fat deposit in the body cavity and perivisceral organs (e.g. liver) in lean fishes while fatty fishes deposit in muscles. Therefore, the liver of lean fish is commonly fattier than fatty fishes. In spring- spawning fishes such as pikeperch, gonads mature during winter when food supplies are limited and feeding rate is reduced. As a result of that, the nutrients for gonad maturation have to be drawn from other organs such as muscles, liver and visceral fats. The lipid reserve in fish eggs has been identified to play major role in larval development, both as substrate for metabolism and as structural components in membrane biogenesis (Sargent, 1995).

The n-3 highly unsaturated fatty acids (HUFAs), especially eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA) are essential in broodstock diets, having critical function as the main component of phospholipids of the cell membranes (Izquierdo *et al.*, 2001). Studies by Furuita *et al.* (2000) showed that deficiency of n-3 HUFAs negatively affect egg quality, possibly due to the fact that developing eggs and larval stages of the fish probably have greater requirements for n-3 HUFA, because of the mass concentration of n-3 HUFA in their neural and visual tissue which predominates in the early stages of development. For that reason, any deficiency in these particular fatty acids can cause developmental abnormalities in the neural system and may affect their success as visual predators at the onset of first feeding (Bell *et al.*, 1995). Luo *et al.* (2015) found that Siberian sturgeon (*Acipenser baeri*) broodstocks produced higher DHA, EPA, and total PUFA eggs when fed with higher DHA to EPA ratio diet compared to diets with higher EPA to DHA ratio. Higher DHA to EPA ratio diet also improve fecundity, eggs hatchability, and the quality of larvae hatched for this species. Furthermore, for European eel (*Anguilla anguilla*) male broodstocks, high levels of DHA correlate positively with

sperm volumes, while high level of EPA effects higher sperm motility (Butts *et al.*, 2015).

However, Fernández-Palacios *et al.* (1995) demonstrated that 1.6% dry weight n-3 HUFA in gilthead sea bream diets increased fecundity, hatching and larval survival but inclusion rates of 1.13%, 2.18% and 3.15% resulted in significantly lower performance. Similarly, Furuita *et al.*, (2002) found that high amounts of n-3 HUFA negatively affected egg quality in Japanese flounder (*Paralichthys olivaceus*). Those contradictory results may be due to the competitive elongation and desaturation between n3 and n6 HUFA (Sargent *et al.*, 1997).

Navas *et al.*, (2001) and Sargent (1995) reported that n-6 HUFAs also play an important role in fish reproduction. They demonstrated that both EPA and ARA (arachidonic acid; 20:4 n-6) are involved in cell mediated functions and are precursors of eicosanoids which has an impact on the reproduction. Furuita *et al.* (2003) tested 3 different ARA levels (0.1, 0.6 and 1.2% of diet) on Japanese flounder (*P. olivaceus*) broodstocks. They had found that 0.6% supplementation of ARA improved the reproductive performance of *P. olivaceus* ; however the higher inclusions of ARA at 1.2% had negatively affected the egg and larval quality probably due to the inhibitory effect on EPA bioconversion. Recently, Norberg *et al.* (2017) found that ARA effected the reproductive biology of Atlantic Cod (*Gadus morhua*) female broodstocks through changes in steroid plasma level (E2 and T) and speculate on a possible ARA effect on the endocrine regulation of reproductive development in this species. Meanwhile, for tongue sole (*Cynoglossus semilaevis*), ARA was identified to regulates sex steroid hormone in tongue sole broodstocks, with higher build up in the gonads; depending on fish sex and maturation level (Xu *et al.*, 2017). It seems that dietary ARA supplementation is more beneficial for males compare to females broodstocks.

Sargent *et al.* (2002) described that the eicosanoid production is influenced by the cellular ratio of EPA/ARA. ARA is the chief precursor of the eicosanoids, including the generation of series II prostaglandins (PGs). However, EPA competitively interferes with eicosanoid production from ARA, because both series

II and series III PGs are catalysed by the same cyclooxygenase and lipoxygenase. In short, it is believed that the ARA/EPA ratio in the diets can influence eicosanoid production and consequently physiological processes and broodstock reproductive performance (Sargent *et al.*, 1999). Liang *et al.* (2014) also demonstrate that n-3 and n-6 PUFA ratio influence spawning performance, egg and larval quality for tongue sole, *Cynoglossus semilaevis*, with best ratios for n-3 and n-6 PUFA ranged between 2.8 and 5.2% of total fatty acids.

CHAPTER 3

METHODOLOGY

3.1 Location of Study

Three feeding trials designed to achieve the objectives of this study were conducted at the Mahseer Hatchery, Institute of Tropical Aquaculture (AKUATROP), Universiti Malaysia Terengganu (UMT), Kuala Nerus, Terengganu, Malaysia.

3.2 Source of Sample

Tor tambroides filial 1 (F_1) generation broodstocks were obtained from Tarat Indigenous Fisheries Production and Research Center (IFPRC), Serian, Sarawak through artificial propagation and reared in concrete recirculating aquaculture system (RAS) tanks for 4-5 years at Lu Thian Tack (LTT) Aquaculture Farm, Asajaya, Sarawak. Eggs produced from these boodstocks were reared for two years before being purchased and used in this study. The broodstocks were acclimatized for 30 days before being used for the experiment. During the acclimation period, fish were fed with commercial pellet (ASIA) (40% crude protein and 5% crude fat). Individual fish were tagged using microchip-based passive integrated transponder (PIT) tag (Trovan Ltd., UK) for identification. This is important to identify which fish was used for blood collection and sacrificed for data analysis.

3.3 Diet Preparation

The diet ingredients and compositions are described separately for each feeding trial in the following sub-topic. After formulation, all dry ingredients were ground and sieved with a 600-μm sieve. After that, they were mixed by a vertical mixer for 10 minutes. Once all dry ingredients were completely mixed, oil was added to each diet and mixed for another five minutes. Finally distilled water was poured on the diet until homogenous dough was produced. The moist dough was immediately pelletized using an SLG65-III twin screw extruder (Jinan Saibainuo Technoloogy Development Co., Ltd) with 3mm die size available at the Mahseer Hatchery, AKUATROP, UMT. The extruded pellets were then spread over aluminium trays and oven-dried at 65°C for 6-8 hours. The extruded pellets were stored in sealed plastic bags and stored at -20°C until use. Each diet was subjected to proximate analysis as well as fatty acid analysis to confirm the accuracy of formulation.

3.4 Proximate Analysis

The proximate analysis was performed by adopting standard methods (AOAC, 2006). All diet ingredients and diets prepared were subjected for this analysis. The analytical procedures of the proximate composition are summarized below.

3.4.1 Determination of Moisture

Empty crucibles were dried in the oven at 100°C for 1 hour, cooled down, and weighted. Later, 2 g of sample were placed into the crucible and dried in the oven at 100°C for 6 hours, cooled down, and weighted. The moisture content of the samples was calculated as:

Dry matter = [(W2 – W1) / W3] x 100

% of moisture = 100 - % of dry matter

Where: W1= Weight of empty crucible

W2= Weight of crucible + dried sample

W3= Weight of sample before drying process

3.4.2 Determination of Ash

Empty crucibles were dried in the oven at 100°C for 1 hour, cooled down, and weighted. Later, 2 g of sample were placed into the crucible, and were kept inside the muffle furnace at 600°C for 3 hours and left overnight, cooled down, and weighted. The ash content was calculated as:

% ash = [(W2 – W1) / W3] x 100

Where: W1= Weight of empty crucible

W2= Weight of crucible + ash

W3= Weight of original sample

3.4.3 Determination of Crude Protein

Crude protein of the diets was analyzed using the Kjeldahl's method. In summary, the samples were digested with H_2SO_4 in a digestion tube at 450°C for 60 minutes. The digested samples were then neutralized with 40% NaOH before being distilled with a steam. Steam liberated was collected in 5 ml of 4% boric acid solution with a drop of methyl red indicator. The amount of nitrogen in the samples was then be determined by titration with (0.01 N) HCl and calculated as below:

Weight of titrate sample (W2) = [(W1 x V2) / V1] x 1000 mg

% N in the sample = [(T x N x 14.007) / W2] x 100

% Protein in the sample = %N x F

Where: W1= Weight of sample

V1= sample after digestion (100ml)

V2= subsample from sample after digestion (10ml)

N= Normality of HCl (0.01N)

F= Protein Factor (6.25)

T= Volume of HCl during titration

3.4.4 Determination of Crude Lipid

The crude fat was determined by exhaustive Soxhlet extraction method using petroleum ether on a Soxtec 2043 System. Empty extraction cup was washed, dried in the oven at 100°C for 1 hour, cooled down, and weighted. Later, 2 g of sample was put in a Soxhlet extraction thimble, and 50 ml of petroleum ether (40-60C, BP) were filled into the extraction cup. The thimble and extraction cup were then moved into the Soxtec System for extraction process. After extraction process, the extraction cup was removed and placed into an oven at 110°C for 2 hours, cooled down, and weighted. The residue in the thimble was kept for fiber analysis. The lipid percentages were calculated as below:

% of lipid = [(W3 - W1) / W2] x 100

Where: W1= Weight of extraction cup (g)

W2= Weight of sample (g)

W3= Weight of extraction cup with oil essence (g)

3.4.5 Determination of Crude Fibre

Crude fibre was determined using FIBRETHERM FT 12. FibreBags and crucibles were dried inside the oven at 105°C for 1 hour, cooled down, and weighted. Sample (1 g) was put into the FibreBags. After defatting, the drained FibreBags was put into separate crucibles and dried for 4 hours before being weighted. The FibreBags was then incinerated at 600°C for 3 hours and left overnight. Then the samples were discarded from the furnace, cooled to room temperature in desiccators before being reweighed. The crude fibre was calculated as follow:

% fibre = ([W3 – W1 – W6 – B) / W2] x 100

Where: W1= Initial weight of FibreBags

W2= Weight of sample (g)

W3= Weight of FibreBags after drying for 4 hours (g)

W4= Initial weight of crucibles (g)

W5= Weight of crucibles with ash (g)

W6= Weight of Ash (W5 - W4) (g)

B= Weight of blank FibreBags after drying for 4 hours (g)

3.4.6 Determination of Nitrogen-free Extract (NFE)

Nitrogen-free extract (carbohydrate) was calculated by subtracting 100 with the added value of moisture, crude protein, crude fat, crude fibre and ash values.

NFE(%) = 100 – (moisture* + protein + lipid + ash + fibre**)

* In case of dry matter basis, moisture was excluded.

** Fibre was included here so that NFE represents potentially-available carbohydrate.

3.5 Experimental Design

3.5.1 Effects of Different Fish Oil to Corn Oil Ratios on Female Gonad Development of *Tor tambroides* Broodstock

Three isonitrogenous and isolipidic diets were formulated to contain fish oil (FO) and corn oil (CO) at ratio of 1:0 (Diet 1:0), 1:1 (Diet 1:1), and 0:1 (Diet 0:1), respectively (Table 3.1) where Diet 1:0 serves as control with 100% FO as oil sources. Ninety female broodstocks with average weights of 0.91± 0.13 kg were equally and randomly assigned to nine rectangular tanks (five ton capacity) (three replicates per treatment) in a Recirculating Aquaculture System (RAS). The outlet from each tanks were stock into a reservoir tank, filtered using sand filter, which were then being pump into the stock tank, before being supplied to each tank by using gravity. Water temperature, dissolved oxygen (DO) and pH levels were monitored every week. Due to the size of the tanks and the nature of tropics environment in Terengganu, the water temperature was maintained naturally and were recorded between 26 to 29°C. Meanwhile, the DO and pH levels were recorded between 6.22 to 7.21 mg L^{-1} and 6.0 to 7.24, respectively. The feeding trial was conducted for five months from January to May 2013 and during this period; fish were fed twice per day (at 0900 and 1600h).

Table 3.1: Ingredients and proximate composition (g kg^{-1}) of formulated diets containing different fish oil (FO) to corn oil (CO) ratio over the five months feeding trial.

Ingredients (g kg^{-1})	Diet 1:0 (FO:CO; 1:0)	Diet 1:1 (FO:CO;1:1)	Diet 0:1 (FO:CO; 0:1)
Fish Meal	500.0	500.0	500.0
Soy bean Meal	92.0	92.0	92.0
Wheat Flour	250.0	250.0	250.0
Rice Bran	68.0	68.0	68.0
Fish Oil	50.0	25.0	0.0
Corn Oil	0.0	25.0	50.0
Vitamin Premix[1]	20.0	20.0	20.0
Mineral Premix[2]	20.0	20.0	20.0
Proximate composition			
Protein	455.6	457.9	453.3
Lipid	104.1	104.2	103.6
Fibre	38.4	37.6	36.5
Ash	97.3	98.0	98.4
Moisture	84.8	82.4	83.6
NFE[3]	219.7	219.9	224.5
Energy[4]	18.64	18.70	18.65

[1]Vitamin premix (mg kg^{-1}): Thiamine-HCl, 8.0; Riboflavin, 8.0; Niacin mix, 100.0; Pyridoxine-HCl, 20.0; Cyanocobalamine, 0.1; Pantothenate, 20.0; Biotin, 1.0; Inositol, 100.0; Folic acid, 5.0; Ascorbic acid, 250.0; Vitamin A, 20.0; Vitamin D, 8.0; Vitamin E, 150.0; Vitamin K, 10.0; BHT,10.0; α-cellulose, 1289.9.

[2]Mineral premix (mg kg^{-1}): $MgSO_4 \cdot 7H_2O$, 300.0; $FeSO_4 \cdot 7H_2O$, 180.0; $ZnSO_4 \cdot 7H_2O$, 120.0; $MnSO_4 \cdot 7H_2O$, 35.0; KI, 0.65; Na_2SeO_3, 0.5; $CoCl \cdot 6H_2O$ (1%), 7.0; $CuSO_4 \cdot 5H_2O$, 5.0; Zeolite, 7351.85

[3]NFE= nitrogen free extracts calculated as 1000 – (protein + lipid + ash + fibre) g kg^{-1}
[4]Energy value was calculated based on known value of energy from protein (23.6 KJ g^{-1}), lipid (39.5 KJ g^{-1}) and carbohydrate (17.2 KJ g^{-1}).

3.5.2 Effects of Varying Lipid Levels on Gonad Development of Female *Tor tambroides* Broodstock

Four isonitrogenous (450 g kg^{-1} crude protein) with increasing percentages of lipid inclusions were formulated with percentages of 0% (Diet 60 g kg^{-1}), 2.5% (Diet 82 g kg^{-1}), 5.0% (Diet 105 g kg^{-1}), and 7.5% (Diet 128 g kg^{-1}). Diet 60 g kg^{-1} served as control where no oil was added in the pellet (Table 3.2). The lipid ratio used in this experiments was based on the best results from the previous experiment which is Diet 1:1 (FO:CO; 1:1). Forty female broodstocks with average weights of 1.85 $\pm$ 0.13 kg were equally and randomly assigned to eight tanks (five ton each) (two replicates per treatment) in a same RAS as the first experiment. Water temperature, DO and pH were monitored every week. The water temperature was maintained naturally and was recorded between 28.95 to 30.95°C while the DO and pH level were recorded between 6.14 to 7.13 mg L^{-1} and 6.06 to 7.89, respectively. The feeding trial was conducted for 5 months from February to June 2015 and fish were hand fed ad libitum twice per day (at 0900 and 1600h).

Table 3.2: Ingredients and proximate composition (g kg^{-1}) of formulated diets containing varying lipid levels over the five months feeding trial.

Ingredients (g kg-1)	Diet 60 (0%)	Diet 82 (2.5%)	Diet 105 (5.0%)	Diet 128 (7.5%)
Fish Meal	500.0	500.0	500.0	500.0
Soy bean Meal	65.0	79.0	92.0	107.0
Wheat Flour	250.0	250.0	250.0	250.0
Rice Bran	145.0	106.0	68.0	28.0
Fish Oil	0	12.5	25.0	37.5
Corn Oil	0	12.5	25.0	37.5
Vitamin Premix[1]	20.0	20.0	20.0	20.0
Mineral Premix[2]	20.0	20.0	20.0	20.0
Proximate composition				
Protein	454.1	460.2	453.2	450.3
Lipid	60.1	83.7	110.1	137.2
Fibre	48.8	47.0	37.6	26.5
Ash	97.7	97.6	97.1	96.8
Moisture	92.7	90.5	90.8	93.7
NFE[3]	246.7	221.1	211.2	195.5
Energy[4]	17.33	17.97	18.68	19.41

[1]Vitamin premix (mg kg^{-1}): Thiamine-HCl, 8.0; Riboflavin, 8.0; Niacin mix, 100.0; Pyridoxine-HCl, 20.0; Cyanocobalamine, 0.1; Pantothenate, 20.0; Biotin, 1.0; Inositol, 100.0; Folic acid, 5.0; Ascorbic acid, 250.0; Vitamin A, 20.0; Vitamin D, 8.0; Vitamin E, 150.0; Vitamin K, 10.0; BHT,10.0; α-cellulose, 1289.9.

[2]Mineral premix (mg kg^{-1}): $MgSO_4 \cdot 7H_2O$, 300.0; $FeSO_4 \cdot 7H_2O$, 180.0; $ZnSO_4 \cdot 7H_2O$, 120.0; $MnSO_4 \cdot 7H_2O$, 35.0; KI, 0.65; Na_2SeO_3, 0.5; $CoCl \cdot 6H_2O$ (1%), 7.0; $CuSO_4 \cdot 5H_2O$, 5.0; Zeolite, 7351.85

[3]NFE= nitrogen free extracts calculated as 1000 – (protein + lipid + ash + fibre) g kg^{-1}

[4]Energy value was calculated based on known value of energy from protein (23.6 KJ g^{-1}), lipid (39.5 KJ g^{-1}) and carbohydrate (17.2 KJ g^{-1}).

3.5.3 Effects of Varying Lipid Levels on Gonad Development of Male *Tor tambroides* Broodstock

The diet formulation used in the second feeding trial was also used for the third feeding trial. Forty male broodstocks with average weights of 2.17 ± 0.15 kg were equally and randomly assign to eight tanks (two replicates per treatment) equipped with the same RAS as previous experiments. Water temperature, dissolved oxygen and pH were monitored and documented every week. During the feeding trial, the temperature, DO and pH ranged between 28.95 to 30.95°C, 6.14 to 7.13 mg L^{-1} and 6.06 to 7.89, respectively. The feeding trial was conducted for 5 months from February to June 2015 and fish were hand fed twice per day (at 0900 and 1600h) until satiation.

3.6 Data Collection

At the end of the feeding trials, three fish from each treatment were randomly selected, anesthesized (with 40ppm of clove oil), which was later humanely sacrificed (via clove oil overdosed) and the same fish were used for all the data analysis. The gonad and liver were extracted for gonadosomatic index (GSI) and hepatosomatic index (HSI). The GSI and HSI were calculated using the following formulae:

GSI (%) = [Gonad weight (g) / Body weight (g)] x 100
HSI (%) = [Liver weight (g) / Body weight (g)] x 100

Development of gonad in all three feeding trials was observed via histological analysis. At the end of each feeding trials, the gonads were taken from three fish from each treatment for histological examination. The gonads were fixed in Bouin's solution (Ismail *et al.*, 2011) until further histological analysis procedure.

For sperm quality assessment, fish were turned upside down and covered in wet towel and the ventral part of the brood fish was gently blotted dry with clean and

dry towel to remove water, mucus, urine and fecal matter. Semen samples were collected by hand stripping by gently pressing along both sides of the belly to expel the semen into centrifuge tubes. The collected sperm were put in ice until further analysis.

Fresh samples were taken at the end of all three feeding trials. The dissected fish were successively dressed and the muscles from area between the lateral and dorsal line were removed. Samples of experimental feed and fresh sample (muscle, liver and gonad) were subjected for fatty acid analysis. All fresh samples were freeze dried and kept in -80°C refrigerator prior to fatty acid analysis.

One month before the end of the experiments, all female fish were injected with Ovaplant. At the end of all feeding trials, the broodstocks from each treatment were injected with Ovaprim (Syndel) (0.5 ml kg^{-1} (female) and 0.25ml kg^{-1} (male) of body weight). One ml of blood sample were collected from caudal vein of three randomly selected individual fish from each treatments using heparinized syringe fitted with 22-gauge needle at 0 h (prior to injection), 12 and 24 h post injection of Ovaprim. The same fish were used for this blood sampling, hence the need to use the PIT tag for identification. The blood samples were centrifuged at 7000 rpm for 10 minutes and plasma sample were collected. The plasma samples were stored at -20°C till assayed.

3.7 Gonad Analysis

3.7.1 Histological Analysis

The histological study was conducted using routine paraffin histology and embedded in paraffin wax. The embedded gonads were trimmed and sectioned into 5μm thick slices and stained with hematoxylin-eosin to study the developments of the gonadal tissues. The gonads were divided into two parts: anterior and posterior from the vent opening. The slides containing the sections were then cover slipped before being observed under a compound microscope (Nikon eclipse 80i, Japan) equipped with Motic camera and analysed with Motic Image software using a computer. The gonad stages were determined under light microscope according to method from Ismail *et al.* (2011).

3.7.2 Oocyte Diameter Analysis

In order to assess the oocyte size frequency distribution, 100 oocytes (from histological analysis picture) were randomly measured by using ImageJ software for each specimen from two sampling sides (anterior and posterior) of gonads, using the methods from West (1990), Grande *et al.* (2012) and Fernandes *et al.*, (2016). When the oocyte diameter was measured as the average of the major and minor axes, and the limit for each stage was identified as the limits of confidence intervals which did not overlap the mean value to segregate the oocyte stages.

3.7.3 Sperm Quality Assessment

Sperm qualities were observed immediately after collection. Sperm volume (mL), concentration and motility were carried out following the protocol from Basavaraja and Hedge (2005) and Oguntuase and Adebayo (2014). Motility percentages were determined by observing water activated milt placed on a glass slide under microscope. The motile sperms were observed and expressed as a percent

of total sperm. Spermatozoa density was determined immediately after their collection using haemocytometer counts. Prior to density determination, the milt was diluted 1:7 (sperm to diluent ratio) in an extender solution comprises of 202 mM D(+)-glucose monohydrate, 51.5 mM sodium chloride and 6 mM sodium bicarbonate, with pH 7.1–7.8 and an osmolality of 309 ± 30 mOsmol kg^{-1} (Chew *et al.*, 2010). The spermatozoa were then counted by taking aliquots and expressed as the total number of spermatozoa per mL milt, using the formula by Quinito *et al.*, (1984):

$$\text{Sperm motility} = \frac{\text{Total number of motile sperm}}{\text{Total number of sperm}} \times 100$$

$$\text{Spermatozoa density} = \frac{\text{Total number of spermatozoa in four counting blocks}}{\text{Number of counting blocks}} \times 10\,000.$$

3.8 Fatty Acids Analysis

Fatty acid (FA) analysis was conducted based on one-step method by Abdulkadir and Tsuchiya (2008). The internal standard solution was prepared by dissolving 100 mg of 19:0 (Nonadecanoic acid, 99.5% purity Brand Fluka, 74208 Puriss) in 100 ml hexane to obtain a final concentration of 1 mg/ml of C 19:0. A 200 mg of each samples (freezed dried for fresh samples) were mixed with 4 ml of hexane and 1 ml of internal standard solution in a 50 ml centrifuge tube. Two ml of 14% boron triflouride in methanol were then added. After that, the head space of tube was flushed with nitrogen gas and then closed tightly with a Teflon-lined screw-cap. The capped tube was heated on a hot water at 100 °C for 120 min under continuous stirring. After cooling to room temperature, 1 ml of hexane was added followed by 2 ml of distilled water. The tube was then shaken vigorously for 1 min and centrifuged for 3 min at 2500 rpm. The upper phase was hexane layer containing the FAMEs. Finally, 1–2 ml of the hexane layer was transferred using a Pasteur pipette into a clean sample vial to be injected into the GC for FAME analysis. The

FAMEs were separated and quantified using a gas chromatograph (GC 14-B Shimadzu) equipped with flame ionization detector.

FAMEs were analysed in an Agilent 7890N gas chromatograph (Agilent Technologies, Inc., Santa Clara CA, USA) equipped with a split/splitless injector, a flame ionization detector and a Supelco SP-2330 capillary column (30m×0.25mm ID, 0.20 μm film thickness) (Supelco Inc., Bellefonte PA, USA). One μL of sample was injected to the gas chromatography by an automatic sampler unit. High purity nitrogen (Malaysian Oxygen Bhd., Malaysia) at a rate of 40 mL min^{-1} was used as the carrier gas while high purity hydrogen, produced by a high purity hydrogen generator (Domnick Hunter Industrial Division, Parker Hannifin Ltd., England), and compressed air (Malaysian Oxygen Bhd., Malaysia) were used for the flame ionization detector. Column temperature was set at 100 °C for the first 2 min, and then increased to 170 °C at 10°C min^{-1} with a holding time of 2 min, followed by an increase to 200 °C at 7.5 °C min^{-1} with a holding time of 20 min. Injector port and detector temperature were set at 250 and 300 °C respectively.

Fatty acids were identified by comparing relative FAMEs peak retention time of samples with those of known standard. Areas beneath the identified chromatographic peaks were determined with computer integrator software (Hewlett-Packard, Avondale, PA). Automatic expression of the peak areas as percentage amounts of each fatty acid was obtained with a programmed PC under Microsoft Excel 2000 (Microsoft Corp. Redmond, USA).

3.9 Hormone Analysis

The 17β-estradiol (E2) was measured only for the female feeding trial, while testosterone (T) was measured for the male feeding trial. The E2 and T concentrations were measured using commercially available enzyme linked immunosorbent assay (ELISA) kits from Cayman Chemical Company (Ann Arbor, MI, USA). E2 was extracted from plasma using methylene chloride while diethyl ether was used to extract T and were measured in duplicate. The E2 and T levels were quantified by EIA using Gauthier-Clerc *et al.* (2006) methods with some modification. The standard and sample absorbances were read at 405nm by using microplate reader. The absorbance values were analyzed by using a computer spreadsheet (by Cayman) as suggested by the ELISA kits. The hormone concentrations were expressed as mean and standard error.

3.10 Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM) unless otherwise stated. The data were tested for normality (Shapiro-Wilk normality test) and differences between GSI, HSI, fatty acid concentration and hormone levels after Ovaprim injection were analyzed by using one-way Analysis of Variance (ANOVA) followed by Duncan post-hoc test where applicable. Differences were considered to be significant at $p < 0.05$. Pearson correlation was used to evaluate any significant ($p < 0.01$ and $p < 0.05$) positive or negative correlations among parameters. When the data appear to be not normally distributed, as in data for oocyte diameter analysis, a non-parametric Kruskal-Wallis test was used while the distribution of oocyte diameter between anterior and posterior were tested with Mann-Whitney ($P < 0.05$) test to verify statistically significant differences (Fernandes *et al.*, 2016). All data were analyzed using IBM SPSS Statistics software (version 23).

CHAPTER 4

RESULTS

4.1 Effects of Different Fish Oil to Corn Oil Ratios on Female *Tor tambroides*

4.1.1 Gonadosomatic Index (GSI) and Hepatosomatic Index (HSI)

The ANOVA test showed that there are no significant differences for the gonadosomatic index (GSI) and hepatosomatic index (HSI) values ($p>0.05$) between treatments after the five month of different source of lipid feeding trial (Table 4.1). Treated fishes shown varied GSI values from 0.18 to 0.31% while HSI values varied from 0.54 to 0.60%.

Table 4.1: Gonadosomatic index (GSI) and hepatosomatic index (HSI) percentage of female *Tor tambroides* cultured for five months and fed with diets containing different fish oil (FO) to corn oil (CO) ratio.

Group of treatment	Diet 1:0 (FO:CO; 1:0)	Diet 1:1 (FO:CO; 1:1)	Diet 0:1 (FO:CO; 0:1)
GSI (%)	0.18±0.02	0.31±0.17	0.22±0.08
HSI (%)	0.59±0.07	0.60±0.06	0.54±0.05

Values are (mean + SE).

4.1.2 Gonad Analysis

4.1.2.1 Histological Analysis

The gonad stages are determined according to gonad characterization of *T. tambroides* females from Ismail *et al.* (2011) and Wibowo and Kaban, (2014). The gonad of *T. tambroides* female showed Stage 1 (S1), Stage 2 (S2) and Stage 3 (S3) of oocyte stages. Based on the histological studies, the gonads in all treated fish are dominated by previtellogenic oocytes in the anterior part (Figure 4.1a, 4.2a and 4.3a) and also posterior part (Figure 4.1b, 4.2b and 4.3b). The gonads anterior part are dominated by S2 and S3 oocytes with no S1 stage oocyte was found meanwhile in the posterior part, some S1 oocytes were found. However, fish fed with the diet containing fish oil to corn oil at a ratio of 1:1 showed higher amount of S3 oocytes compared to other two groups with Stage 4 (S4) and Stage 5 (S5) oocytes started to develop (Figure 4.2b).

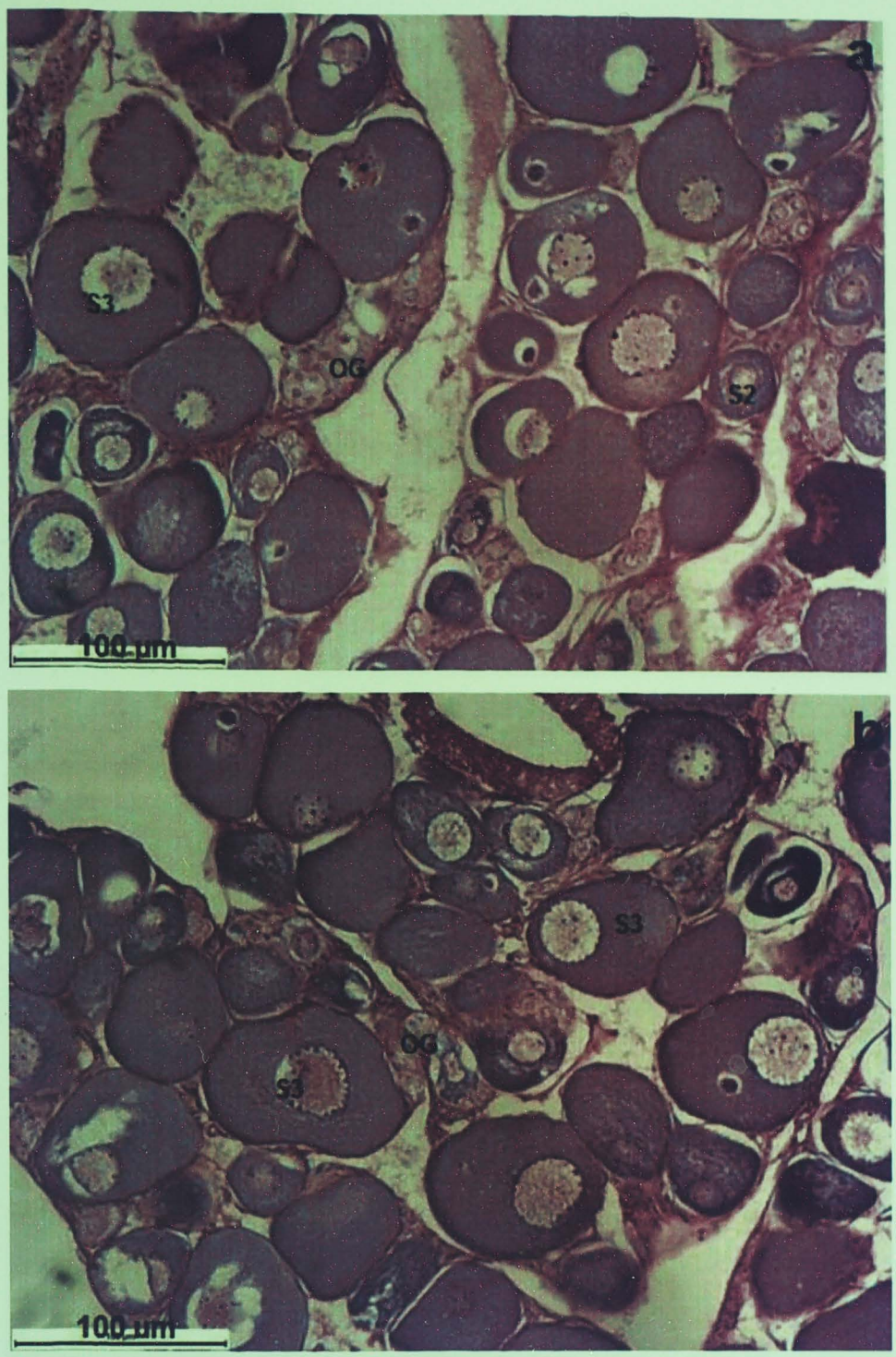


Figure 4.1: Gonadal cross-section of *Tor tambroides* female fed with diet containing fish oil and corn oil at a ratio of 1:0 after five month of dietary treatment for different fish oil to corn oil ratios from different part of the gonad: a) anterior and b) posterior. OG: oogonia; S2: stage 2 oocytes and S3: stage 3 oocytes.

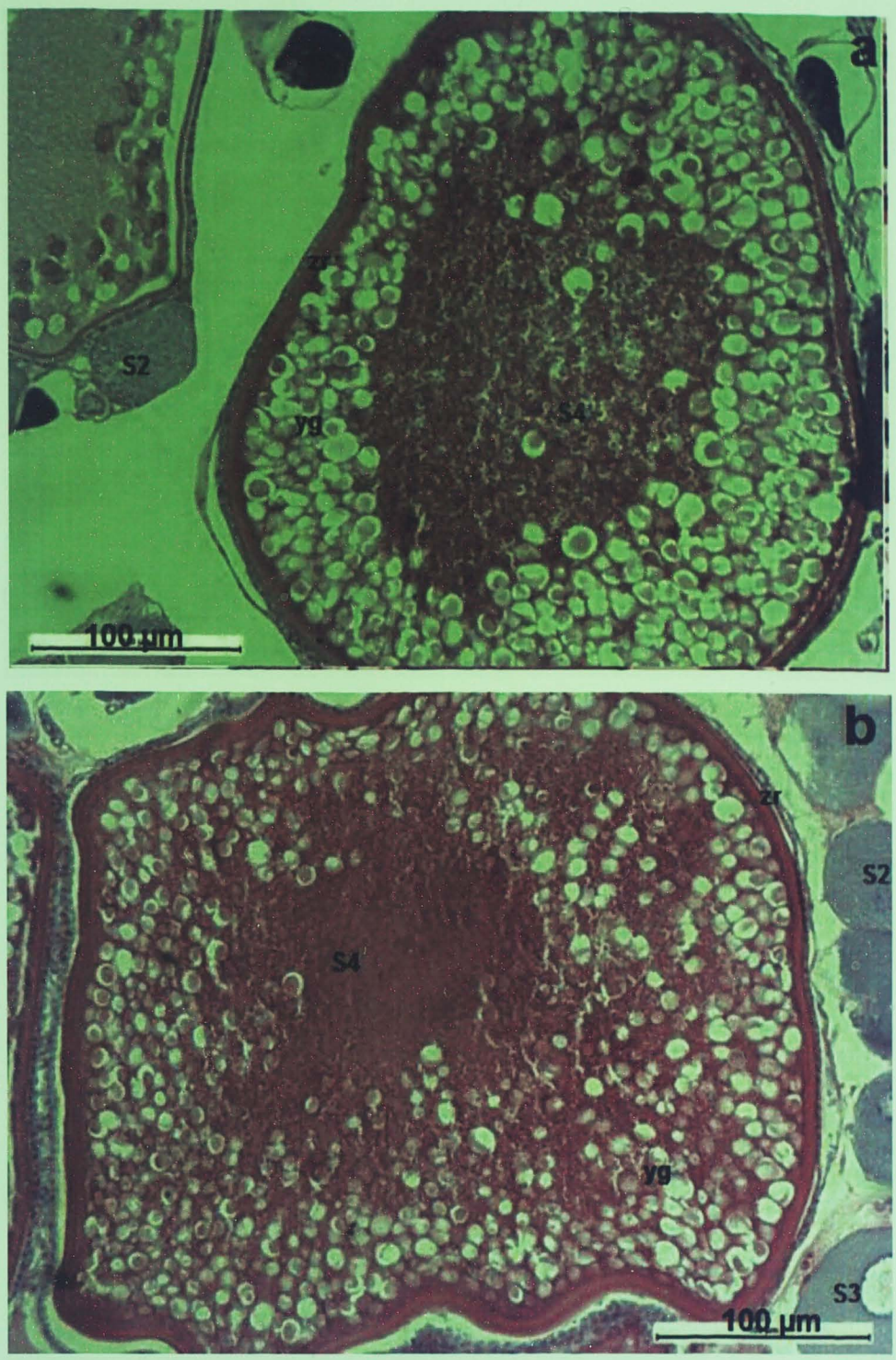


Figure 4.2: Gonadal cross-section of *Tor tambroides* female fed with diet containing fish oil and corn oil at a ratio of 1:1 after five month of dietary treatment for different fish oil to corn oil ratios from different part of the gonad: a) anterior and b) posterior. OG: oogonia; S2: stage 2 oocytes and S3: stage 3 oocytes.

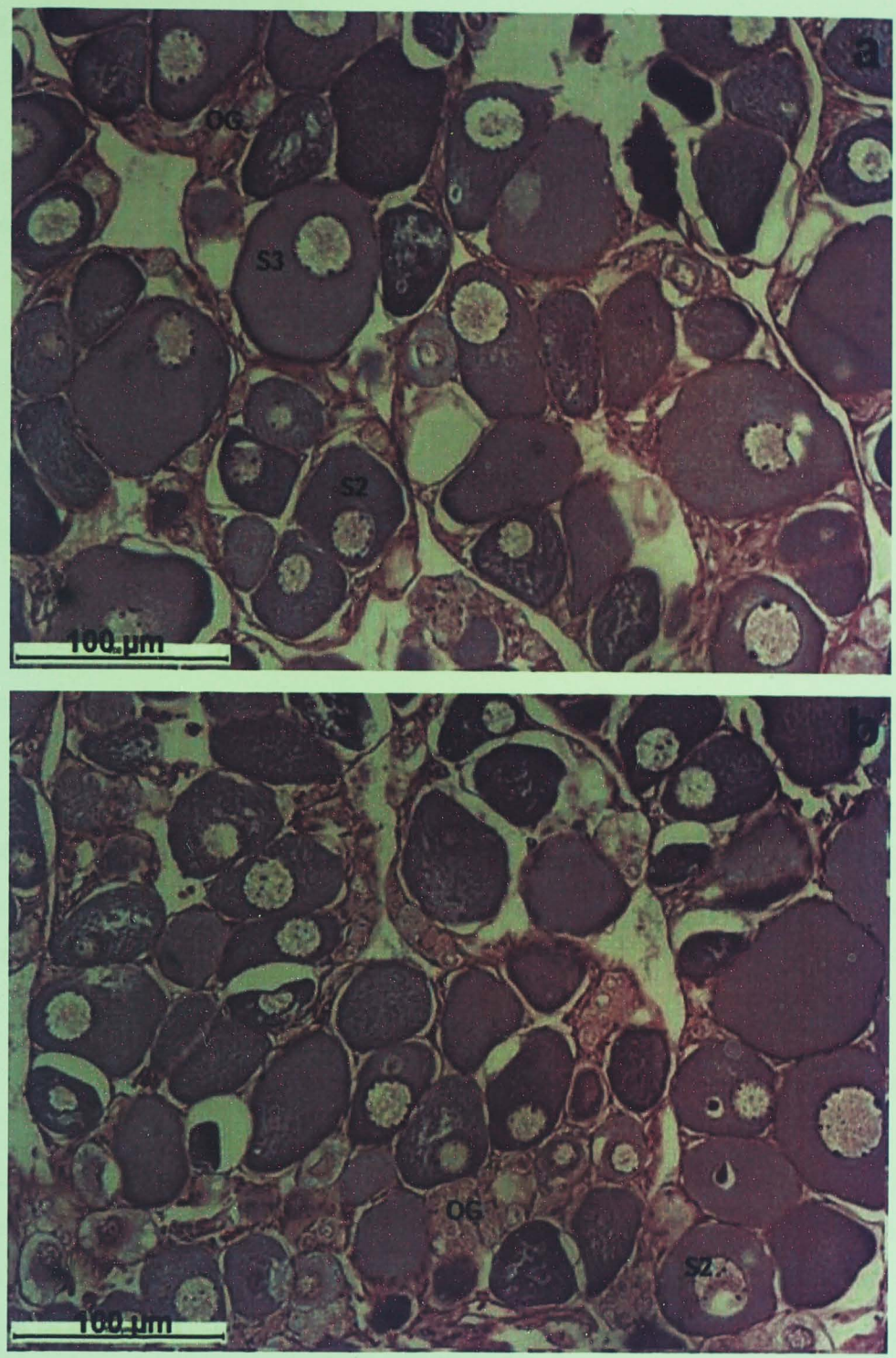


Figure 4.3: Gonadal cross-section of *Tor tambroides* female fed with diet containing fish oil and corn oil at a ratio of 0:1 after five month of dietary treatment for different fish oil to corn oil ratios from different part of the gonad: a) anterior and b) posterior. OG: oogonia; S2: stage 2 oocytes and S3: stage 3 oocytes.

4.1.2.2 Oocyte Size-Frequency Distribution

The oocyte size-frequency distributions of oocyte diameter in all dietary treated groups are illustrated in Figure 4.4. In the anterior part (Figure 4.4a), all gonads from each groups were dominated by S2 oocytes with values of 97.7 ± 0.33, 90.3 ± 1.20 and 89.3 ± 0.88% from the total of counted eggs for fish fed with fish oil to corn oil ratio of 1:0, 1:1 and 0:1, respectively. Meanwhile, the frequency of S3 oocytes is increasing between treated groups with 2.3 ± 0.33, 7.3 ± 1.86 and 10.3 ± 0.67 % respectively. Conversely, only fish fed with fish oil to corn oil ratio of 1:1 and 0:1 shows presence of S4 oocytes with values of 2.3±0.67 and 0.3±0.33% respectively.

A Kruskal-Wallis H test is run to determine if there are differences in percentages of oocyte stages between the dietary treated groups. The distributions of oocytes stages score are statistically significantly different between dietary treated groups, for S3 ($X^2(3) = 6.214$, $p = 0.045$) and S4 oocytes stages($X^2(3) = 6.231$, $p = 0.044$). Subsequently, pairwise comparisons were performed, however, no statistically significant pairwise comparisons are found for both the S3 and S4 oocyte stage for all dietary treated groups.

As for the posterior part of the gonad (Figure 4.4b), the same pattern can be seen with S2 oocytes dominating the gonad of fish fed with fish oil to corn oil ratio of 1:0, 1:1 and 0:1 with values of 95.3 ± 2.73, 78.3 ± 11.05 and 91 ± 0.00% respectively. S1 oocytes are also found in all dietary treated groups with value of 0.3 ± 0.33, 0.7 ± 0.67 and 0.7 ± 0.67% respectively. S3 oocytes is higher in fish fed with fish oil to corn oil ratio of 1:1 (12.7 ± 3.52%) compared to fish fed with fish oil to corn oil ratio of 1:0 (4.3 ± 2.96%) and fish fed with fish oil to corn oil ratio of 0:1 (8.33 ± 0.67%). Furthermore, only fish fed with fish oil to corn oil ratio of 1:1 shows presence of S4 (6 ± 6.0%) and S5 (2.3 ± 2.33%) oocytes. Subsequently, a Kruskal-Wallis test is run to determine if there are differences in percentages of oocyte stages score between all three dietary treated groups, but there are no significant differences found ($p>0.05$). Finally, with a Mann-Whitney test, there are no significant

differences ($p>0.05$) between anterior and posterior oocyte size-frequency distribution for all dietary treated groups.

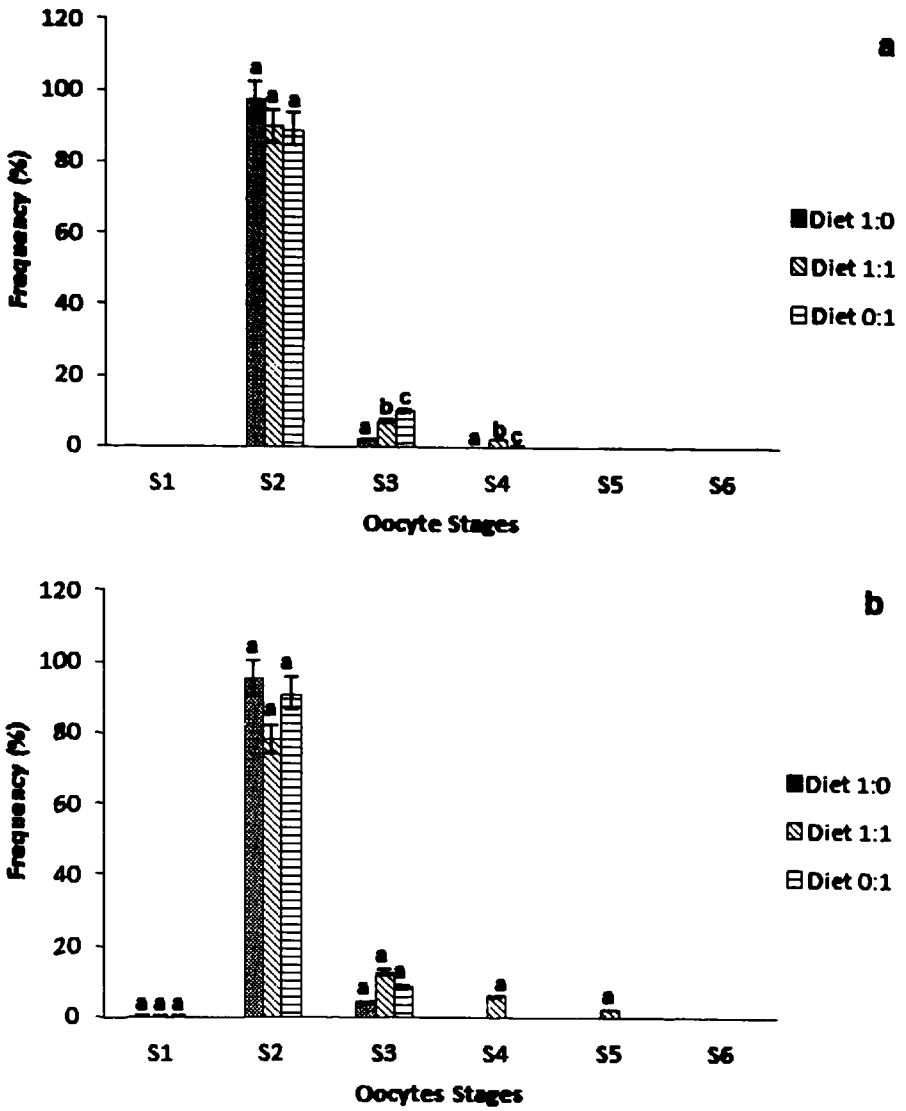


Figure 4.4: Frequency distribution of *Tor tambroides* oocytes for each oocyte stages after five months of feeding trial for different ratios of fish oil to corn oil from different part of the gonad: a) anterior ; and b) posterior.

4.1.3 Fatty Acid Analysis

4.1.3.1 Fatty Acid Analysis of the Diets

Fatty acid (FA) concentrations (mg g^{-1}) of the diets used in different lipid sources experiment are shown in Table 4.2. Higher level of total saturated fatty acids (SFA) was found in diet containing fish oil to corn oil ratio of 0:1 with 5.71 mg g^{-1} compared to 2.43 and 4.08 mg g^{-1} in fish fed with diet containing fish oil to corn oil ratio of 1:0 and 1:1 respectively. The total monounsaturated fatty acids (MUFA) increase from diet containing fish oil to corn oil ratio of 1:0 to 0:1, with oleic acid (OA) values of 0.66 to 3.62 mg g^{-1} respectively. The ANOVA test showed that there are a significant increase ($p<0.05$) in linoleic acids (LA), total PUFA and total FA values from diet containing fish oil to corn oil ratio of 1:0 to 0:1. The values range between 0.19 to 2.46 mg g^{-1}, 1.76 to 3.4 mg g^{-1} and 5.68 to 14.05 mg g^{-1} respectively. Conversely, the values of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) decrease significantly ($p<0.05$) from diet containing fish oil to corn oil ratio of 1:0 to 0:1 based on the results from ANOVA test. The values varied from 0.36 to 0.57 mg g^{-1} and 0.23 to 0.35 mg g^{-1} respectively. There are also a significant decrease ($p<0.05$) on the n3 to n6 PUFA ratio with values of 3.39, 0.74 and 0.21 mg g^{-1} respectively.

Table 4.2: Fatty acid concentrations (mg g^{-1}± SEM) of the diets used (Diet 1:0, Diet 1:1 and Diet 0:1) in the feeding trial for different fish oil to corn oil ratios.

Fatty acid	Diet 1:1 (FO:CO; 1:0)	Diet 1:1 (FO:CO; 1:1)	Diet 0:1 (FO:CO; 0:1)
C16:0	0.48±0.00[a]	1.61±0.00[b]	2.73±0.30[c]
C18:0	1.69±0.00[a]	1.83±0.00[b]	1.97±0.02[c]
C16:1	0.13±0.00[a]	0.29±0.00[b]	0.45±0.01[c]
C18:1n9	0.66±0.00[a]	2.14±0.00[b]	3.62±0.04[c]
C18:2n6	0.19±0.00[a]	1.03±0.00[b]	2.46±0.03[c]
C18:3n3	0.08±0.00	0.04±0.00	-
C20:4n6	0.03±0.00	0.02±0.00	0.01±0.00
C20:5n3	0.57±0.00[a]	0.42±0.00[b]	0.36±0.00[c]
C22:6n3	0.35±0.00[a]	0.29±0.00[b]	0.23±0.00[c]
∑ SFA	2.43±0.00[a]	4.08±0.01[b]	5.71±0.62[c]
∑ MUFA	1.49±0.01[a]	3.22±0.01[b]	4.94±0.06[c]
∑ PUFA	0.30±0.00[a]	1.16±0.00[b]	2.73±0.03[c]
∑ HUFA	1.46±0.00[a]	1.02±0.00[b]	0.68±0.01[c]
∑ n3	1.36±0.00[a]	0.93±0.00[b]	0.59±0.01[c]
∑ n6	0.4±0.00[a]	1.25±0.00[b]	2.81±0.03[c]
n3:n6	3.39±0.01[a]	0.74±0.00[b]	0.21±0.00[c]
∑ FA	5.68±0.02[a]	9.48±0.01[b]	14.06±0.16[c]

Values are (mean + SE); '-'= not detected
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

4.1.3.2 Fatty Acid Analysis of the Muscles

The FA concentration of the muscle of *T. tambroides* females fed with diet containing fish oil to corn oil ratios are given in Table 4.3. There are trend of increment in all the analysed FA concentration from fish fed with diet containing fish oil to corn oil ratio of 1:0 to 0:1. The total SFA values showed a significant increase ($p>0.05$) between fish fed with diet containing fish oil to corn oil ratio of 1:0 and 0:1 based on the results from ANOVA test. The values are 4.74 and 9.11 mg g^{-1} respectively. There are significant differences found in the total MUFA values ($p>0.05$) between fish fed with diet containing fish oil to corn oil ratio of 1:0 and 0:1, with the concentration of OA of 1.68 and 4.15 mg g^{-1} respectively. The ANOVA test also showed that the values for LA,DHA, total HUFA and total FA for fish fed with diet containing fish oil to corn oil ratio of 1:0 are significantly lower ($p<0.05$) compared to fish fed with diet containing fish oil to corn oil ratio of 0:1.

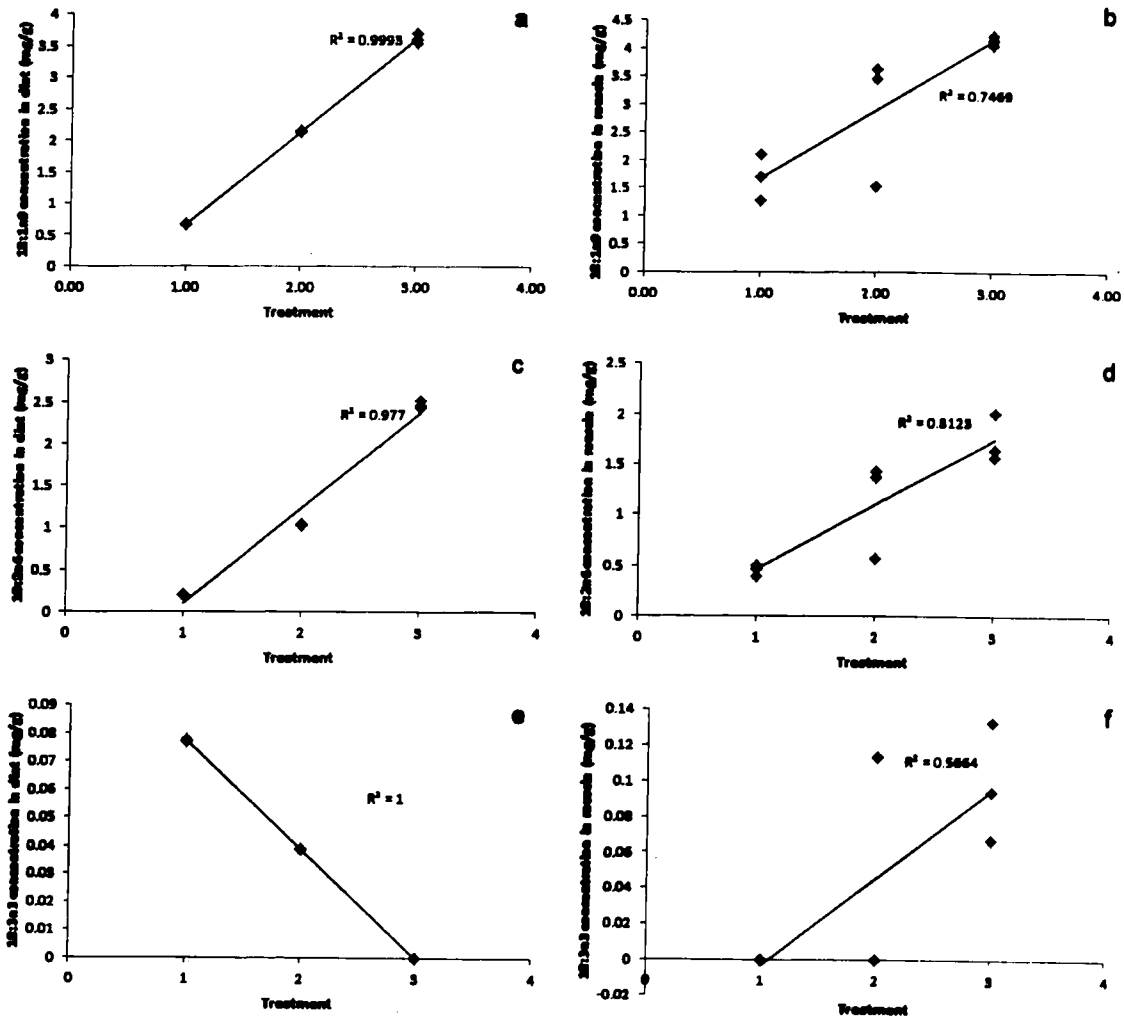
Table 4.3: Fatty acid concentrations (mg g^{-1} ± SEM) of the muscle of *Tor tambroides* females cultured for five months and fed diets with different fish oil and corn oil ratios.

Fatty acid	Diet 1:0 (FO:CO; 1:0)	Diet 1:1 (FO:CO; 1:1)	Diet 0:1 (FO:CO; 0:1)
C16:0	1.97±0.52	2.17±0.46	3.05±0.06
C18:0	2.09±0.81^{a}	3.83±0.73^{ab}	4.79±0.02^{b}
C16:1	0.20±0.03^{a}	0.37±0.08^{a}	0.60±0.03^{b}
C18:1n9	1.68±0.24^{a}	2.88±0.67^{ab}	4.15±0.05^{b}
C18:2n6	0.44±0.03^{a}	1.12±0.28^{b}	1.74±0.14^{b}
C18:3n3	-	0.04±0.04	0.10±0.02
C20:4n6	0.21±0.07	0.28±0.02	0.33±0.03
C20:5n3	0.07±0.04	0.13±0.07	0.17±0.01
C22:6n3	1.45±0.05^{a}	2.22±0.51^{ab}	2.96±0.15^{b}
∑ SFA	4.74±0.41^{a}	7.80±1.89^{ab}	9.11±0.06^{b}
∑ MUFA	3.25±0.13^{a}	4.68±1.14^{ab}	6.02±0.18^{b}
∑ PUFA	0.66±0.12^{a}	1.30±0.23^{b}	1.87±0.13^{b}
∑ HUFA	1.80±0.10^{a}	2.75±0.57^{ab}	3.63±0.19^{b}
∑ n3	1.55±0.11^{a}	2.38±0.54^{ab}	3.29±0.18^{b}
∑ n6	0.92±0.05^{a}	1.67±0.29^{b}	2.21±0.12^{b}
n3:n6	1.70±0.19	1.41±0.19	1.49±0.07
∑ FA	10.45±0.92^{a}	16.53±3.78^{ab}	20.59±0.47^{b}

Values are (mean + SE); '-'= not detected
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

4.1.3.3 Relationship between Fatty Acid Concentrations from Different Sources of Diet and Muscle with Treatments

The correlations between FA concentrations from different sources with treatments are illustrated in Figure 4.5. There are strong positive correlation of OA ($r(7)=1.000$; $p<0.01$; Figure 4.5a), LA ($r(7)=0.988$; $p<0.01$; Figure 4.5c) and total FA ($r(7)=0.998$; $p<0.01$; Figure 4.5i) between diet and treatment. Conversely, strong negative correlations are found in ALA ($r(7)=-1.000$; $p<0.01$; Figure 4.5e) and DHA ($r(7)=-0.999$; $p<0.01$; Figure 4.5g). Strong positive correlation is also found between muscle and treatment for oleic acid ($r(7)=0.864$; $p<0.01$; Figure 4.5b), LA ($r(7)=0.901$; $p<0.01$; Figure 4.5d), ALA ($r(7)=0.761$; $p<0.05$; Figure 4.5f), DHA ($r(7)=0.788$; $p<0.05$; Figure 4.5h) and total FA ($r(7)= 0.788$; $p<0.05$; Figure 4.5j).



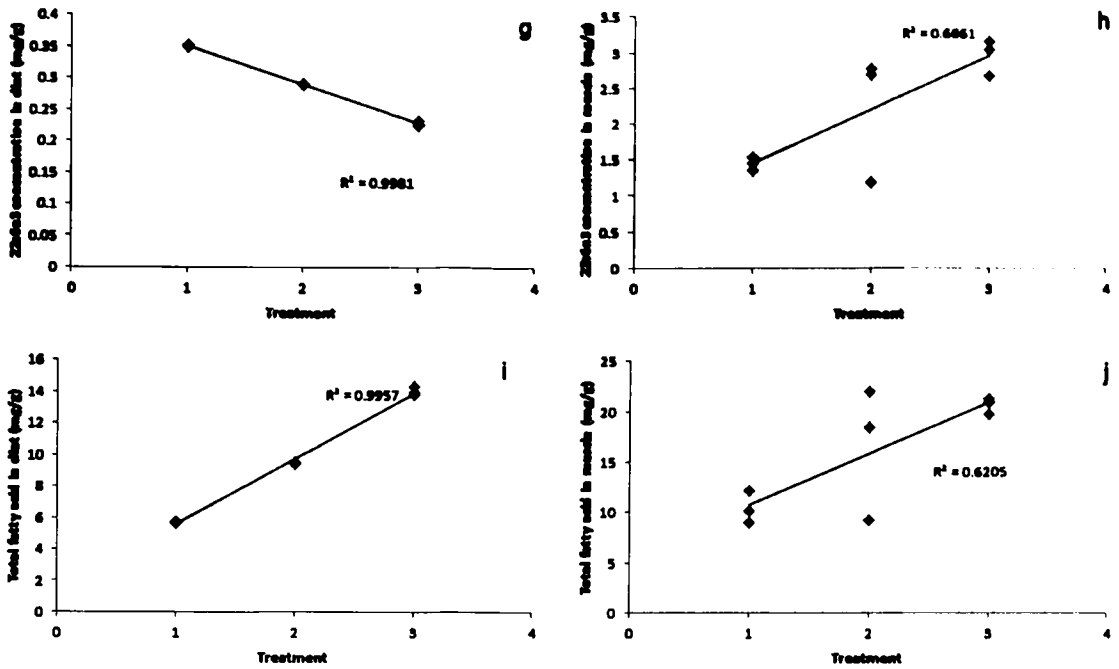


Figure 4.5: Relationship between (a) oleic acid (OA) in diet with treatment; (b) oleic acid (OA) in muscle with treatment; (c) linoleic acid (LA) in diet with treatment; (d) linoleic acid (LA) in muscle with treatment; (e) linolenic acid (ALA) in diet with treatment; (f) linolenic acid (ALA) in muscle with treatment; (g) docosahexanoic acid (DHA) in diet with treatment; (h) docosahexanoic acid (DHA) in muscle with treatment; (i) total fatty acid in diet with treatment and (j) total fatty acid in muscle with treatment

4.1.4 Hormone Analysis

The concentrations of 17β estradiol (E2) (ng ml^{-1}) in blood plasma of females *T. tambroides* observed at 0 h (prior to Ovaprim injection), 12 and 24 h post injection are shown in Figure 4.6. The E2 concentration pattern shows an increase from 0h and then peaked at 12h before decreasing at 24h. Fish fed with diet containing fish oil to corn oil ratio of 1:1 exhibit higher E2 levels at every blood sampling compared to the other two dietary treated groups. At 0h, fish fed with diet containing fish oil to corn oil ratio of 1:1 display values of 0.01 ng ml^{-1} compared to fish fed with diet containing fish oil to corn oil ratio of 1:0 (0.006 ng ml^{-1}) and 0:1 (0.008 ng ml^{-1}). The E2 concentrations peaked at 12h for all dietary treated fish with values of 0.015, 0.035 and 0.024 ng ml^{-1} for fish fed with diet containing fish oil to corn oil ratio of 1:0, 1:1 and 0:1, respectively. Subsequently, the E2 concentrations decrease to 0.008, 0.012 and 0.003 for fish fed with diet containing fish oil to corn oil ratio of 1:0, 1:1 and 0:1, respectively at 24h post injection. However, there are no significant differences for values of E2 in all blood sampling intervals between all diet treated fish based on the results from ANOVA test ($p>0.05$) .

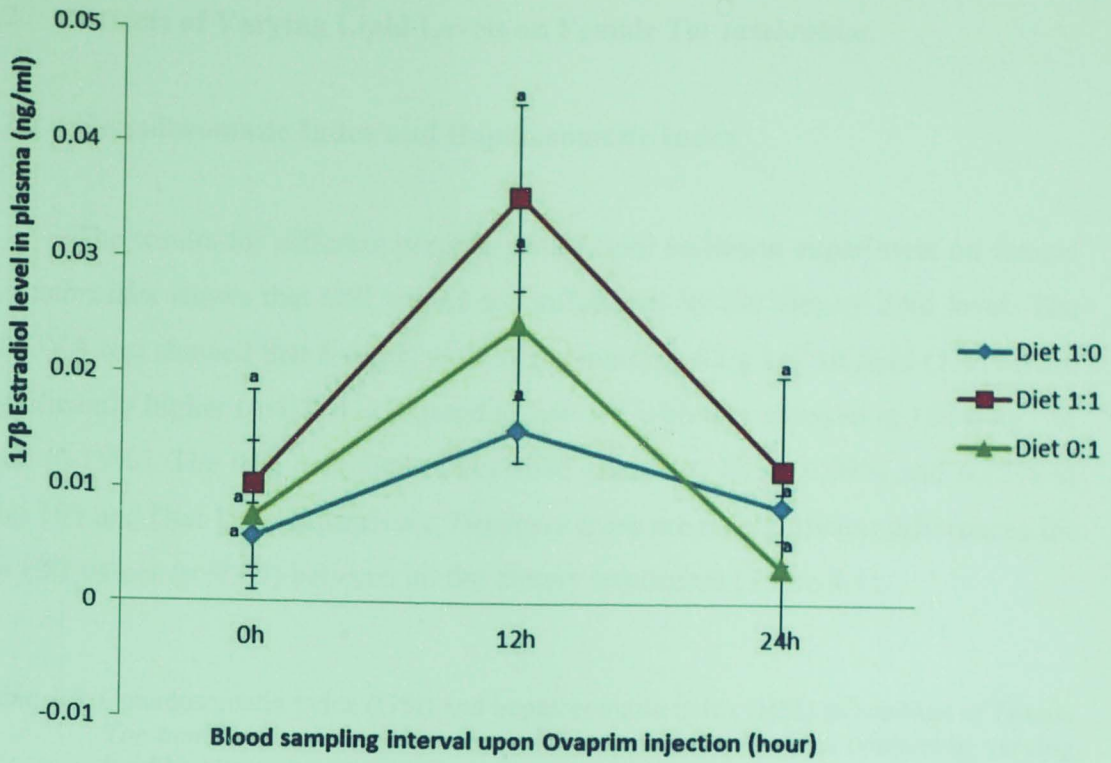


Figure 4.6: 17β estradiol changes in *Tor tambroides* female broodstocks in 24-h artificially induced ovulation with ovaprim after five months of dietary treatment with diets containing different fish oil to corn oil ratios (Diet 1:0, 1:1 and 0:1).

4.2 Effects of Varying Lipid Levels on Female *Tor tambroides.*

4.2.1 Gonadosomatic Index and Hepatosomatic Index

The results for different percentages of lipid inclusion experiment on female *T. tambroides* shows that GSI values are influenced by the dietary lipid level. The ANOVA test showed that fish fed with diet containing 82 g kg^{-1} of lipid (1.47%) are significantly higher ($p<0.05$) compared to fish fed with diet containing 128 g kg^{-1} of lipid (0.15%). The GSI percentages decrease after Diet 82 to 0.59% and 0.15% in Diet 105 and Diet 128, respectively. However there are no significant differences for the HSI values ($p>0.05$) between all the dietary treatments (Table 4.4).

Table 4.4: Gonadosomatic index (GSI) and hepatosomatic index (HSI) percentage of female *Tor tambroides* cultured for five months and fed with diets containing varying lipid levels.

Group of treatment	Diet 60 (0%)	Diet 82 (2.5%)	Diet 105 (5.0%)	Diet 128 (7.5%)
GSI (%)	0.98 ± 0.34^{ab}	1.47 ± 0.57^{b}	0.59 ± 0.25^{ab}	0.15 ± 0.06^{a}
HSI (%)	0.70±0.15	0.68±0.03	0.47±0.06	0.49±0.20

Values are (mean + SE).
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

4.2.2 Gonad Analysis

4.2.2.1 Histological Analysis

At the end of dietary treatment, the gonad of *T. tambroides* female contained S2, S3, S4 and S5 oocyte stages (Figure 4.7, 4.8, 4.9 and 4.10). Based on the histological studies, the gonads are dominated by vitellogenic oocytes in the anterior from the vent opening and also posterior part. The ovarian cells are dominated by S2 and S3 oocytes with presence of S4 and S5 oocytes in fish fed with diet containing 60, 82, and 105 g kg^{-1} of lipid but not for fish fed with 128 g kg^{-1} of lipid. Furthermore, low amount of S6 oocytes is only found in fish fed with diet containing 82 g kg^{-1} of lipid.

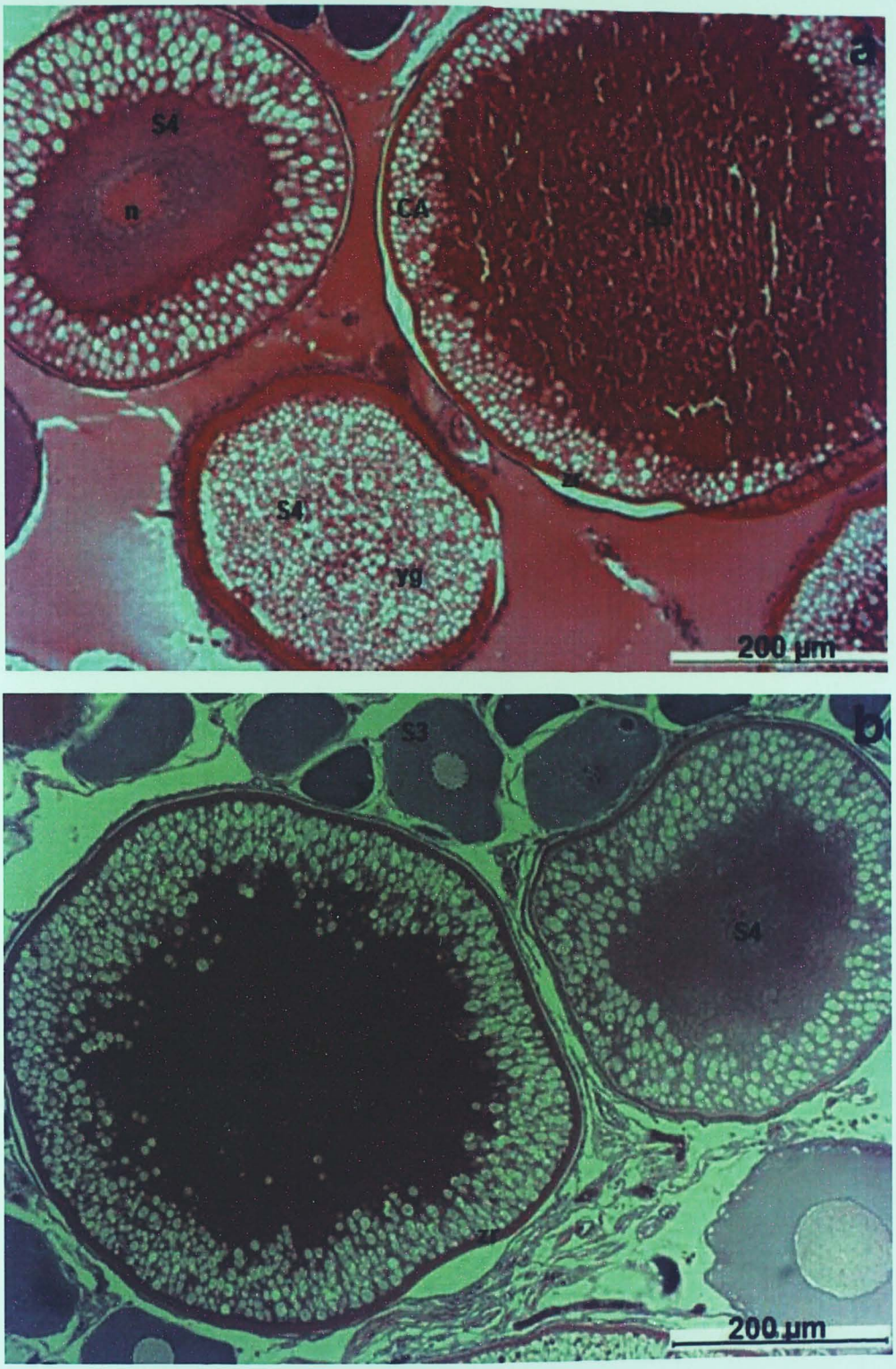


Figure 4.7: Gonadal cross-section of *Tor tambroides* female fed with diet containing 60 g kg^{-1} of lipid after five month of dietary treatment for varying lipid levels from different part of the gonad: a) anterior and b) posterior. (10x magnification). OG: oogonia; S3: stage 3 oocytes; S4: stage 4 oocytes; S5: stage 5 oocytes; ca: cortisol alveolus; n= nucleus; yg: yolk globule; and zr: zona radiata.

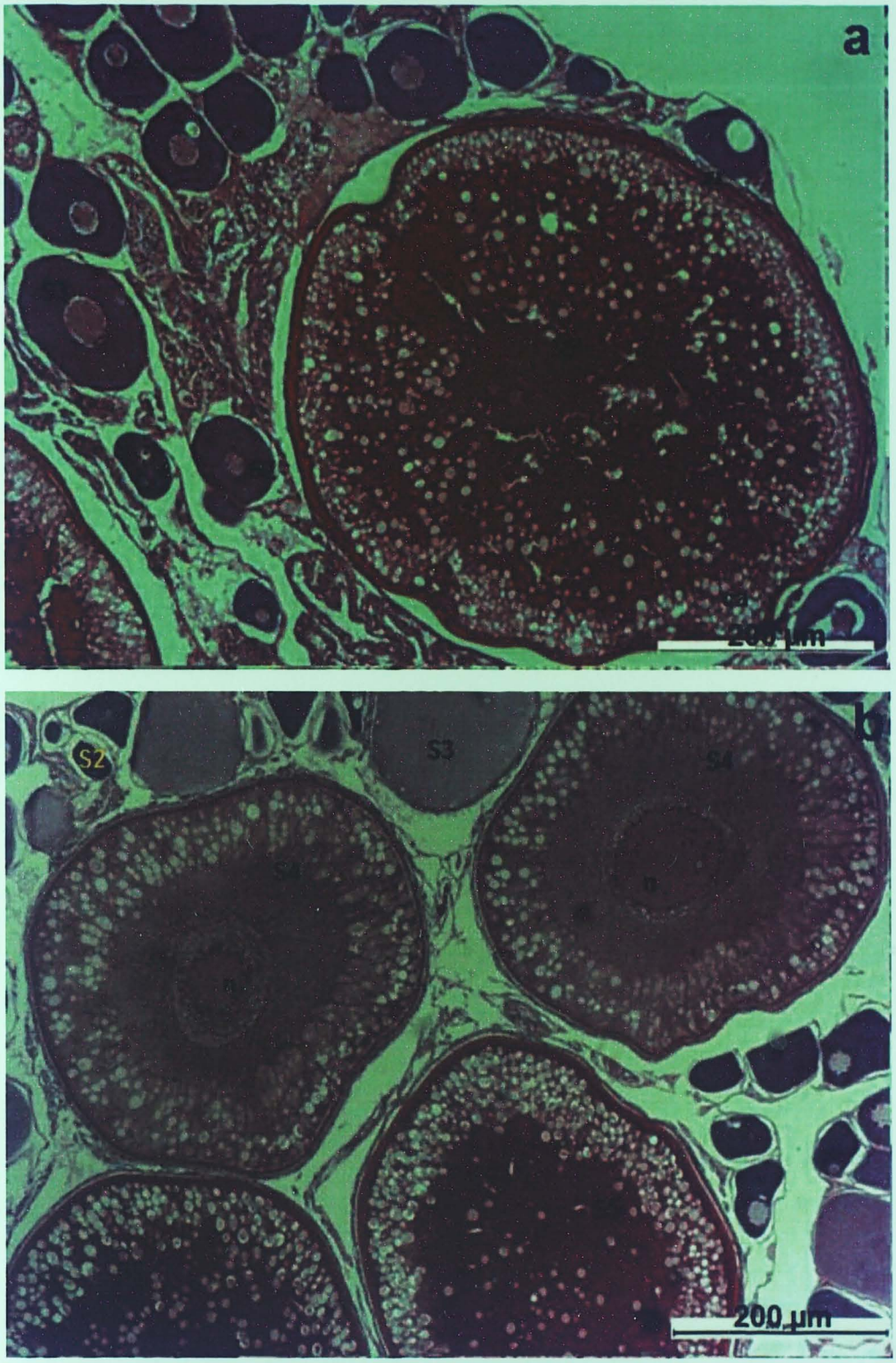


Figure 4.8: Gonadal cross-section of *Tor tambroides* female fed with diet containing 82 g kg^{-1} of lipid after five month of dietary treatment for varying lipid levels from different part of the gonad: a) anterior and b) posterior. (10x magnification). OG: oogonia; S2: stage 2 oocytes; S3: stage 3 oocytes; S4: stage 4 oocytes; S5: stage 5 oocytes; ca: cortisol alveolus; n= nucleus; and zr: zona radiata.

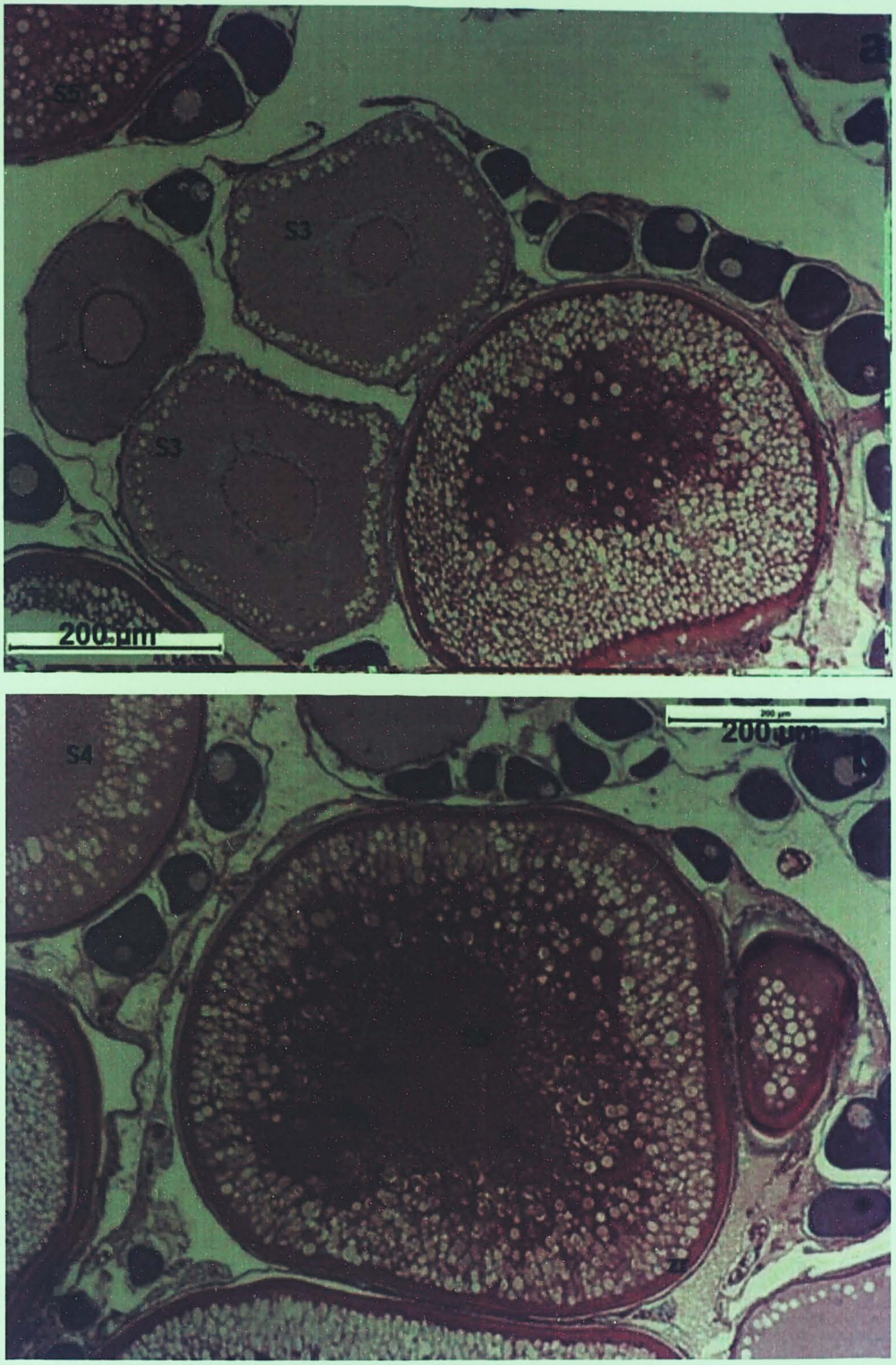


Figure 4.9: Gonadal cross-section of *Tor tambroides* female fed with diet containing 105 g kg^{-1} of lipid after five month of dietary treatment for varying lipid levels from different part of the gonad: a) anterior and b) posterior. (10x magnification). OG: oogonia; S2: stage 2 oocytes; S3: stage 3 oocytes; S4: stage 4 oocytes; S5: stage 5 oocytes; ca: cortisol alveolus; and zr: zona radiata.

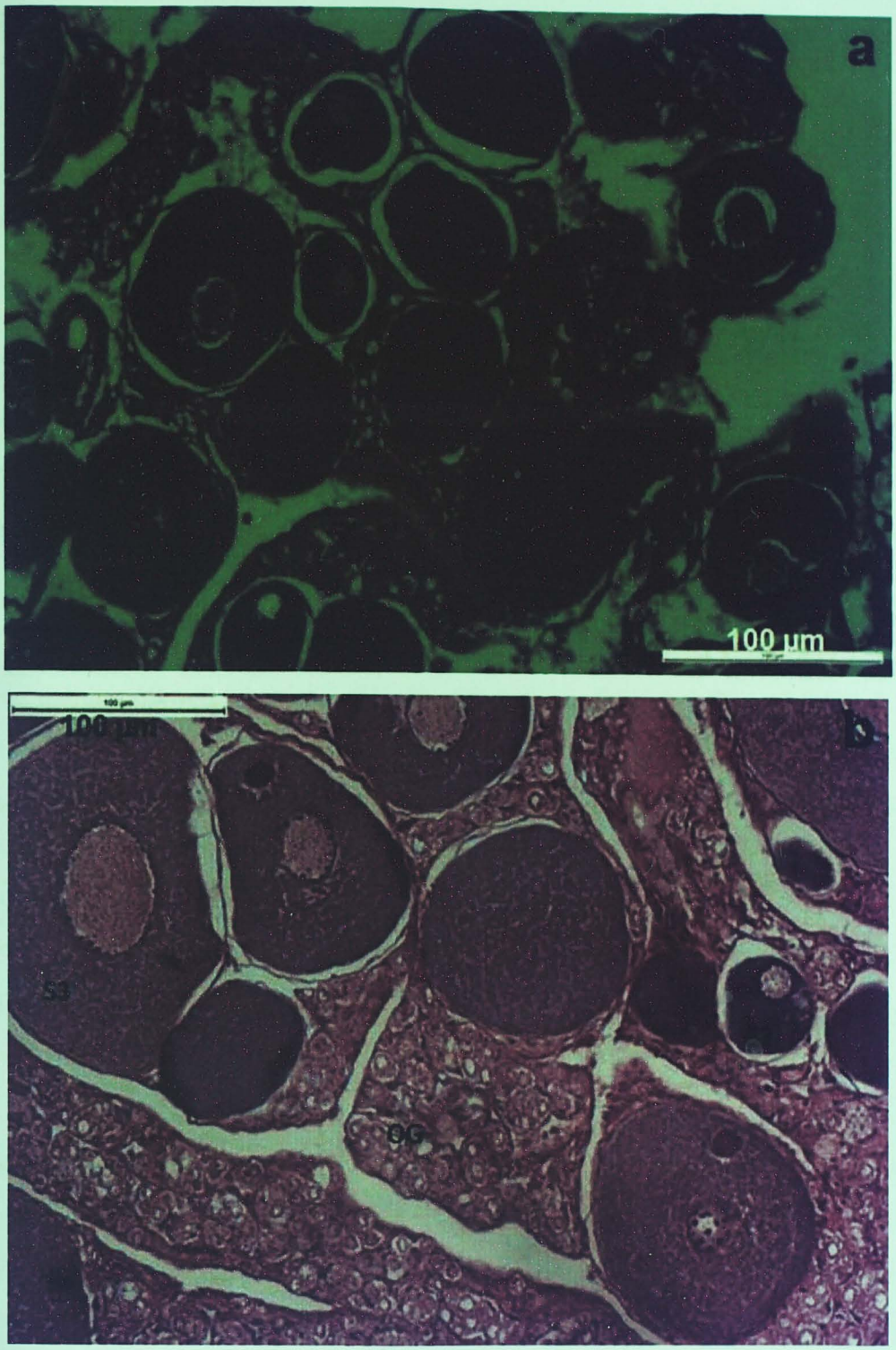


Figure 4.10: Gonadal cross-section of *Tor tambroides* female fed with diet containing 128 g kg^{-1} of lipid after five month of dietary treatment for varying lipid levels from different part of the gonad: a) anterior and b) posterior. (20x magnification). OG: oogonia; S2: stage 2 oocytes; and S3: stage 3 oocytes.

4.2.2.2 Oocyte Size-Frequency Distribution

The oocyte size-frequency distributions of oocyte diameter in all dietary treated groups are illustrated in Figure 4.10. In the anterior part from the vent opening (Figure 4.11a), the gonads of fish fed with diet containing 60 and 82 g kg^{-1} of lipid showed almost similar percentages between S2 and S3 oocytes with 48.3 ± 3.17 to 46.7 ± 5.36% and 48 ± 2.08 to 42 ± 1.67% respectively. Higher value of S3 oocyte (63 ± 6.66%) is found in fish fed with diet containing 105 g kg^{-1} of lipid compared to S2 oocyte (33 ± 3.79%). Conversely, higher percentages of S2 (64.7 ± 3.84%) are demonstrated in fish fed with diet containing 128 g kg^{-1} of lipid compared to S3 oocyte (35.3 ± 3.84%).

An increasing trend of S4 oocytes percentages are seen from fish fed with diet containing 60 and 82 g kg^{-1} of lipid with values of 2.7 ± 1.76 and 6.0 ± 0.58% before dropping to 3.0 ± 2.0% (fish fed with diet containing 105 g kg^{-1} of lipid) and 0% (fish fed with diet containing 128 g kg^{-1} of lipid). A similar pattern are shown for S5 oocytes percentages where the value increase from 2.3 ± 2.33% (fish fed with diet containing 60 g kg^{-1} of lipid) to 3.7 ± 1.20% (fish fed with diet containing 82 g kg^{-1} of lipid) before decreasing to 1.0 ± 1.0% (fish fed with diet containing 105 g kg^{-1} of lipid) and 0% (fish fed with diet containing 128 g kg^{-1} of lipid).

A Kruskal-Wallis test has shown that the distributions of oocytes stages are statistically significantly different between groups, for S2 ($X^2(4) = 9.359$, $p = 0.025$). A post hoc analysis revealed statistically significant differences in percentages of S2 oocyte stages scores between Diet 3 (2.00) and Diet 4 (11.00) ($p=0.013$) but not in other group combination.

As for the posterior part of the gonad (Figure 4.11b), higher amounts of S4, S5 and S6 of oocytes can be found in fish fed with diet containing 60, 82 and 105 g kg^{-1} of lipid. Fish fed with diet containing 82 g kg^{-1} of lipid show the lowest percentages of S2 oocyte with 26.0 ± 11.26% compared to fish fed with diet containing 60 g kg^{-1} of lipid (44.3 ± 8.29%) and fish fed with diet containing 105 g kg^{-1} of lipid (33.0 ± 2.51%). A same pattern can be seen for S3 oocyte with values of

52.7 ± 9.20, 41.7 ± 1.20 and 61.0 ± 4.58% respectively. However, fish fed with diet containing 82 g kg^{-1} of lipid shows the highest percentages of S4, S5 and S6 with 13.3 ± 4.18, 17.7 ± 7.84 and 1.3 ± 0.88% respectively. Fish fed with diet containing 128 g kg^{-1} of lipid posterior gonad only consist of S2 (52.7 ± 4.36%) and S3 oocytes (47.3 ± 4.26%).

Accordingly, a Kruskal-Wallis test had shown that the distributions of oocytes stages are statistically significantly different between groups, for S5 oocyte stage ($X^2(4) = 9.143$, $p = 0.027$). However, no statistically significant pairwise comparisons are found for the S5 oocyte stage scores. A Mann-Whitney test is later run to determine if there were differences in oocytes stage percentages between anterior and posterior gonad samples. There is no statistically significantly difference between anterior and posterior for all dietary treated fish.

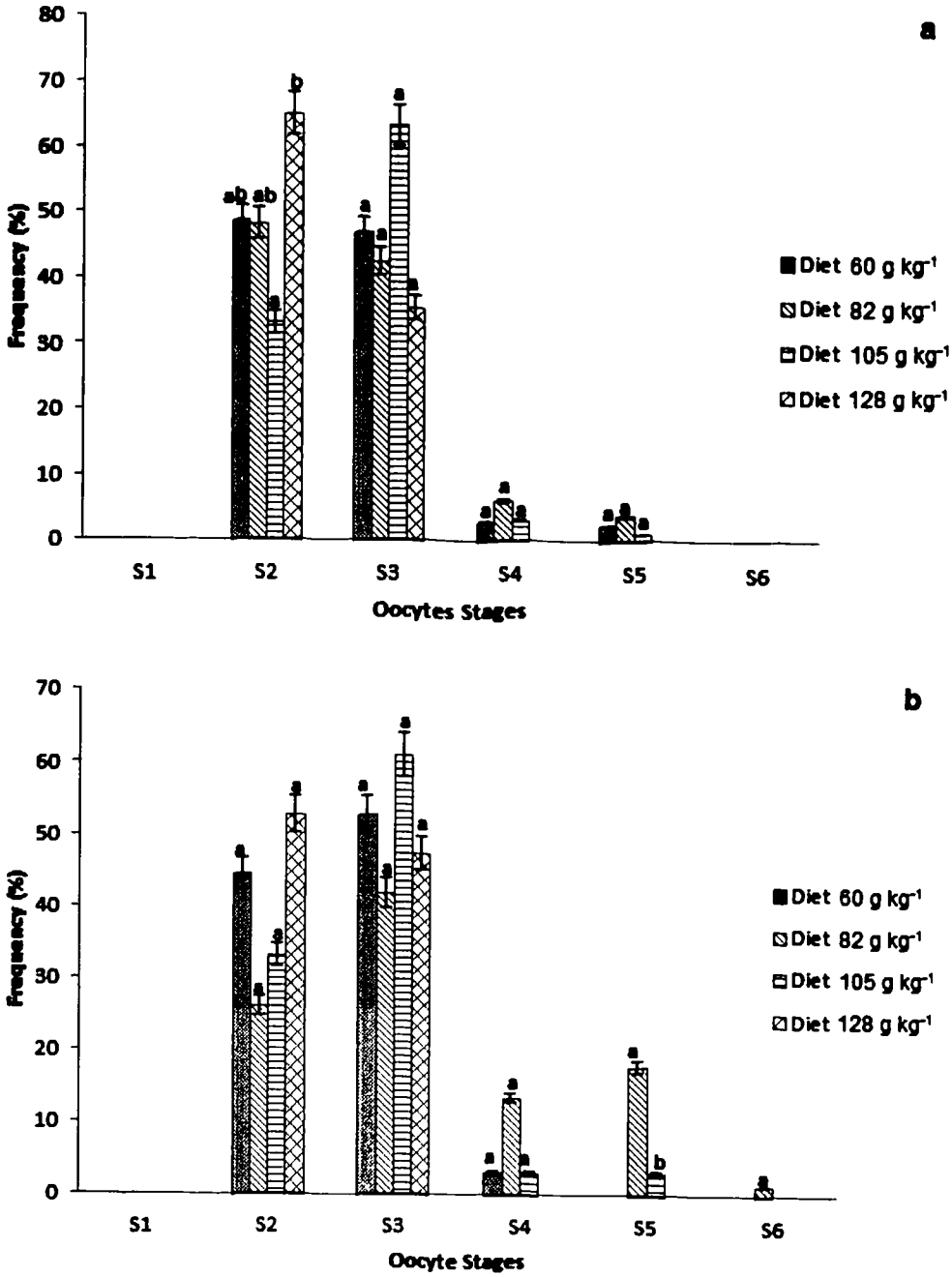


Figure 4.11: Frequency distribution of *Tor tambroides* oocytes for each oocyte stages after five months of feeding trial for varying lipid levels from different part of the gonad: a) anterior ; and b) posterior.

4.2.3 Fatty Acid Analysis

4.2.3.1 Fatty Acid Analysis of the Diets

Fatty acid concentrations (mg g^{-1}) of the diets used in varying lipid levels experiment are shown in Table 4.5. Higher level of total SFA was found in fish fed with diet containing 128 g kg^{-1} of lipid at 10.99 mg g^{-1} compared to other treatments. Based on the ANOVA test conducted, the total MUFA values increase significantly ($p<0.05$) from fish fed with diet containing 60 g kg^{-1} of lipid to fish fed with diet containing 128 g kg^{-1} of lipid, with the value of 4.35, 8.11, 11.29 and 14.09 mg g^{-1} respectively. There are significant differences ($p<0.05$) between all the diets for LA, ALA, ARA, EPA, DHA, total PUFA, total HUFA, total FA, total n3 PUFA, total n6 PUFA and the n3 to n6 ratio values. The total FA increases as the lipid inclusions increases with values varied from 11.55 to 33.64 mg g^{-1}. Although the total n3 and total n6 PUFA increases, however the n3 to n6 PUFA ratio values significantly decreases ($p<0.05$) from 1.32 to 0.58 mg g^{-1}.

Table 4.5: Fatty acid concentrations (mg g^{-1}± SEM) of the diets used (60, 82, 105 and 128 g kg^{-1} of lipid) in the feeding trial for varying lipid levels in the diet.

Fatty acid	Diet 60 (0%)	Diet 82 (2.5%)	Diet 105 (5.0%)	Diet 128 (7.5%)
C16:0	2.21±0.01^a	4.27±0.02^b	5.95±0.01^c	7.61±0.01^d
C18:0	1.56±0.01^a	2.44±0.01^b	2.46±0.00^c	2.01±0.00^d
C16:1	0.59±0.003^a	1.09±0.01^b	1.38±0.00^c	1.21±0.00^d
C18:1n9	1.97±0.01^a	5.10±0.03^b	8.42±0.02^c	10.66±0.01^d
C18:2n6	0.23±0.00^a	2.08±0.01^b	3.72±0.01^c	5.24±0.00^d
C18:3n3	0.09±0.00^a	0.06±0.00^b	0.09±0.00^c	0.13±0.00^d
C20:4n6	0.73±0.00^a	0.05±0.00^b	0.19±0.00^c	0.08±0.00^d
C20:5n3	0.42±0.00^a	0.68±0.00^b	0.62±0.00^c	0.85±0.00^d
C22:6n3	0.86±0.01^a	1.58±0.01^b	1.67±0.00^c	1.77±0.00^d
∑ SFA	4.80±0.03^a	8.16±0.04^b	9.77±0.02^c	10.99±0.01^d
∑ MUFA	4.35±0.02^a	8.11±0.04^b	11.29±0.02^c	14.09±0.01^d
∑ PUFA	0.39±0.00^a	2.14±0.01^b	3.80±0.01^c	5.44±0.01^d
∑ HUFA	2.02±0.01^a	2.64±0.01^b	2.94±0.01^c	3.11±0.00^d
∑ n3	1.37±0.01^a	2.65±0.01^b	2.69±0.01^c	3.14±0.00^d
∑ n6	1.04±0.01^a	2.13±0.01^b	4.05±0.01^c	5.42±0.01^d
n3:n6	1.32±0.01^a	1.25±0.01^b	0.66±0.00^c	0.58±0.00^d
∑ FA	11.55±0.07^a	21.06±0.11^b	27.81±0.05^c	33.64±0.03^d

Values are (mean + SE).
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

4.2.3.2 Fatty Acid Analysis of the Muscles

Fatty acid concentrations (mg g^{-1}) of the muscle of *T. tambroides* females fed with diet containing varying lipid levels are given in Table 4.6. The total SFA values increase from 5.66 mg g^{-1} for fish fed with diet containing 60 g kg^{-1} of lipid to 6.21 mg g^{-1} for fish fed with diet containing 82 g kg^{-1} of lipid before declining to 4.73 and 4.29 mg g^{-1} for fish fed with diet containing 105 and 128 g kg^{-1} of lipid. A similar pattern can be seen in the total FA values with 12.93, 13.33, 11.45 and 10.52 mg g^{-1} for fish fed with diet containing 60, 82, 105 and 128 g kg^{-1} of lipid respectively. The total PUFA decrease from fish fed with diet containing 60 to 128 g kg^{-1} of lipid with values of 1.47 to 0.90 mg g^{-1}. The ANOVA test showed that, there are no significant differences ($p>0.05$) between all the muscle samples for LA, ALA, ARA, EPA, DHA, total SFA, total MUFA, total PUFA, total HUFA, total FA, total n3 PUFA, total n6 PUFA and the n3 to n6 ratio values.

Table 4.6: Fatty acid concentrations (mg g^{-1} ± SEM) of the muscle of *Tor tambroides* females cultured for five months and fed diets with varying lipid levels.

Fatty acid	Diet 60 (0%)	Diet 82 (2.5%)	Diet 105 (5.0%)	Diet 128 (7.5%)
C16:0	1.33±0.36	1.41±0.37	1.07±0.35	0.88±0.15
C18:0	3.25±0.67	3.74±0.78	2.80±0.62	2.54±0.68
C16:1	0.26±0.09	0.17±0.05	0.23±0.11	0.12±0.01
C18:1n9	2.13±0.63	2.05±0.44	1.98±0.51	1.52±0.25
C18:2n6	1.07±0.28	0.87±0.15	0.82±0.36	0.74±0.05
C18:3n3	-	0.05±0.05	0.02±0.02	0.03±0.03
C20:4n6	0.21±0.03	0.33±0.06	0.20±0.08	0.37±0.01
C20:5n3	0.03±0.01	0.13±0.02	0.11±0.07	0.05±0.03
C22:6n3	1.75±0.27	1.87±0.45	1.37±0.32	1.58±0.60
∑ SFA	5.66±1.11	6.21±1.23	4.73±1.05	4.29±1.06
∑ MUFA	3.73±0.43	3.20±0.61	3.68±0.70	3.24±0.80
∑ PUFA	1.47±0.51	1.24±0.40	1.22±0.53	0.90±0.10
∑ HUFA	2.07±0.31	2.69±0.39	1.81±0.30	2.10±0.57
∑ n3	1.80±0.24	2.29±0.43	1.52±0.24	1.70±0.55
∑ n6	1.74±0.58	1.59±0.42	1.49±0.49	1.29±0.09
n3:n6	1.23±0.32	1.65±0.56	1.17±0.28	1.34±0.44
∑ FA	12.93±2.35	13.33±2.26	11.45±1.73	10.52±2.40

Values are (mean + SE); '-'= not detected

4.2.3.3 Fatty Acid Analysis of the Livers

Fatty acid concentrations (mg g^{-1}) of the liver of *T. tambroides* females fed with diet containing varying lipid levels are illustrated in Table 4.7. An increasing value of total SFA is seen between each group with significant difference ($p<0.05$) between fish fed with diet containing 60 and 82 g kg^{-1} of lipid with fish fed with diet containing 128 g kg^{-1} of lipid based on the results from ANOVA test. The values were 15.22, 17.59 and 29.58 mg g^{-1} respectively. A same pattern can be seen in oleic acid values, while for total MUFA values, significant differences ($p<0.05$) can be seen between fish fed with diet containing 60 and 82 g kg^{-1} of lipid (7.76 and 9.34mg g^{-1}) with fish fed with diet containing 105 and 128 g kg^{-1} of lipid (11.97 and 16.28 mg g^{-1}). As for the PUFAs value, increasing values are observed in LA and DHA. Highest values for both are found in fish fed with diet containing 128 g kg^{-1} of lipid with 4.42 and 15.68 mg g^{-1} respectively. There are significantly different ($p<0.05$) values of total HUFA between fish fed with diet containing 60, 82 and 105 g kg^{-1} of lipid with fish fed with diet containing 128 g kg^{-1} of lipid with values of 7.95, 10.42, 14.56 and 17.21 mg g^{-1} respectively. Furthermore, significantly different ($p<0.05$) values of total FA are found between fish fed with diet containing 60 g kg^{-1} of lipid with fish fed with diet containing 105 and 128 g kg^{-1} of lipid. The values are 32.54, 53.12 and 67.88 mg g^{-1} respectively. However, there are no significant differences ($p>0.05$) found in the n3 to n6 PUFA ratio between all the groups.

Table 4.7: Fatty acid concentrations (mg g^{-1} ± SEM) of the liver of *Tor tambroides* females cultured for five months and fed diets with varying lipid levels.

Fatty acid	Diet 60 (0%)	Diet 82 (2.5%)	Diet 105 (5.0%)	Diet 128 (7.5%)
C16:0	7.36±0.77^{a}	8.05±1.76^{a}	12.92±2.18^{ab}	16.46±1.70^{b}
C18:0	6.39±0.78^{a}	8.19±1.78^{ab}	8.98±0.92^{ab}	10.53±0.08^{b}
C16:1	0.65±0.10^{a}	0.70±0.14^{a}	0.86±0.16^{a}	1.67±0.06^{b}
C18:1n9	5.32±0.64^{a}	7.39±1.19^{a}	8.35±1.35^{a}	13.28±0.30^{b}
C18:2n6	1.47±0.03^{a}	1.84±0.86^{a}	2.55±0.73^{ab}	4.42±0.24^{b}
C18:3n3	0.18±0.03^{a}	0.04±0.04^{b}	0.11±0.06^{ab}	0.23±0.00^{a}
C20:4n6	0.55±0.01^{a}	0.73±0.21^{ab}	0.60±0.21^{ab}	1.10±0.11^{b}
C20:5n3	0.20±0.06^{a}	0.08±0.07^{ab}	-	0.06±0.06^{ab}
C22:6n3	6.95±1.35^{a}	9.45±2.12^{ab}	13.59±2.09^{bc}	15.68±0.74^{c}
∑ SFA	15.22±1.42^{a}	17.59±3.69^{a}	23.86±3.41^{ab}	29.58±1.77^{b}
∑ MUFA	7.76±0.60^{a}	9.34±1.19^{a}	11.97±0.62^{b}	16.28±0.43^{c}
∑ PUFA	1.72±0.04^{a}	1.98±0.90^{a}	2.70±0.76^{a}	1.81±0.27^{b}
∑ HUFA	7.95±1.24^{a}	10.42±2.29^{ab}	14.56±2.11^{bc}	17.21±0.87^{c}
∑ n3	7.36±1.27^{a}	9.57±2.06^{ab}	13.77±2.17^{bc}	15.97±0.78^{c}
∑ n6	2.31±0.00^{a}	2.82±1.11^{a}	3.32±0.95^{a}	5.93±0.26^{b}
n3:n6	3.18±0.54	5.04±2.09	4.95±1.84	2.69±0.02
∑ FA	32.54±3.30^{a}	39.33±8.03^{ab}	53.12±5.72^{bc}	67.88±2.47^{c}

Values are (mean + SE); '-'= not detected
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

4.2.3.4 Fatty Acid Analysis of the Gonads

Fatty acid concentrations (mg g^{-1}) of the gonad of *T. tambroides* females fed with varying lipid levels are presented in Table 4.8. The ANOVA test showed that there are no significant differences ($p>0.05$) between all the gonad samples for LA, ALA, ARA, EPA, DHA, total SFA, total MUFA, total PUFA, total HUFA, total FA, total n3 PUFA, total n6 PUFA and the n3 to n6 ratio values.

Table 4.8: Fatty acid concentrations (mg g^{-1} ± SEM) of the gonad of *Tor tambroides* females cultured for five months and fed diets with varying lipid levels.

Fatty acid	Diet 60 (0%)	Diet 82 (2.5%)	Diet 105 (5.0%)	Diet 128 (7.5%)
C16:0	6.10±1.33	6.93±1.09	4.94±0.10	3.60±0.15
C18:0	6.59±0.51	6.18±0.91	7.17±1.85	6.08±0.14
C16:1	0.45±0.05	0.40±0.08	0.35±0.06	0.34±0.02
C18:1n9	6.37±1.06	6.62±0.75	4.83±1.00	3.88±0.07
C18:2n6	1.44±0.13	1.54±0.32	1.53±0.56	1.21±0.15
C18:3n3	0.05±0.03	0.09±0.06	0.05±0.02	0.03±0.02
C20:4n6	0.90±0.05	1.07±0.19	1.88±0.81	1.14±0.03
C20:5n3	0.10±0.06	0.05±0.03	0.10±0.07	-
C22:6n3	9.27±1.43	10.80±2.24	11.69±3.61	9.07±0.33
∑ SFA	14.94±1.69	15.06±2.56	16.01±4.06	12.59±0.05
∑ MUFA	8.24±1.44	8.27±0.71	6.79±1.59	5.54±0.19
∑ PUFA	1.64±0.09	1.75±0.39	1.74±0.60	1.32±0.13
∑ HUFA	10.49±1.46	12.42±2.35	13.89±4.43	10.51±0.39
∑ n3	9.46±1.52	10.97±2.32	11.84±3.58	9.20±0.37
∑ n6	2.67±0.16	3.18±0.43	3.79±1.46	2.55±0.15
n3:n6	3.64±0.80	3.38±0.26	3.33±0.35	3.66±0.36
∑ FA	35.31±4.49	37.51±6.00	38.43±10.64	29.85±0.02

Values are (mean + SE); '-'= not detected

4.2.3.5 Differences of Fatty Acid Concentrations between the Diet given, Muscle, Liver and Gonad of Female *Tor tambroides*

The differences of FAs concentration (mg g^{-1}) between sources of *T. tambroides* females fed with diet containing varying lipid levels are shown in Table 4.9. For fish fed with diet containing 60 g kg^{-1} of lipid, the concentrations of FA given through the diet reflects in the muscle, liver and gonad sample for oleic acid, LA and DHA values. Each sample demonstrates higher values of referred fatty acids compared in the diet. There are significant difference in total FA between diet with liver and gonad with values of 11.55, 32.64 and 35.32 mg g^{-1} respectively.

In fish fed with diet containing 82 g kg^{-1} of lipid, higher values of ARA and DHA are found in muscle, liver and gonad samples; meanwhile lower levels of LA and EPA are detected compared to the diet given. For the total FA concentration, the muscle exhibit lowest concentration with 13.33 mg g^{-1} compared to 39.33 mg g^{-1} which are shown in the liver.

For fish fed with diet containing 105 g kg^{-1} of lipid, values of oleic acid expressed by the liver are significantly different compared to the muscle and gonad. The FAs concentration in the muscle is significantly lower for oleic acid, LA, and EPA values compared to the diet. The total FA values are higher for the liver (53.12 mg g^{-1}; $p<0.05$) and gonad (38.43 mg g^{-1}) compared to the diet (28.31 mg g^{-1}).

The ANOVA test showed that there are significant differences between muscle, liver and gonad sample with the diet given for the OA, LA, ALA, ARA and EPA concentrations for fish fed with diet containing 128 g kg^{-1} of lipid. The value of total FA in liver (67.88 mg g^{-1}) is significantly higher compared to the diets given (33.64 mg g^{-1}).

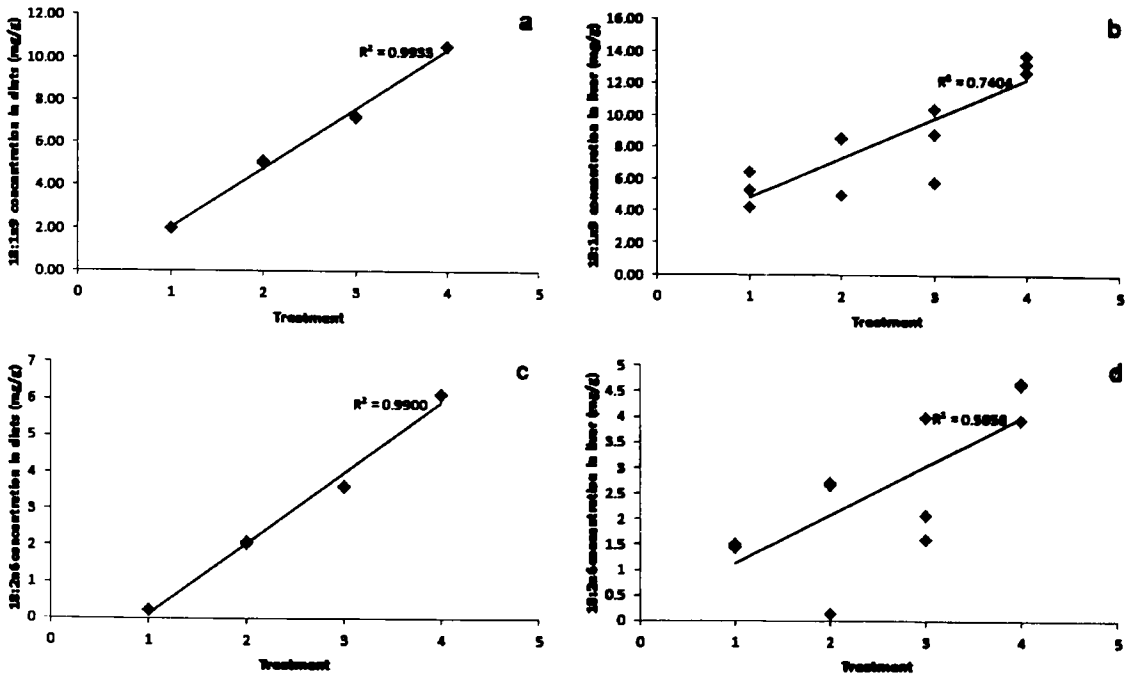
Table 4.9: Fatty acid concentrations (mg g^{-1} ± SEM) of the diet, muscle, liver and gonad of *Tor tambroides* females fed with varying lipid levels diet for five months.

Fatty acid	Diet	Muscle	Liver	Gonad
Diet 60 g kg^{-1}				
C18:1n9	1.97±0.01^{a}	2.13±0.63^{a}	5.32±0.64^{b}	6.37±1.06^{b}
C18:2n6	0.23±0.003^{a}	1.07±0.28^{b}	1.47±0.03^{b}	1.44±0.13^{b}
C18:3n3	0.09±0.0005^{a}	-	0.18±0.03^{b}	0.05±0.03^{ac}
C20:4n6	0.74±0.003^{a}	0.21±0.03^{b}	0.55±0.01^{c}	0.90±0.05^{d}
C20:5n3	0.42±0.003^{a}	0.03±0.01^{b}	0.20±0.06^{c}	0.10±0.06^{bc}
C22:6n3	0.86±0.01^{a}	1.75±0.27^{a}	6.95±1.35^{b}	9.27±1.43^{b}
Total FA	11.55±0.07^{a}	12.93±2.35^{a}	32.64±3.30^{b}	35.32±4.49^{b}
Diet 82 g kg^{-1}				
C18:1n9	5.10±0.03^{a}	2.05±0.44^{b}	7.39±1.19^{a}	6.62±0.75^{a}
C18:2n6	2.08±0.01	0.87±0.15	1.84±0.86	1.54±0.32
C18:3n3	0.06±0.0002	0.05±0.05	0.04±0.04	0.09±0.06
C20:4n6	0.05±0.0005^{a}	0.33±0.06^{ab}	0.73±0.21^{bc}	1.07±0.19^{c}
C20:5n3	0.68±0.003^{a}	0.13±0.02^{b}	0.08±0.07^{b}	0.05±0.03^{b}
C22:6n3	1.58±0.01^{a}	1.87±0.45^{a}	9.45±2.12^{b}	10.80±2.24^{b}
Total FA	21.06±0.10^{ab}	13.33±2.26^{b}	39.33±8.03^{c}	37.51±6.00^{ac}
Diet 105 g kg^{-1}				
C18:1n9	7.22±0.02^{ac}	1.98±0.51^{b}	8.35±1.35^{c}	4.83±1.00^{ab}
C18:2n6	3.62±0.01^{a}	0.82±0.36^{b}	2.55±0.73^{ac}	1.54±0.56^{bc}
C18:3n3	0.09±0.0001	0.02±0.02	0.11±0.06	0.05±0.02
C20:4n6	0.19±0.0005^{a}	0.20±0.08^{a}	0.60±0.21^{ab}	1.88±0.81^{b}
C20:5n3	0.62±0.0005^{a}	0.11±0.07^{b}	-	0.10±0.07^{b}
C22:6n3	1.57±0.003^{a}	1.37±0.32^{a}	13.59±2.09^{b}	11.69±3.61^{ab}
Total FA	28.31±0.05^{ab}	11.47±1.73^{b}	53.12±5.72^{c}	38.43±10.64^{ac}
Diet 128 g kg^{-1}				
C18:1n9	10.55±0.01^{a}	1.52±0.25^{b}	13.28±0.30^{c}	3.88±0.07^{d}
C18:2n6	6.14±0.13^{a}	0.74±0.05^{b}	4.42±0.24^{c}	1.21±0.15^{d}
C18:3n3	0.13±0.0001^{a}	0.03±0.03^{b}	0.23±0.004^{c}	0.03±0.02^{b}
C20:4n6	0.08±0.0005^{a}	0.37±0.01^{b}	1.10±0.11^{c}	1.14±0.03^{c}
C20:5n3	0.85±0.0005^{a}	0.05±0.03^{b}	0.06±0.06^{b}	-
C22:6n3	1.57±0.0005^{a}	1.58±0.60^{a}	15.68±0.74^{b}	9.07±0.33^{c}
Total FA	33.64±0.03^{a}	10.53±2.40^{b}	67.88±2.47^{c}	23.85±0.02^{a}

Values are (mean + SE); '-'= not detected
Rows assign different letters were significantly different (ANOVA, $p<0.05$)

4.2.3.6 Relationship between Fatty Acid Concentrations from Different Sources of Diet, Muscle, Liver and Gonad with Treatments

The correlation between FA concentrations from different source of diet, muscle, liver and gonad with treatments are illustrated in Figure 4.12. There are strong positive correlation of oleic acid ($r(10)=0.997$; $p<0.01$; Figure 4.11a), LA ($r(10)=0.995$; $p<0.01$; Figure 4.11c), DHA ($r(10)=0.580$; $p<0.05$; Figure 4.11e) and total FA ($r(10)=0.992$; $p<0.01$; Figure 4.11g) between diet and treatment. Strong positive correlation is also found between liver and treatment for oleic acid ($r(10)=0.860$; $p<0.01$; Figure 4.11b), LA ($r(10)=0.765$; $p<0.01$; Figure 4.11d),DHA ($r(10)=0.815$; $p<0.01$; Figure 4.11f) and total FA ($r(10)=0.862$; p 0.01; Figure 4.11h).



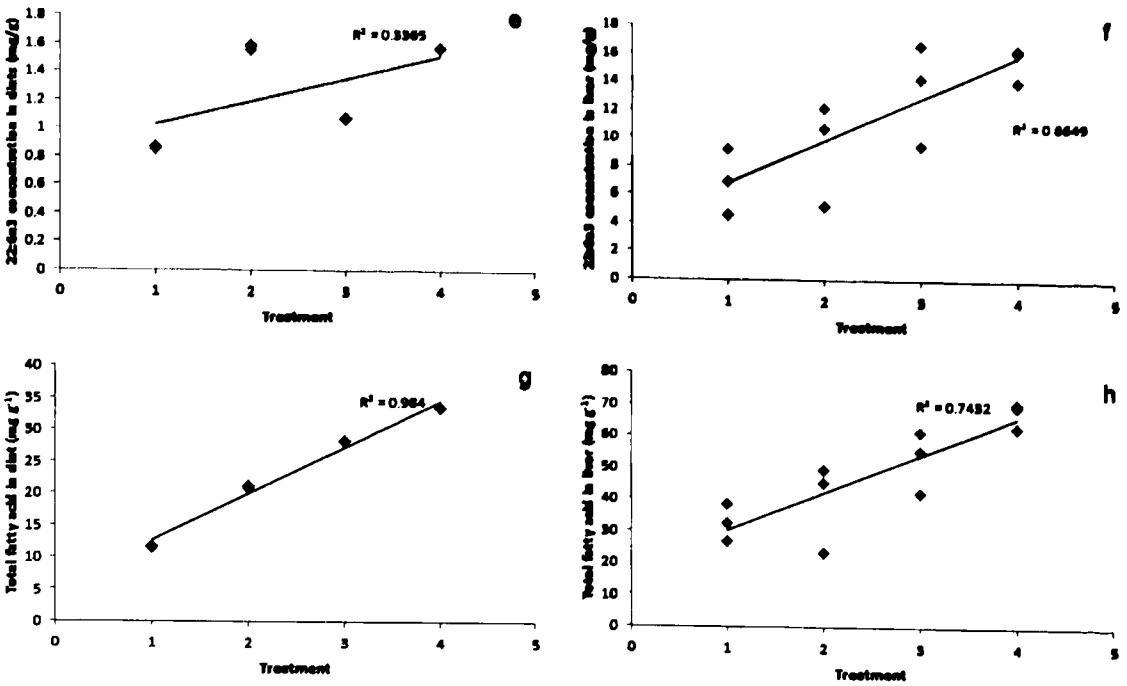


Figure 4.12: Relationship between (a) oleic acid (OA) in diet with treatment; (b) oleic acid (OA) in liver with treatment; (c) linoleic acid (LA) in diet with treatment; (d) linoleic acid (LA) in liver with treatment; (e) docosahexanoic acid (DHA) in diet with treatment; (f) docosahexanoic acid (DHA) in liver with treatment; (g) total fatty acid in diet with treatment and (h) total fatty acid in liver with treatment.

4.2.4 Hormone Analysis

The concentrations of E2 (ng ml^{-1}) in blood plasma of females *T. tambroides* after five months of feeding trial with diets containing varying lipid levels are shown in Figure 4.13. The E2 concentration increases from 0h to 12h, before decreasing at 24h. The E2 concentrations of fish fed with diet containing 82 g kg^{-1} are higher than the other three dietary treatments at every blood sampling. At 0h, the ANOVA test showed that there are significant differences ($p<0.05$) between fish fed with diet containing 82 g kg^{-1} of lipid with fish fed with diet containing 60 and 105 g kg^{-1} of lipid treated fish with values of 0.14, 0.03 and 0.07 ng ml^{-1} respectively. Meanwhile fish fed with diet containing 128 g kg^{-1} of lipid shows values of 0.12 ng ml^{-1}.

Subsequently, the E2 concentrations peaked at 12h with fish fed with diet containing 82 g kg^{-1} of lipid is significantly different ($p<0.05$) compared to other dietary treatments with values of 0.75 ng ml^{-1}. The values of E2 for fish fed with diet containing 60, 105 and 128 g kg^{-1} of lipid are 0.36, 0.29 and 0.28 ng ml^{-1} respectively. Meanwhile, at 24h, the concentrations of E2 are rapidly decreasing from the 12h values. The values of E2 for fish fed with diet containing 60 g kg^{-1} (0.1 ng ml^{-1}) and fish fed with diet containing 82 g kg^{-1} of lipid (0.1 ng ml^{-1}) are significantly different ($p<0.05$) compared to fish fed with diet containing 105 g kg^{-1} of lipid (0.061 ng ml^{-1}), and fish fed with diet containing 128 g kg^{-1} of lipid (0.034 ng ml^{-1}).

In this experiment, the E2 levels of female broodstock which are also reared in the hatchery and had ovulated previously (FCE) are also taken for comparison purposes. The values of E2 were 0.67, 1.47 and 0.41 ng ml^{-1} for 0h, 12h and 24h respectively. These values are significantly higher ($p<0.05$) compared to all diet treated fish based on the ANOVA test results.

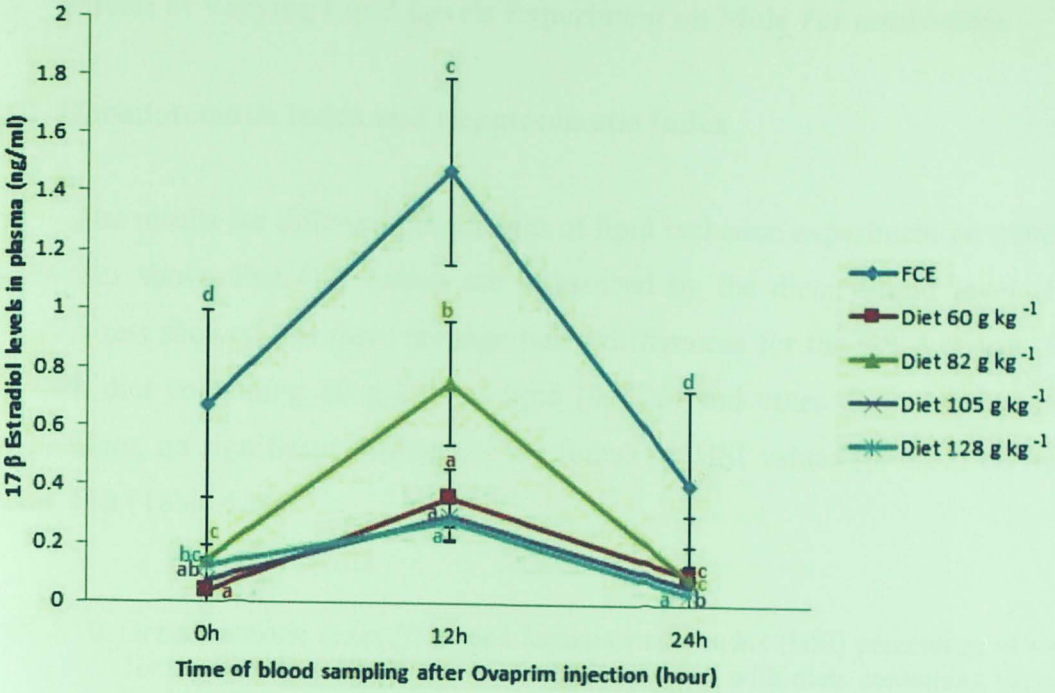


Figure 4.13: 17β estradiol changes in *Tor tambroides* female broodstocks in 24-h artificial induced ovulation with ovaprim after five month of dietary treatment with diets containing varying lipid levels (Diet 60, 82, 105 and 128 g kg $^{-1}$) and females with confirm eggs released (FCE)

4.3 Effects of Varying Lipid Levels Experiment on Male *Tor tambroides.*

4.3.1 Gonadosomatic Index and Hepatosomatic Index

The results for different percentages of lipid inclusion experiment on male *T. tambroides* shows that GSI values are influenced by the dietary lipid level. The ANOVA test showed that there are significant differences for the GSI between fish fed with diet containing 82 g kg^{-1} of lipid (0.67%) and other dietary treatments. Furthermore, no significant differences are found for HSI values ($p>0.05$) between treated fish (Table 4.10).

Table 4.10: Gonadosomatic index (GSI) and hepatosomatic index (HSI) percentage of male *Tor tambroides* cultured for five months and fed with diets containing varying lipid levels.

Group of treatment	Diet 60 (0%)	Diet 82 (2.5%)	Diet 105 (5.0%)	Diet 128 (7.5%)
GSI (%)	0.11 ± 0.02^{a}	0.67 ± 0.17^{b}	0.27 ± 0.02^{a}	0.28 ± 0.04^{a}
HSI (%)	0.43±0.0002	0.42±0.03	0.49±0.08	0.43±0.02

Values are (mean + SE).
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

4.3.2 Gonad Analysis

4.3.2.1 Histological Analysis

Phases of spermatogenesis are defined in male *T.tambroides* based on histological changes following Ismail *et al.* (2011) and Wibowo and Kaban., (2014). Observation on the gonad shows that all stages of spermatogenesis are found in all samples. All anterior samples which are nearer to the vents opening show only free spermatozoa and some spermatocytes. Higher density of spermatozoa can be seen in fish fed with diet containing 82 g kg^{-1} of lipid (Figure 4.14a) and fish fed with diet containing 105 g kg^{-1} of lipid (Figure 4.15a) compared to fish fed with diet containing 60 and 128 g kg^{-1} of lipid (Figure 4.13a and 4.16a respectively). Meanwhile, in the posterior, all stages of spermatogenesis are exhibit by the treated fish (Figure 4.13b, 4.14b, 4.15b and 4.16b). These stages of spermatogenesis are usually found in the periphery of the gonad. The size of each spermatogenesis stages are stage dependent, where the size decreases from spermatocyte, to spermatid and to spermatozoa.

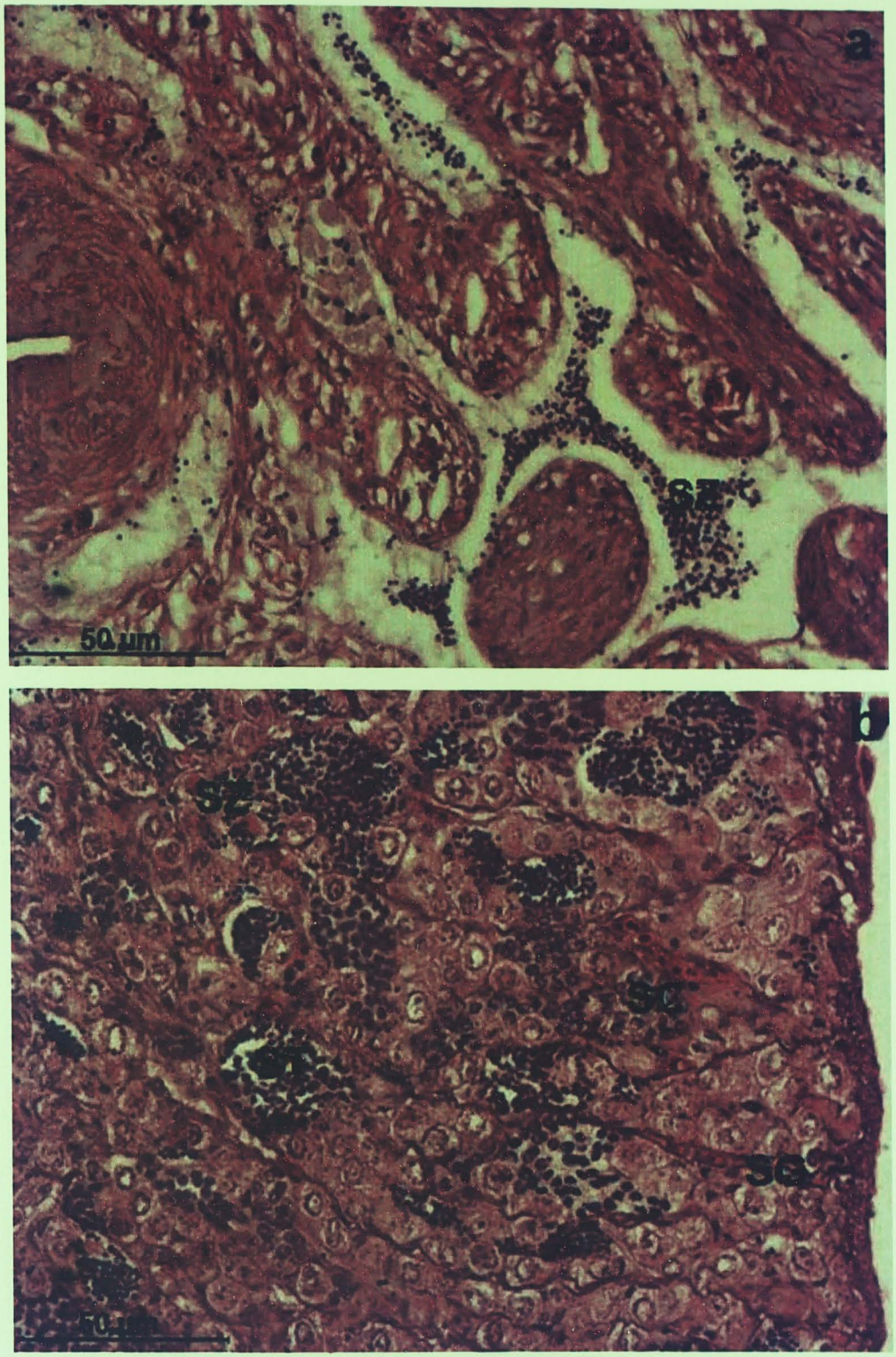


Figure 4.14: Gonadal cross-section of *Tor tambroides* male fed with diet containing 60 g kg^{-1} of lipid after five month of dietary treatment for varying lipid levels from different part of the gonad: a) anterior and b) posterior. (40x magnification).SG: spermatogonia; SC: spermatocyte; ST: spermatid and SZ: spermatozoa.

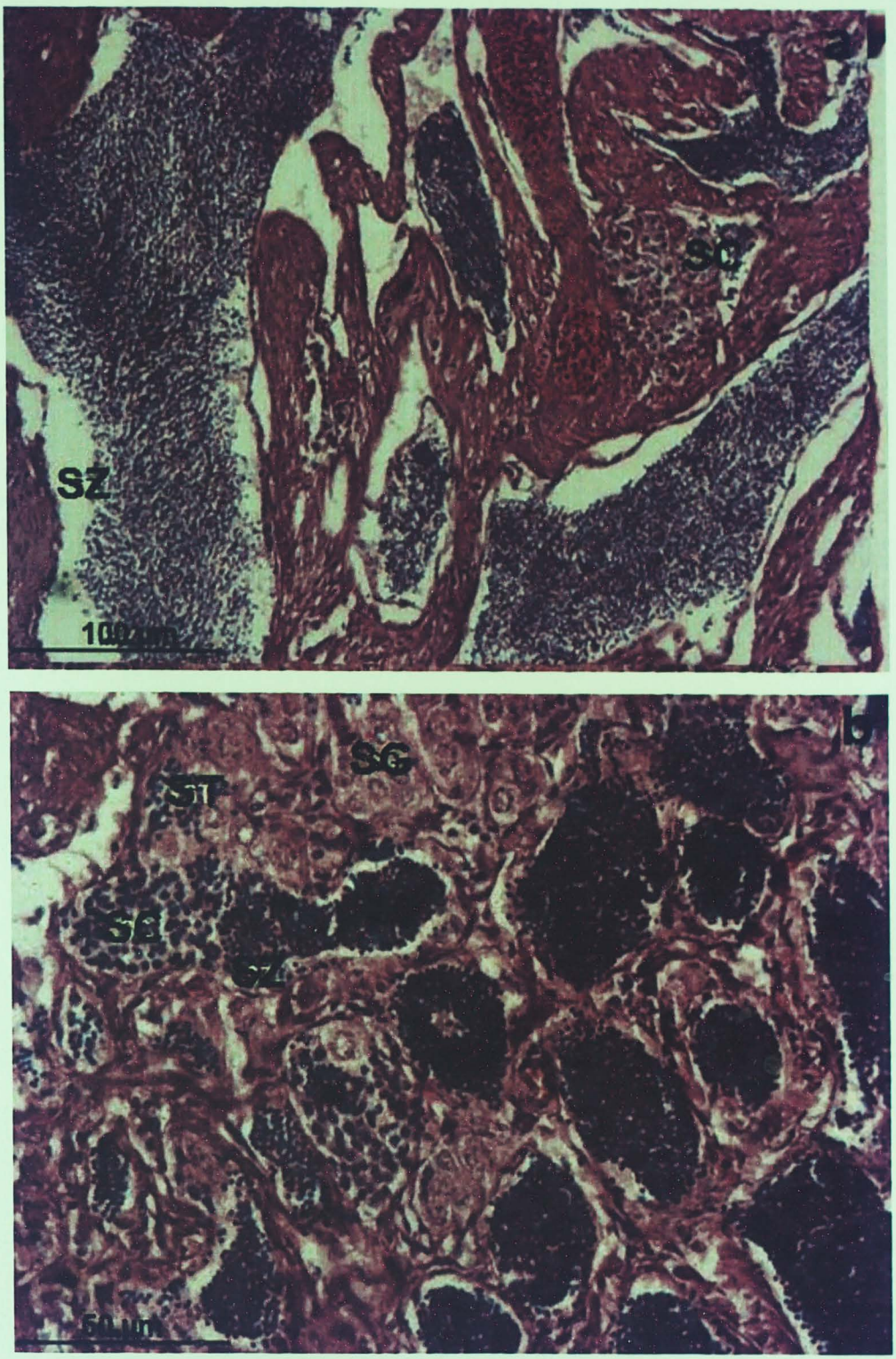


Figure 4.15: Gonadal cross-section of *Tor tambroides* male fed with diet containing 82 g kg^{-1} of lipid after five month of dietary treatment for varying lipid levels from different part of the gonad: a) anterior (20x magnification) and b) posterior (40x magnification). SG: spermatogonia; SC: spermatocyte; ST: spermatid and SZ: spermatozoa.

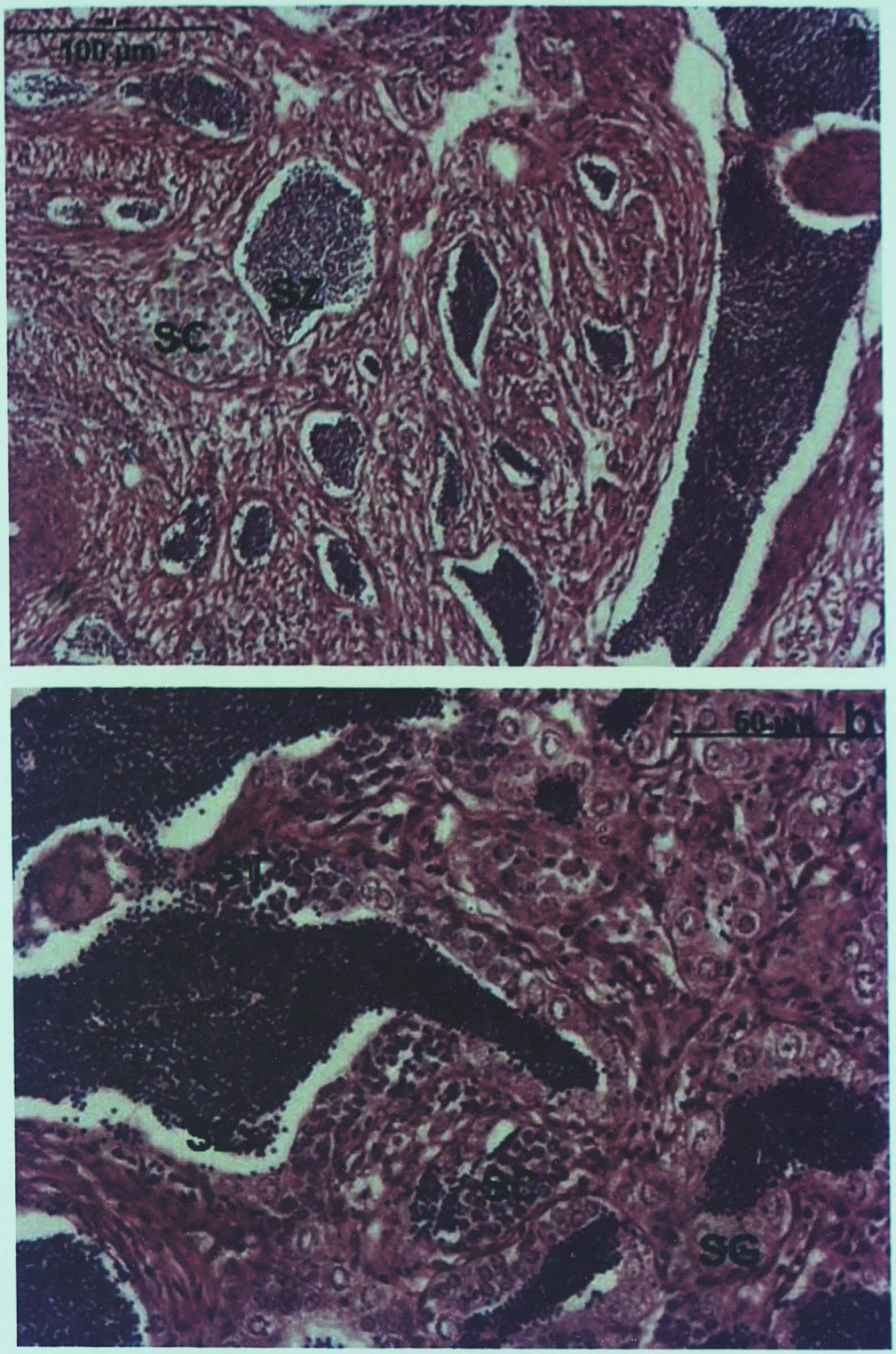


Figure 4.16: Gonadal cross-section of *Tor tambroides* male fed with diet containing 105 g kg^{-1} of lipid after five month of dietary treatment for varying lipid levels from different part of the gonad: a) anterior (20x magnification) and b) posterior. (40x magnification). SG: spermatogonia; SC: spermatocyte; ST: spermatid and SZ: spermatozoa.

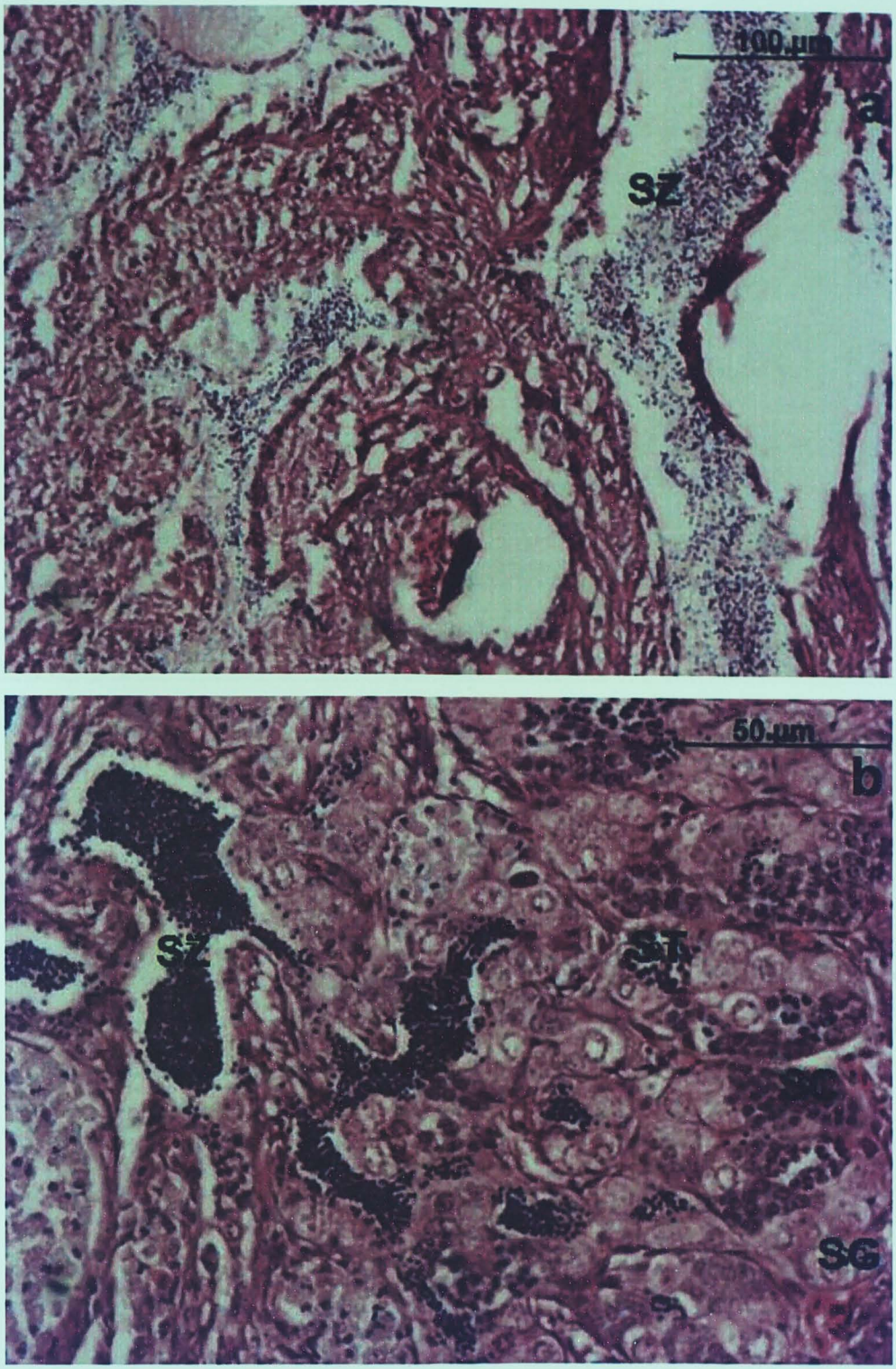


Figure 4.17: Gonadal cross-section of *Tor tambroides* male fed with diet containing 128 g kg^{-1} of lipid after five month of dietary treatment for varying lipid levels from different part of the gonad: a) anterior (20x magnification) and b) posterior. (40x magnification). SG: spermatogonia; SC: spermatocyte; ST: spermatid and SZ: spermatozoa.

4.3.2.2 Sperm Quality Assessment

Assessments on the sperm quality on volume, concentration and motility of all the treated fish are conducted. However, only three fish are able to exert measureable sperm at the end of the experiment; two from fish fed with 82 g kg^{-1} of lipid, and one from fish fed with 128 g kg^{-1} of lipid. Therefore, no statistical analysis is conducted. The results are shown in Table 4.11.

Table 4.11: Sperm quality assessment on the sperm of *Tor tambroides* after five month of dietary treatment with varying lipid levels.

Sperm quality parameter	Fish 1 (Diet 82 g kg^{-1})	Fish 2 (Diet 82 g kg^{-1})	Fish 3 (Diet 128 g kg^{-1})
Sperm volume (ml)	0.3	2.0	0.5
Sperm concentration	5.51×10^{9}	1.64×10^{10}	3.72×10^{9}
Sperm motility	70%	80%	50%

4.3.3 Fatty Acid Analysis

4.3.3.1 Fatty Acid Analysis of the Muscles

Fatty acid concentrations (mg g^{-1}) of the diets used in varying lipid levels experiment are shown in Table 4.5 and had been explained in chapter 4.2.3.1. Fatty acid concentrations (mg g^{-1}) of the muscle of *T. tambroides* males fed with diet containing varying lipid levels are given in Table 4.12. There is a pattern where the FA concentration increases from fish fed with 60g kg^{-1} to fish fed with 82 g kg^{-1} of lipid where it is the highest concentration. The FA concentrations then were lower for fish fed with105 and 128 g kg^{-1} of lipid samples. The FAs are palmitic acid, stearic acid, LA and DHA. This pattern is also illustrated by the value of total SFA, total MUFA, total n3, total n6, n3 to n6 ratios and total FA. However, the ANOVA test showed that significant differences ($p<0.05$) are only found between fish fed with 82 g kg^{-1} and fish fed with 128 g kg^{-1} of lipid for DHA (2.19 and 1.16 mg g^{-1}), total HUFA (2.81 and 1.91 mg g^{-1}), total n3 (2.29 and 1.38 mg g^{-1}) and n3 to n6 ratio values (1.20 and 0.81).

Table 4.12: Fatty acid concentrations (mg g^{-1} ± SEM) of the muscle of *Tor tambroides* males cultured for five months and fed diets with varying lipid levels.

Fatty acid	Diet 60 (0%)	Diet 82 (2.5%)	Diet 105 (5.0%)	Diet 128 (7.5%)
C16:0	1.39±0.18	1.42±0.28	1.23±0.40	1.13±0.01
C18:0	3.78±0.54	4.22±0.74	3.23±1.08	2.81±0.03
C16:1	0.20±0.003	0.19±0.02	0.21±0.08	0.19±0.001
C18:1n9	2.42±0.24	2.40±0.38	2.09±0.64	1.68±0.01
C18:2n6	1.10±0.01	1.12±0.16	1.04±0.31	0.99±0.02
C18:3n3	$0.02±0.01^{a}$	$0.04±0.004^{b}$	$0.05±0.00002^{b}$	-
C20:4n6	0.41±0.10	0.49±0.23	0.25±0.02	0.47±0.22
C20:5n3	$0.09±0.01^{a}$	$0.06±0.03^{a}$	$0.10±0.02^{a}$	$0.18±0.01^{b}$
C22:6n3	$1.68±0.24^{ab}$	$2.19±0.16^{b}$	$1.43±0.27^{a}$	$1.16±0.19^{a}$
∑ SFA	6.18±0.83	6.94±1.09	5.42±1.70	4.68±0.004
∑ MUFA	3.54±0.15	3.88±0.39	3.12±0.67	3.50±0.12
∑ PUFA	1.42±0.18	1.42±0.29	1.44±0.48	1.16±0.09
∑ HUFA	$2.34±0.16^{ab}$	$2.81±0.08^{b}$	$1.88±0.29^{a}$	$1.91±0.39^{a}$
∑ n3	$1.89±0.22^{ab}$	$2.29±0.14^{b}$	$1.65±0.29^{ab}$	$1.38±0.16^{a}$
∑ n6	1.87±0.12	1.93±0.15	1.67±0.48	1.69±0.14
n3:n6	$1.00±0.05^{ab}$	$1.20±0.10^{b}$	$1.07±0.15^{ab}$	$0.81±0.03^{a}$
∑ FA	13.48±1.32	15.04±1.34	11.87±3.14	11.24±0.42

Values are (mean + SE); '-'= not detected
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

4.3.3.2 Fatty Acid Analysis of the Livers

Fatty acid concentrations (mg g^{-1}) of the liver of *T. tambroides* males fed with diets containing varying lipid levels are illustrated in Table 4.13. There is a decreasing pattern for OA, total MUFA and total FA levels as the values of lipid levels increases. The values are 8.23 to 5.63 mg g^{-1}, 13.32 to 8.89 mg g^{-1}, and 47.72 to 35.94 mg g^{-1}, respectively. The ANOVA test showed that total MUFA concentrations are significantly different ($p<0.05$) between each dietary treatment.

Highest value of palmitoleic acid (C16:1), LA, ALA, ARA, total PUFA and total n6 can be found in fish fed with diet containing 82 g kg^{-1} of lipid with values of 1.07, 3.92, 0.22, 0.97, 4.95 and 5.90 mg g^{-1} respectively. However, only LA, total PUFA and total n6 concentrations are significantly different between fish fed with 82 g kg^{-1} of lipid with other dietary treatment.

Table 4.13: Fatty acid concentrations (mg g^{-1} ± SEM) of the liver of *Tor tambroides* males cultured for five months and fed diets with varying lipid levels.

Fatty acid	Diet 60 (0%)	Diet 82 (2.5%)	Diet 105 (5.0%)	Diet 128 (7.5%)
C16:0	9.35±1.93	8.41±0.52	8.69±1.03	7.76±1.10
C18:0	8.36±1.20	5.99±0.71	8.02±0.54	7.43±1.00
C16:1	0.90±0.13^{a}	1.07±0.06^{a}	0.90±0.01^{a}	0.51±0.08^{b}
C18:1n9	8.23±1.06	7.63±0.42	7.11±0.41	5.63±1.02
C18:2n6	2.99±0.27^{a}	3.92±0.03^{b}	2.76±0.36^{ac}	2.07±0.27^{c}
C18:3n3	0.11±0.06^{ab}	0.22±0.04^{b}	0.06±0.04^{a}	-
C20:4n6	0.69±0.12	0.97±0.32	0.48±0.02	0.56±0.12
C20:5n3	0.11±0.07	0.06±0.03	0.05±0.03	0.08±0.05
C22:6n3	10.74±2.34	7.44±0.74	6.06±0.09	6.53±1.47
∑ SFA	19.37±3.32	17.90±2.14	20.64±0.48	17.62±1.80
∑ MUFA	13.32±0.33^{a}	10.91±0.35^{b}	9.90±0.35^{c}	8.89±0.06^{d}
∑ PUFA	3.28±0.29^{a}	4.95±0.74^{b}	2.91±0.35^{a}	2.14±0.32^{a}
∑ HUFA	11.75±2.51	8.67±0.63	6.78±0.002	7.29±165
∑ n3	10.96±2.47	7.72±0.70	6.23±0.06	6.61±1.52
∑ n6	4.06±0.33^{a}	5.90±0.73^{b}	3.46±0.28^{a}	2.82±0.45^{a}
n3:n6	2.60±0.40^{a}	1.33±0.11^{b}	1.83±0.13^{bc}	2.26±0.17^{ac}
∑ FA	47.72±5.79	42.44±2.06	40.22±0.48	35.94±3.83

Values are (mean + SE); '-'= not detected
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

4.3.3.3 Fatty Acid Analysis of the Gonads

Fatty acid concentrations (mg g^{-1}) of the gonad of *T. tambroides* males fed with diets containing varying lipid levels are presented in Table 4.14. Fish fed with diet containing 60 g kg^{-1} of lipid demonstrate the lowest palmitic acid (C16:0) concentration compared to fish fed with diet containing 128 g kg^{-1} of lipid. Similarly, the same pattern is found for the concentration of other FA concentration except for concentrations of palmitoleic acid (C16:1) and LA. However, significant differences ($p<0.05$) are only found for concentration of palmitic acid (C16:0), stearic acid (C18:0), ARA, DHA, total SFA, total MUFA, total HUFA, total n3, total n6 and total FA concentrations. The values for total FA between all dietary treated groups were 15.05, 23.96, 24.05 and 33.70 mg g^{-1} respectively. The ANOVA test showed that there is no significant difference between all dietary treated groups found in the n3 to n6 PUFA ratio values.

Table 4.14: Fatty acid concentrations (mg g^{-1} ± SEM) of the gonad of *Tor tambroides* males cultured for five months and fed diets with varying lipid levels.

Fatty acid	Diet 60 (0%)	Diet 82 (2.5%)	Diet 105 (5.0%)	Diet 128 (7.5%)
C16:0	1.65±0.05^{a}	3.18±0.52^{b}	3.27±0.40^{b}	4.45±0.53^{b}
C18:0	2.86±0.07^{a}	4.52±1.09^{ab}	4.18±0.33^{ab}	5.59±0.84^{b}
C16:1	0.23±0.05	0.20±0.19	0.35±0.06	0.34±0.02
C18:1n9	2.08±0.17	4.07±1.24	3.07±0.11	4.38±0.53
C18:2n6	1.07±0.18	1.88±0.65	0.18±0.14	1.61±0.01
C18:3n3	0.02±0.01	0.06±0.03	0.06±0.03	0.03±0.02
C20:4n6	0.79±0.09^{a}	0.81±0.13^{a}	1.34±0.28^{ab}	1.75±0.06^{b}
C20:5n3	0.06±0.03	0.13±0.07	0.12±0.05	0.22±0.06
C22:6n3	2.71±1.23^{a}	5.14±0.51^{ab}	6.88±2.10^{ab}	10.75±2.41^{b}
∑ SFA	6.34±0.70^{a}	9.73±1.60^{ab}	9.41±0.97^{ab}	12.72±1.5^{b}
∑ MUFA	3.77±0.23^{a}	5.96±1.27^{ab}	4.85±0.09^{ab}	6.26±0.32^{b}
∑ PUFA	1.27±0.27	2.00±0.66	1.33±0.12	1.78±0.03
∑ HUFA	3.67±1.27^{a}	6.28±0.32^{a}	8.46±2.34^{b}	12.94±2.41^{b}
∑ n3	2.86±1.15^{a}	5.33±0.45^{a}	7.05±2.01^{ab}	11.07±2.35^{b}
∑ n6	2.08±0.15^{a}	2.93±0.63^{ab}	2.73±0.21^{ab}	3.65±0.03^{b}
n3:n6	1.52±0.66	2.09±0.65	2.45±0.55	3.01±0.62
∑ FA	15.05±1.46^{a}	23.96±3.25^{ab}	24.05±3.10^{ab}	33.70±2.41^{b}

Values are (mean + SE).
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

4.3.3.4 Differences of Fatty Acid Concentrations between the Diet given, Muscle, Liver and Gonad of Male *Tor tambroides*

The differences of FAs concentration (mg g^{-1}) between sources of *T. tambroides* males fed with different percentages of lipid inclusions are shown in Table 4.15. For fish fed with diet containing 60 g kg^{-1} of lipid, the concentrations of FA given thorough the diet reflects in the muscle, liver and gonad sample for oleic acid, LA, DHA and total FA values. Each sample demonstrates higher values of referred fatty acids compared in the diet. There is significant difference ($p<0.05$) of total FA between diet with liver with values of 11.55 and 47.12 mg g^{-1} respectively.

In fish fed with diet containing 82 g kg^{-1} of lipid, higher values of ARA and DHA are found in muscle, liver and gonad samples compared to the diet given. Higher levels of oleic acid, LA and ALA are detected in the liver compared to the diet given. For the total FA concentration, the muscle exhibit lowest concentration with 15.04 mg g^{-1} ($p>0.05$) compared to the diets given with 21.06 mg g^{-1}, where the highest value is shown by the liver with 42.44 mg g^{-1} ($p<0.05$).

The concentration of OA expressed by the liver is significantly different ($p<0.05$) compared to the muscle and gonad for fish fed with diet containing 105 g kg^{-1} of lipid. The FAs concentration in the muscle are significantly ($p<0.05$) lower for OA, LA, ALA, EPA, DHA and total FA values compared to the diet given. The total FA values are higher for the liver (40.22 mg g^{-1}; $p<0.05$) and lower for the gonad (11.87 mg g^{-1}, $p<0.05$) compared to the diet given (28.31 mg g^{-1}).

For fish fed with diet containing 128 g kg^{-1} of lipid, there are significant differences ($p<0.05$) between muscle, liver and gonad sample with the diet given for the OA, LA, ARA and EPA concentrations. Meanwhile, there are significant differences ($p<0.05$) in DHA values between diet given (1.57 mg g^{-1}) with liver (6.53 mg g^{-1}) and gonad (10.75 mg g^{-1}). The value of total FA in muscle (11.24 mg g^{-1}) is significantly lower ($p<0.05$) compared to the diets (33.64 mg g^{-1}).

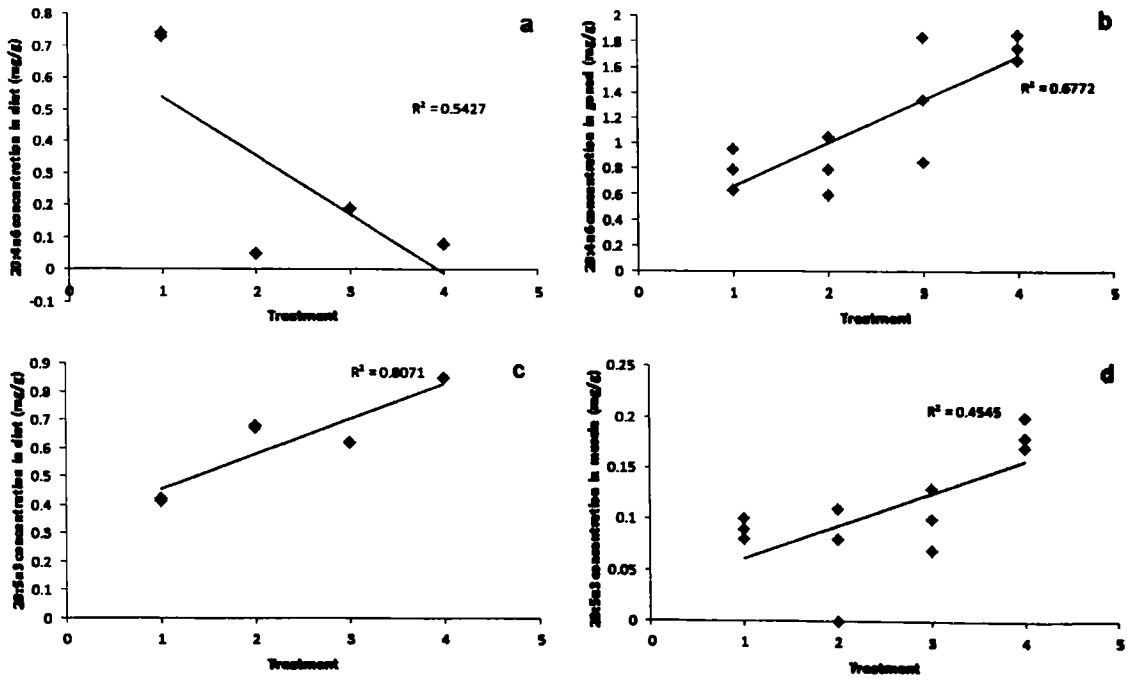
Table 4.15: Fatty acid concentrations (mg g^{-1} ± SEM) of the gonad of *Tor tambroides* males cultured for five months and fed diets with varying lipid levels.

Fatty acid	Diet	Muscle	Liver	Gonad
Diet 60 g kg^{-1}				
C18:1n9	1.97±0.01^{a}	2.42±0.24^{a}	8.23±1.06^{b}	2.08±0.17^{a}
C18:2n6	0.23±0.003^{a}	1.10±0.01^{b}	2.99±0.27^{c}	1.07±0.18^{b}
C18:3n3	0.09±0.0005	0.02±0.01	0.11±0.06	0.02±0.01
C20:4n6	0.74±0.003^{b}	0.41±0.10^{a}	0.69±0.12^{ab}	0.79±0.09^{b}
C20:5n3	0.42±0.003^{b}	0.09±0.01^{a}	0.11±0.07^{a}	0.06±0.03^{a}
C22:6n3	0.86±0.01^{a}	1.68±0.24^{a}	10.74±2.34^{b}	2.71±1.23^{a}
Total FA	11.55±0.07^{a}	13.48±1.32^{a}	47.12±5.79^{b}	15.05±1.46^{a}
Diet 82 g kg^{-1}				
C18:1n9	5.10±0.03^{b}	2.40±0.38^{a}	7.63±0.42^{c}	4.07±1.24^{ab}
C18:2n6	2.08±0.01^{a}	1.12±0.16^{a}	3.92±0.03^{b}	1.88±0.65^{a}
C18:3n3	0.06±0.0002^{a}	0.04±0.004^{a}	0.22±0.04^{b}	0.06±0.03^{a}
C20:4n6	0.05±0.0005^{a}	0.49±0.23^{ab}	0.97±0.32^{b}	0.81±0.13^{b}
C20:5n3	0.68±0.003^{b}	0.06±0.03^{a}	0.06±0.03^{a}	0.13±0.07^{a}
C22:6n3	1.58±0.01^{a}	2.19±0.16^{a}	7.44±0.74^{b}	5.14±0.51^{c}
Total FA	21.06±0.10^{ab}	15.04±1.34^{a}	42.44±2.06^{c}	23.96±3.25^{b}
Diet 105 g kg^{-1}				
C18:1n9	7.22±0.02^{b}	2.09±0.64^{a}	7.11±0.41^{b}	3.07±0.11^{a}
C18:2n6	3.62±0.01^{c}	1.04±0.31^{a}	2.76±0.36^{b}	0.18±0.14^{a}
C18:3n3	0.09±0.0001	0.05±0.00002	0.06±0.04	0.06±0.03
C20:4n6	0.19±0.0005^{a}	0.25±0.02^{a}	0.48±0.02^{a}	1.34±0.28^{b}
C20:5n3	0.62±0.0005^{b}	0.10±0.02^{a}	0.05±0.03^{a}	0.12±0.05^{a}
C22:6n3	1.57±0.003^{a}	1.43±0.27^{a}	6.06±0.09^{b}	6.88±2.10^{b}
Total FA	28.31±0.05^{b}	11.87±3.14^{a}	40.22±0.48^{c}	24.05±3.10^{b}
Diet 128 g kg^{-1}				
C18:1n9	10.55±0.01^{c}	1.68±0.01^{a}	5.63±1.02^{b}	4.38±0.53^{b}
C18:2n6	6.14±0.13^{a}	0.99±0.02^{b}	2.07±0.27^{c}	1.61±0.01^{d}
C18:3n3	0.13±0.0001^{a}	-	-	0.03±0.02^{b}
C20:4n6	0.08±0.0005^{a}	0.47±0.22^{ab}	0.56±0.12^{b}	1.75±0.06^{c}
C20:5n3	0.85±0.0005^{c}	0.18±0.01^{ab}	0.08±0.05^{a}	0.22±0.06^{b}
C22:6n3	1.57±0.0005^{a}	1.16±0.19^{a}	6.53±1.47^{b}	10.75±2.41^{b}
Total FA	33.64±0.03^{b}	11.24±0.42^{a}	35.94±3.83^{b}	33.70±4.27^{b}

Values are (mean + SE); '-'= not detected
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

4.3.3.5 Relationship between Fatty Acid Concentrations from Different Sources of Diet, Muscle, Liver and Gonad with Treatments

The relationships between FA concentrations from different sources with treatments are illustrated in Figure 4.18. There are strong positive correlation of EPA ($r(10)=0.898$; $p<0.01$; Figure 4.17c), DHA ($r(10)=0.580$; $p<0.05$; Figure 4.17e) and total FA ($r(10)=0.992$; $p<0.01$; Figure 4.17g) in the diet with treatment. Conversely, a strong negative correlation is found in ARA ($r(10)=-0.737$; $p<0.01$; Figure 4.17a). Furthermore, strong positive correlations are found between gonad and treatment for ARA ($r(10)=0.823$; $p<0.01$; Figure 4.17b), DHA ($r(10)=0.757$; $p<0.01$; Figure 4.17f) and total FA ($r(10)=0.784$; $p<0.01$; Figure 4.17i). Additionally, positive correlations are found in EPA ($r(10)=0.674$; $p<0.05$; Figure 4.17d) while a negative correlations was found in total FA ($r(10)=0.630$; $p<0.05$; Figure 4.17h) between liver and treatment.



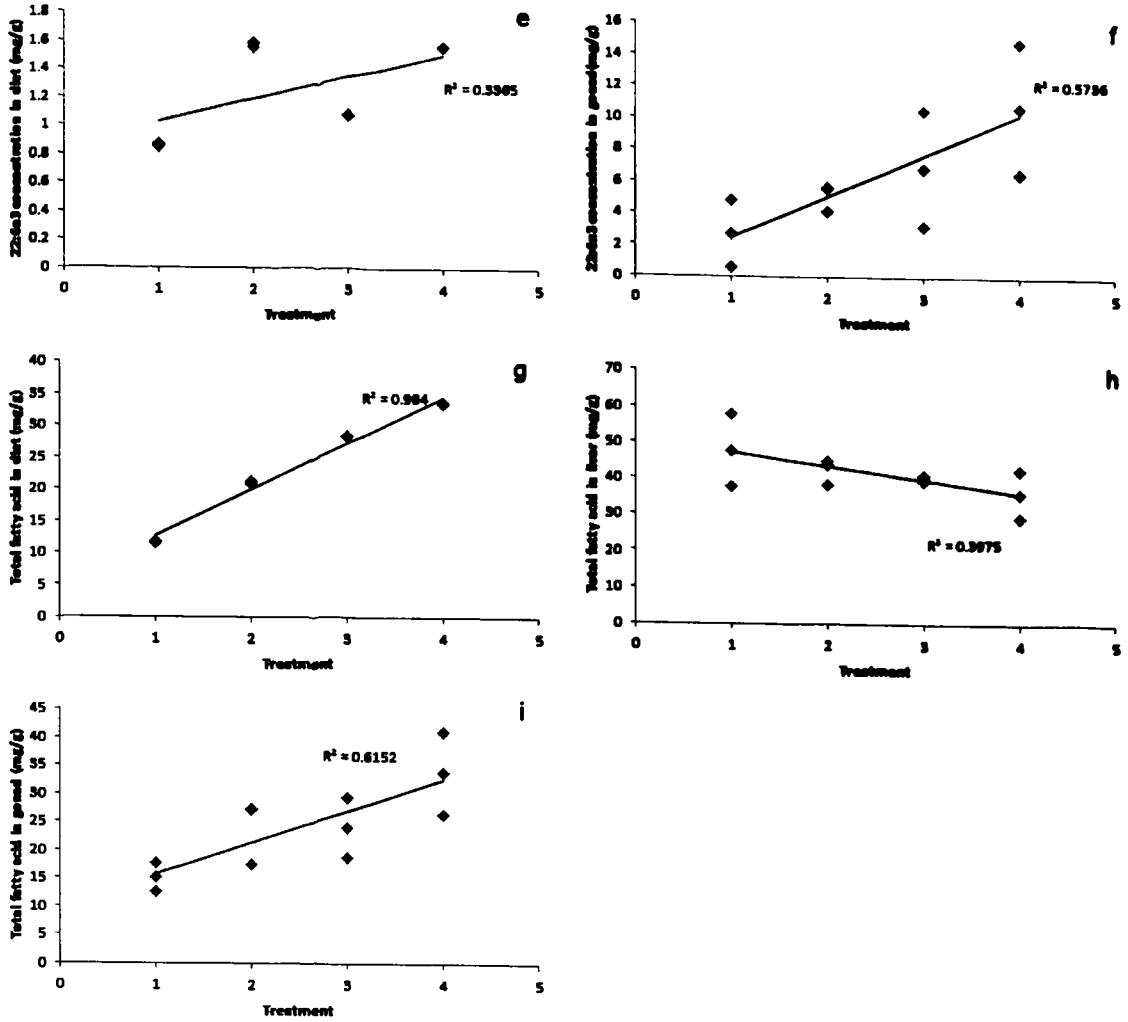


Figure 4.18: Relationship between (a) arachodonic acid (ARA) in diet with treatment; (b) arachidonic acid (ARA) in gonad with treatment; (c) eicosapentanoic acid (EPA) in diet with treatment; (d) eicosapentanoic acid (EPA) in muscle with treatment; (e) docosahexanoic acid (DHA) in diet with treatment; (f) docosahexanoic acid (DHA) in gonad with treatment; (g) total fatty acid in diet with treatment; (h) total fatty acid in liver with treatment and (i) total fatty acid in gonad with treatment.

4.3.4 Hormone Analysis

The levels of testosterone (T) (ng ml^{-1}) in blood plasma of males *T. tambroides* after five months of feeding trial with diets containing varying lipid levels are shown in Figure 4.19. The pattern of hormone fluctuation is similar with E2 concentration in previous experiments. The T levels increased from 0h to 12h, before declining to its lowest values at 24h. Fish fed with diet containing 82 g kg^{-1} of lipid exhibit the highest T concentration in all blood sampling compared to other dietary treatment groups. At 0h, the ANOVA test showed that there are significant differences ($p<0.05$) between fish fed with diet containing 82 g kg^{-1} of lipid (0.57 ng ml^{-1}) and other diet treated fish. The values for fish fed with diet containing 60, 105 and 128 g kg^{-1} of lipid are 0.33, 0.33 and 0.35 ng ml^{-1} respectively.

The highest T concentration at 12h is shown by fish fed with diet containing 82 g kg^{-1} of lipid with 0.69 ng ml^{-1}. This then followed by fish fed with diet containing 128 g kg^{-1} of lipid with 0.66 ng ml^{-1} respectively. However, there are no significant differences ($p>0.05$) between all dietary treated groups with fish fed with diet containing 60 and 105 g kg^{-1} of lipid recording values of 0.513 and 0.507 ng ml^{-1} respectively. Finally, at 24h, the value of T concentration for fish fed with diet containing 82 g kg^{-1} of lipid (0.32 ng ml^{-1}) is significantly different ($p<0.05$) compared to fish fed with diet containing 60 g kg^{-1} of lipid (0.19 ng ml^{-1}) and fish fed with diet containing 128 g kg^{-1} of lipid (0.16 ng ml^{-1}). Meanwhile, T value for fish fed with diet containing 105 g kg^{-1} of lipid is 0.29 ng ml^{-1}.

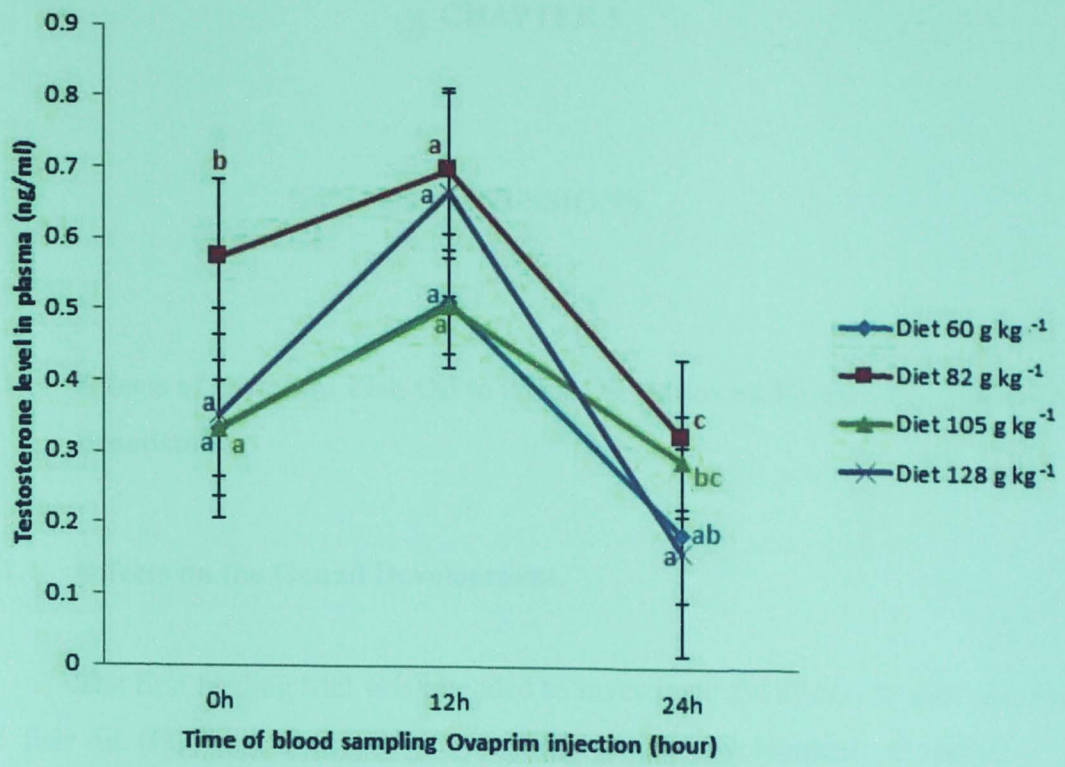


Figure 4.19: Testosterone changes in *Tor tambroides* male broodstocks in 24-h artificial induced spermiation with ovaprim after five month of dietary treatment with diets containing varying lipid levels (Diet 60, 82, 105 and 128 g kg^{-1}).

CHAPTER 5

DISCUSSIONS

5.1 Effects of Different Fish Oil to Corn Oil Ratios on Female *Tor tambroides* Broodstocks

5.1.1 Effects on the Gonad Development

The first feeding trial was intended to investigate the effects of different ratio of fish oil (FO) and corn oil (CO) on the gonad development of female *T or tambroides* broodstocks. There were no significant differences between the GSI values of all dietary treated groups. The sampling size for statistical analysis might be the cause for this. From this experiment, only three fish from each treatment were sacrificed and used for all the data analysis. This is due to the fact that the broodstock were expensive and thus not possible to be purchased in large number from the same batch. However, GSI value was highest in fish fed with diet containing fish oil to corn oil ratios of 1:1. This was followed by fish fed with diet containing fish oil to corn oil ratios of 0:1 and 1:0, respectively (Table 4.1).

Gonadosomatic index was correlated positively with gonadal development and more often is used as reliable indicator for fish maturation (Nunes *et al.*, 2011). Wibowo & Kaban (2014) found out that the GSI of wild *T.tambroides* in Western Sumateran River varied between two rivers, Manna (0.5-2.1%) and Batang Tarusan River (0.1-0.8%). The GSI values for the first feeding trial seem to be in the range of fish from Batang Tarusan River.

Hepatosomatic index (HSI) which was used as an indicator for energy status has been observed to show an inverse relationship with GSI suggesting transportation of energy stored from the liver into the gonad for egg production in matured fish (Rueda-Jasso *et al.*, 2013). However, this seems to only applied to synchronous spawners like Atlantic cod, *Gadus morhua* L. where vitellogenesis ceased prior to spawning (Dahle *et al.*, 2003), whereas in asynchronous spawners, with the presence of all oocyte stages in the gonad, the vitellogenesis process continued longer (Nunes *et al.*, 2011).

The liver of *T. tambroides* females functions as lipid store and is also an organ with high metabolic activity, where lipids were accumulated and transferred in the form of lipoproteins throughout the body with special attention to the developing oocytes (Tocher 2003 and Johnson 2009). HSI can also be used to determine spawning season in fish in association with GSI and oocyte maturation stages (Hismayasari *et al.*, 2015). However in the first feeding trial, there were no significant differences in the HSI values between dietary treated groups (Table 4.1). Similar findings were found by Ng & Wang (2011) on Nile tilapia, *Oreochromis niloticus*, which is an asynchronous spawner, where no significant differences were found in the HSI values in broodstock fed with different lipid source (fish oil, palm oil and linseed oil) although it has significant differences in the GSI values. This might due to the fact that asynchronous spawners undergo continuous vitellogenesis due to the presence of previtellogenic oocyte which also continuously undergoes maturation.

Based on histological study, it shows that *T.tambroides* is an asynchronous spawner. Several stages of oocyte stages were observed in the gonad. It is the same with studies by Ismail *et al.*, 2011 and Wibowo & Kaban (2014), done on *T. tambroides*. This phenomenon was also observed in the Brazilian snapper, *Lutjanus alexandrei* (Fernandes *et al.*, 2016), the skipjack, *Katsuwonus pelamis* (Grande *et al.*, 2012) and common carp, *Cyprinus carpio* (Smith & Walker, 2004). Based on the oocyte diameter distribution in the gonad, female *T. tambroides* can be define as indeterminate spawners (Fernandes *et al.*, 2016; Grande *et al.*, 2012) where they can

spawned in batches with continuous oocyte recruitment due to presence of all stages of oocytes (Ingram *et al.*, 2005 and Ismail *et al.*, 2011).

In the first feeding trial, only fish fed with diet containing fish oil to corn oil ratios of 1:1 exhibit development until vittellogenic oocytes phase compared to fish fed with diet containing fish oil to corn oil ratios of 1:0 and 0:1 (Figure 4.1, 4.2 and 4.3), hence the higher GSI value although there were no significant differences between treatment. There were no significant differences of oocyte stages (based on oocyte diameter) found when comparing the stages between the anterior and posterior part of the gonad for this feeding trial (Figure 4.5). This differ with results from (Ismail *et al.*, 2011) which found that the earlier oocyte stages (stage 1-3) were found mostly in the posterior part of the gonad, while later stage (stage 4-6) were found mostly at the anterior part. This might due the method of observation with no statistical analysis conducted to see the differences between these two parts in the gonad in that study compared to this current study.

Studies conducted on the effect of different lipid sources shows that freshwater fish prefer mixture from both n3 and n6 lipid source (Nandi *et al.*, 2001; Furuita *et al.*, 2007 and Xu *et al.*, 2016). Mixture of soy bean oil and fish oil with n3 to n6 ratio of 0.7, gives better reproductive performance for carp, *Catla catla* in terms of advance maturation of the oocyte with higher fecundity and fertilization rate (Nandi *et al.*, 2001). Following this, research by Furuita *et al.* (2007) found that mixture of corn oil and fish oil with n3 to n6 ratio of 0.8, shows better egg quality and FA composition in broodstock and eggs of Japanese eel, *Anguila japonica*. Most recently, Xu *et al.*, (2016) found that mixture of safflower oil and fish oil improved gonadal maturation, and breeding performance in carp, *Cyprinus carpio*, compared to supplementation from perilla oil, safflower oil and fish oil alone.

Whereas in this current study, *T. tambroides* females fed with diet containing fish oil to corn oil ratios of 1:1,with n3 to n6 ratios of 0.74 shows numerically higher GSI values (although not significantly different) and qualitatively has the farthest oocyte development compared to fish fed with fish oil and corn oil ratios of 1:0 and 0:1. However, for white bass, *Morone chrysops*, Lane & Kohler (2006) found that

increasing corn oil substitution of fish oil decrease the reproductive performance and egg hatchability. Interestingly, in yellowfin sea bream, *Acanthopagrus latus* broodstock fed with fish oil supplement only shows better spawning performance compared to fish supplemented with mixture of fish oil and sunflower oil and sunflower oil only (Zakeri *et al.*, 2011).

This shows that freshwater and marine fish exhibit different effects of n3 to n6 ratios. Santiago and Reyes (1993) found out that the reproductive performances of Nile tilapia broodstock, *Oreochromis niloticus* improved considerably when fed with diet containing fish oil to corn oil ratio of 1:1 compared to fish given diet with only fish oil. This is due to the high n3 fatty acids (added by residual oil in fish meal) and low n6 fatty acids in the diet. In this current study, we can see that fish fed with diet containing fish oil to corn oil ratios of 0:1 might gain benefit from the n3 fatty acids from the residual oil in fish meal. The GSI percentages in fish fed with diet containing fish oil to corn oil ratios of 0:1 was slightly higher (although not significantly different) compared to fish fed with diet containing fish oil to corn oil ratios of 1:0. It also shows in the histological study where fish fed with diet containing fish oil to corn oil ratios of 0:1 show until stage 4 oocyte compared to fish fed with diet containing fish oil to corn oil ratios of 1:0. Although only numerical differences were seen, but these results comply with the fact that in general, carp needs input from both n3 and n6 fatty acid (Nandi *et al.*, 2007; Xu *et al.*, 2016).

5.1.2 Effects on the Fatty Acid Concentration

In the first feeding trial, only muscle sample were collected for fatty acid analysis due to insufficient amount of sample for liver and gonad samples. The concentration of FA in the muscle usually mirrored the concentration in the diet given (Furuita *et al.*, 2007; Kamarudin *et al.*, 2012; Ramezani Fard *et al.*, 2014). It is the same with the first feeding trial with some exceptions. For the first feeding trial, the concentration of oleic acid, LA and total FA in the muscle increases as the values in the diet increases (Figure 4.5). However, the concentration of ALA in the muscle increased even though the value decreases in the diets. This shows that *T. tambroides* broodstock selectively retain ALA in its muscle. This is in agreement with studies done by Ramezani-Fard *et al.*, (2014) on *T. tambroides* juveniles fed different ratios of ALA to LA in the diet and also Nile tilapia, *Oreochromis niloticus* (Ng & Wang, 2011).

PUFA conversions are known to occur in a variety of freshwater fish species (Buzzi *et al.*, 1996, 1997; Tocher *et al.*, 2006), including common carp (Xu *et al.*, 2016) and mahseer (Kamarudin *et al.*, 2012; Ramezani-Fard *et al.*, 2012a). It was also demonstrated that dietary LA and ALA stimulate gene expression of desaturases and elongases hence increasing the HUFA biosynthesis activity (Zheng *et al.*, 2005). However, for many freshwater fish species, the conversion of C18 PUFA to long-chain PUFA seems to occur at a very slow rate (Tocher *et al.*, 2006) because of limiting delta-6 and delta-5 desaturation steps (Buzzi *et al.*, 1996, 1997; Tocher *et al.*, 2006; Vagner & Santigosa, 2011) where both delta-6 and delta-5 desaturates were used competitively in the elongation for n3, n6 and n9 C20 HUFA.

Interestingly, the first feeding trial shows that female *T. tambroides* broodstocks were able to elongate and desaturase LA and ALA into HUFA efficiently. This was shown in DHA values, where DHA given in the diets were decreasing (Table 4.2), however the DHA values in the muscle was increasing (Table 4.3).This shows that DHA was an important FA in *T. tambroides* that it can elongate in from its precursors. The diminished concentration of ALA in the muscles of fish fed with diet containing fish oil to corn oil ratio of 1:0 maybe indicate that the

higher levels of EPA and DHA in the diet were enough, up until ALA were utilized as energy source compared to the other two treatments. A study on common carp, *Cyprinus carpio* L., reported that supply of short chain PUFA resulted in higher short chain but not long chain PUFA indicating little PUFA conversion in carp (Schultz *et al.*, 2015).

This is in contrast with this current study where fish fed with diet containing fish oil to corn oil ratio of 0:1 received lower concentration of DHA from the diet but exhibit significantly higher DHA level in the muscle compared to fish fed with diet containing fish oil to corn oil ratio of 1:0 (Table 4.3). It is possibly due to the preferential DHA deposition in the tissue, irrespective of their concentration in the diet, and its poor utilization as a substrate for beta-oxidation due to its complex catabolism (Sargent et al., 2002). In rainbow trout, *Oncorhynchus mykiss*, fish which were fed with diet containing two fish oil (capelin and anchovy) with different vegetables oil (soybean, rapeseed, palm and olive), the muscle DHA levels were higher than the dietary concentration of this fatty acid, irrespective of the type and degree of substitution (Caballero *et al.* 2002). Izquierdo *et al.* (2005) found that muscle EPA and DHA concentration decreased when a substitution of 60 or 80% of fish oil by vegetable oils was performed on gilthead sea bream, *Sparus aurata*. This decrease was significantly larger in EPA than in DHA. As mentioned earlier, with the exception of DHA, all other fatty acids are possibly used for energy purposes, especially when the dietary lipid level increases (Bell *et al.*, 2003).

The reduction of HUFA levels from the diets given causing alterations in the mechanisms involved in the biosynthesis of fatty acids, by increasing the utilization of ALA for elongation and desaturation (Bell *et al.*, 2001; Caballero *et al.*, 2002), which could explain the increased DHA content in the muscle of fish fed diets supplemented with vegetable oils. The n3 to n6 ratio was also affected where significantly different ratios in the diets resulted in a non-significantly different ratio in the muscle of treated fish with the increase of DHA in the muscle.

5.1.3 Effects on the 17β estradiol Concentration

In the first feeding trial, the patterns of 17β estradiol (E2) in the plasma were monitored between dietary groups. At the end of the experimental duration, the broodstock were injected with Ovaprim to induce ovulation. The E2 concentrations were monitored at 0, 6, 12 and 24h post hormonal injection of Ovaprim. The concentrations of E2 were elevated from 0h before reaching its peak at 12h and subsequently decrease at 24h post injection. The pattern was the same in all diet treated fish. The results were the same with study done by Azuadi *et al.* (2013b) also on *T. tambroides* where E2 concentration decrease slightly from 12h PI to 24h post injection. This pattern were also seen in other cyprinid fish like tench, *Tinca tinca* (Podhorec *et al.*, 2016) and grass carp, *Ctenopharyngodon idellus* (Mousavi and Yousefian, 2012).

It was possible that the decreasing concentration of E2 after 12h post injection was caused by a shift in the steroidogenic enzymes from the synthesis of P450 aromatase to 20β hydroxysteroid dehydrogenase, resulting in the production of the maturation inducing steroid (MIH), 17α,20β-dihydroxy-4-pregnen-3-one (DHP) (Nagahama and Yamashita, 2008; Ramezani-Fard *et al.*, 2012b). Hence, although DHP concentration was not monitored in this study, it can be assumed that this decrease of E2 levels was caused by the down regulating of E2 and increased production of DHP in the blood plasma.

However, it seems that the pattern of reproductive hormone may perhaps be species specific. This was due to the fact that in catfish, *Heteropneustes fosilis* (Acharjee *et al.*, 2016; Chaube *et al.*, 2014), the concentration of E2 were decreasing from 0h to 24h post injection of Ovaprim, while the concentration of DHP continue to increase. The concentration of E2 in plasma was also affected by the size and stages of gonadal development in female broodstocks which also correlated with GSI (da Silva *et al.*, 2016). Higher GSI might induce higher E2 concentration (Dahle *et al.*, 2003) where in this current study, fish fed with diet containing fish oil to corn oil ratio of 1:1 has numerically higher GSI value (although with no significant

differences) compared to fish fed with diet containing fish oil to corn oil ratio of 1:0 and 0:1.

As mentioned before, *T.tambroides* is an asynchronous spawner; therefore it has different stages of oocyte development in its gonad. The increase up until the 12h post injection in the current study showed that the fish may still have immature oocytes that may not have complete vitellogenesis (Jerez *et al*, 2006) and responded to the hormone injection (Tamaru *et al.*, 1991). In the first feeding trial, fish fed with diet containing fish oil to corn oil ratio of 1:1, numerically has the highest E2 concentration (although with no significant differences) in all observation time. This result correlates with the numerically higher GSI found in this dietary treatment group (Diet 1:1) compared to the other two dietary treated groups (Diet 1:0 and Diet 0:1).

The main hypophysiotropic hormone involved in reproduction in fish is gonadotropic hormone releasing hormone (GnRH). GnRH targets the anterior pituitary (adenohypophysis) to initiate the release of pituitary hormones such as gonadotropic hormone (GtH) – the primary pituitary hormone involved in reproduction. GtH exists in two identifiable forms; GtH I [follicle stimulating hormone (FSH)] and GtH II [luteinizing hormone (LH)] (Ando *et al.*, 2004; Chyb *et al.*, 1999). While both FSH and LH are involved in gametogenesis, they have different physiological functions. FSH is involved in the early stages of gametogenesis and in steroidogenesis whereas LH plays a larger role in the final stages of gametogenesis (Yaron and Levavi-Sivan, 2011).

It has been proven that sGnRH stimulate the release of both FSH and LH, but at different stage of gonad maturation (Ando *et al.*, 2004). As for the first feeding trial, the possible hormonal cascade effect from the injection of Ovaprim was as shown in Figure 5.1. It might be possible that the Ovaprim injection induces the secretion of FSH for vitellogenesis instead of LH due to the physiological condition of the fish. Fish with GSI percentages value which is less than 0.5% were undergoing primary vittelogenic stages (Luquet and Watanabe, 1986), whereas the

GSI percentages of all treated fish were in range between 0.18 to 0.31%.This shows that the treated fish were still developing their ooocytes.

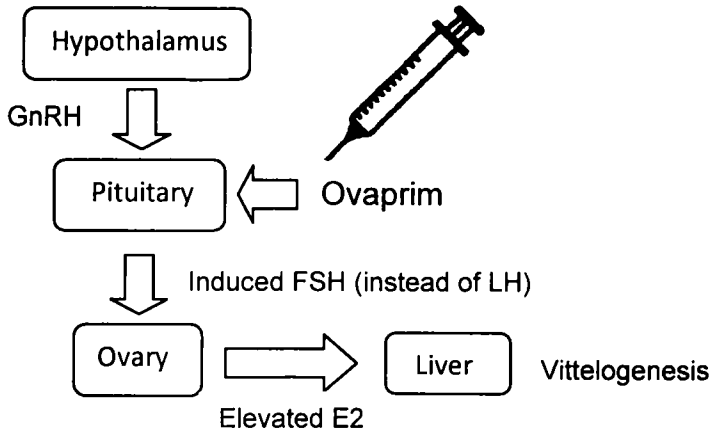


Figure 5.1: Possible hormonal cascade for hormonal induction on female *Tor tambroides* fed with diet containing different fish oil and corn oil ratios.

In the first feeding trial, no eggs were produced after five months of feeding diets containing different fish oil to corn oil ratios and injection of Ovaprim. Azuadi *et al.* (2011, 2013a, 2013b) used F1 generation of female broodstock which were already reared for 4 years in their experiments, while Ingram *et al.* (2005) used fish that had already been reared from fingerling to broodstock for more than 8 years. Ambak *et al.*, 2007 stated that the age of maturation of *T. tambroides* females was 3 years. For female broodstocks, the size of eggs was controlled by fish age and size (Bromage *et al.*, 1990; Kamler, 2005). Study done in common sole, *Solea solea* L. found that smaller and presumed younger broodstocks shows inferior egg quality and reproductive performance compared to wild broodstocks (Lund *et al.*, 2008). They also found that 3 years old cultured broodstocks performed better in reproductive performance than the same breeders at 2 years old.

In the Mahseer Hatchery, AKUATROP, UMT, the author manage to artificially breed *T. tambroides* with broodstocks which has been reared from five inch fingerlings caught from the wild and then reared in the hatchery for more than four years. The broodstocks purchased and used in this current study was already

reared for almost 2 years from eggs hatched from artificial breeding. Findings in the first feeding trial indicate that age may affect reproductive performances of *T. tambroides* broodstocks in captivity.

The reason for the use of virgin fish for this experiment was due to the difficulties in purchasing high quality broodstocks from the same batch. Even if this was possible, this project did not have the financial strength to purchase 90 high quality broodstocks to be use in this experiment. By comparing the age and fish size data from the literature (Ambak et al., 2007, Ismail et al., 2011), the author opt to use the size factor as an indicator in purchasing the broodstocks in this experiment. Although the size of the broodstocks used in the feeding trial was in accordance with Ismail *et al.* (2011) at a range of 0.95 ± 0.36 kg, which also most suited the budget for this study (1 kg of broodstocks= RM 1000), there might be discrepancies in the age of the broodstocks.

Finally, for the first feeding trial, it had shown that different lipid sources affect the gonad development, fatty acid compositions in the muscle and E2 levels in blood plasma of female *T. tambroides* broodstocks. Although there were no significant differences in the results, the author conclude that fish fed with diets containing fish oil to corn oil ratio of 1:1 gives the best results but should be considered with caution and based mostly on the quality of the gonad histology. It has numerically higher GSI percentages, shows the farthest development of oocyte stages, which lead to numerically higher E2 concentration. Therefore, this ratio was chosen for the second and third experiment.

5.2 Effects of Varying Lipid Levels on Female *Tor tambroides* Broodstocks

5.2.1 Effects on the Gonad Development

As the first feeding trial showed that *T.tambroides* females fed with diets containing fish oil to corn oil ratio of 1:1 give the best results, the second feeding trial was conducted to investigate the effects of varying lipid levels on the gonad development of female *T.tambroides* broodstocks. It can be observed that the GSI values increases from fish fed with diet containing 60 g kg^{-1} of lipid and was at its highest in fish fed with diet containing 82 g kg^{-1} of lipid before decreasing in fish fed with diet containing 105 and 128 g kg^{-1} of lipid respectively (Table 4.4). Based on Wibowo & Kaban, (2014) classification of oocyte maturation, fish fed with diet containing 60, 82 and 105 g kg^{-1} of lipid was in the maturing stage compared to fish fed with diet containing 128 g kg^{-1} of lipid (developing stage) (Figure 4.7, 4.8, 4.9 and 4.10). There are also no significant differences of oocyte stages (based on oocyte diameter) found when comparing the stages between the anterior and posterior part of the gonad for the second feeding trial (Figure 4.11).

For the second feeding trial, although there are no significant differences in the HSI values, the values of HSI for fish fed with diet containing 60 and 82 g kg^{-1} of lipid were higher compared to fish fed with diet containing 105 and 128 g kg^{-1} of lipid (Table 4.4). It is the same with studies done on rainbowfish, *Melanotaenia boesemani* (Hismayasari *et al.*, 2015) and Atlantic Sardine, *Sardina pilchardus* (Nunes *et al.*, 2011). This phenomenon is common in asynchronous spawners (like *T. tambroides*), where the oocyte contains all stages of oocyte development, therefore the process of vitellogenesis remain active, hence the need for higher HSI values, as pictured by fish fed with diet containing 60 and 82 g kg^{-1} of lipid. As seen in *S. pilchardus*, the HSI values increased again once vitellogenesis started (Nunes *et al.*, 2011). Whereas in synchronous spawners, the vittellogenic activity ceased during spawning period, as all the eggs have completed vitellogenesis prior to spawning, like Atlantic cod, *Gadus morhua* L. (Dahle *et al.*, 2003).

In the second feeding trial, it shows that fish fed with diet containing 82 g kg^{-1} of lipid gives the best outcome in terms of GSI value. It can be hypothesized that at

level of 8.2% lipid content (2.5% lipid inclusion) was the lipid level requirement for *T. tambroides* female broodstocks in captivity based on the current feeding trial conditions. This is in contrast with findings by Ingram *et al.* (2005) where they improved the *T. tambroides* and *T. douronensis* broodstocks feed with diets formulated for Murray cod, *Maccullochella peelii peelii* (Mitchell) (De Silva *et al.*, 2004a). The protein and lipid level in the diet was 49 and 16% respectively, while the proximate composition of initial feed used were not stated, with remarks that the initial feed did not supply sufficient HUFA levels, in particular, DHA, EPA and ARA. Azuadi *et al.* (2011, 2013a) stated that, the *T. tambroides* broodstocks used for artificial breeding in their study was fed with both self-made diet (62.6% protein and 17.6% lipid) in the morning and commercial tilapia pellet (16% protein and 4% lipid) in the evening. Studies by Ismail *et al.* (2011) only stated the protein level (40%) in the diet fed to *T. tambroides* broodstocks.

Studies on effects of different lipid levels have been conducted on several species. Increasing lipid inclusion in the diet will caused an increase in energy level with the increasing level of oil in the diets. Energy requirement for fish broodstocks differs between species. An excess of energy compared to optimal requirement of certain fish species might cause decrease in fish biology performance and lower feed intake (Wang *et al.*, 2005; Ling *et al.*, 2006; Ramezani-Fard *et al.*,2012a). In sparid, *Acanthopagrus latus*, the best spawning performances were displayed by broodstocks fed with 20% lipid compared to 15 and 25% (Zakeri *et al.*, 2010). Higher lipid level (more than 4.5%) with 30% protein level did not improve the reproductive performances of Brazilian catfish, *Rhambia quelen* (Tessaro *et al.*, 2012). Similarly, in Gulf killifish, *Fundulus grandis*, no significant differences found in reproductive performances with the increasing lipid inclusion percentages (Patterson and Green, 2015). Gonadosomatic index were higher in fish fed with no additional fish oil (4% lipid in diet). However Coldebella *et al.* (2013) suggest that, 8-14% of lipid level with 28% protein level gives better reproductive performances where fish treated with 20% lipid level hinders growth and survival of postlarvae for *R. quelen.*

Results from the second feeding trial shows that female *T. tambroides* broodstocks required lower lipid level of 82 g kg^{-1} (2.5% lipid inclusion) which gives better results compared to the lipid level used in the first feeding trial (105 g kg^{-1} of lipid). This result is supported with study done by Ishak *et al.* (2016) where lipid level between 7.4 and 9.2 % are enough to fulfill the dietary requirement for *T. tambroides* fingerlings. Recently, lipid levels around 7.8% were used in other study on *T. tambroides* fingerlings (Bami *et al.*, 2017a and Kamarudin *et al.*, 2018). This shows that, both fingerlings and female broodstocks of *T. tambroides* requires almost the same lipid levels. However, further studies need to be done to clarify the exact lipid level requirement in female *T. tambroides* broodstocks since fish fed with diet containing 60 g kg^{-1} of lipid also exhibit high GSI values. Therefore, it was suggested that further studies were done on lipid level ranging from 60 to 90 g kg^{-1}, and also the ability to use lower fishmeal amount in the diet compared to present study (500g kg^{-1} of diet) for economical aspect.

5.2.2 Effects on the Fatty Acid Concentration

For the second feeding trial, significantly different FA concentration in the diet did not reflect in the FA concentration in the muscle of treated fish. However different pattern were shown in the FA concentration in the liver. Liver and adipose tissue is the main site for lipogenesis in most animals (Qiu *et al.*, 2017; Rangan and Smith, 2002). The increasing total FA concentration in the diet (Table 4.5) was mirrored with the increasing concentration of total FA in the liver (Table 4.7). The ranges differs greatly with higher concentration in the liver (32.64-67.88 mg g^{-1}) compared to the diet given (11.55-33.64 mg g^{-1}).

Meanwhile, in the gonad, the concentrations were higher in fish fed with diet containing 60, 82 and 105 g kg^{-1} of lipid compared to diet given except for fish fed with diet containing 128 g kg^{-1} of lipid (Table 4.8). The DHA values in the liver and gonad increases as the dietary lipid level increases. This is the same with Ramezani-Fard *et al.* (2012a) where he found that DHA level increases in the liver of *T. tambroides* fingerlings as the dietary lipid level increases. This selective transfer and accumulation of DHA in the eggs has been seen in fish fed high level of DHA and EPA in the diet (Johnson, 2009).

As seen in Table 4.7, it shows that EPA was not detected in liver sample of fish fed with diet containing 105 g kg^{-1} of lipid and the gonad sample of fish fed with diet containing 128 g kg^{-1} of lipid. The concentrations of EPA from all samples (muscle, liver and gonad) were lower than the diet given. It shows that EPA was selectively catabolized as source of energy and/ or incorporated in the oocyte membrane in female *T. tambroides* broodstocks while its n-3 requirement can be satisfied with DHA with/without EPA, and if EPA is required, it can be fulfil with lower concentration. This is in agreement with findings on cobia, *Rachycentron canadum* treated with different level of DHA and EPA supplementation with soybean oil (Trushenski *et al.*, 2012). Emery *et al.* (2016) also found out that n-3 dietary requirement for Atlantic salmon, *Salmo salar* was met with DHA supplementation alone, whereas high EPA supplementation might also give damaging effects. Ramezani-fard *et al.*, (2011) also found that EPA levels in muscle

of fish fed different dietary lipid percentages which continuously decreasing until the end of experiment on *T. tambroides* juveniles while DHA level remain constant.

During vitellogenesis in females, theca cells respond to FSH or LH by producing testosterone (T), which was then converted into 17β-oestradiol (E2) in the granulosa cells (Lubzens *et al.*, 2010, 2017). In turn, E2 regulates oocyte development and stimulates the production of VTG in the liver, which is released into the bloodstream and incorporated into the oocytes (Hiramatsu *et al.*, 2015). In the present study, the total HUFA (ARA, EPA and DHA) concentrations in the liver of fish fed with diet containing 60 and 82 g kg^{-1} of lipid were lower compared to the HUFA concentrations in the gonad. This suggests that successive mobilization of these HUFA from the liver to the gonad, as source of nutrients for gonad development. Both Almansa *et al.*, 2001 and Wassef *et al.*, 2012 reported that female gilthead seabream, *Sparus aurata*, mobilized endogenous PUFA and HUFA from the liver and muscle to ovaries for the purposes of providing energy for metabolism and EFA deposition in the eggs thus might suggest that fed with diet containing 60 and 82 g kg^{-1} may have successfully undergone vitellogenesis. This is in contrast with the total HUFA concentration in the liver of fish fed with diet containing 105 and 128 g kg^{-1}. Their concentration were higher compared to the total HUFA in the gonad especially the DHA concentration. This results also reflects in the GSI percentages where fish fed with diet containing 60 and 82 g kg^{-1} both have numerically higher GSI compared to fed with diet containing 105 and 128 g kg^{-1}.

As stated in previous sub-topic (5.2.1), female *T. tambroides* broodstocks fed with diet containing 82 g kg^{-1} of lipid gives the best outcome in term of GSI values. This diet provides n-3 to n-6 ratio of 1.25. This differs from studies done by Ingram *et al.* (2005) where they used diets for Murray cod, *Maccullochella peelii peelii* (Mitchell) (De Silva *et al.*, 2004a) which has the n-3 to n-6 ratio of 6.1. However, Ng *et al.*, 2007 suggest that *T. tambroides* need equal ratio of n-3 to n-6 throughout its development which is in agreement with the second feeding trial. This was supported by their findings that n-3 to n-6 ratio in eggs and larvae of *T. tambroides* were around 0.9-1.0. Therefore, further studies on the effect of different n-3 to n-6 ratio

(~1.0) should be conducted. The effects of DHA, EPA and ARA level and ratio on the gonad development and breeding performances should also be investigated.

5.2.3 Effects on the Steroid Plasma Concentration

As for the second feeding trial, the E2 patterns are similar with the pattern from the first feeding trial. The E2 concentration peaked at 12h post injection of Ovaprim, before decreasing at 24h post injection. In this feeding trial, the E2 concentration of previously ovulated females *T. tambroides* with confirmed eggs release (FCE) was also examined as a comparison with the feeding trial. As explained before, the E2 concentrations were positively correlated with the GSI values. As for dietary treated fish, fish fed with diet containing 82 g kg^{-1} of lipid exhibit higher E2 levels compared to other dietary treatment, while for FCE fish, the E2 concentrations were significantly higher compared to all diet treated fish in all monitored hours. The values of E2 concentration from FCE fish were comparable to studies done by Ismail *et al.* (2011).

In the second feeding trial, no eggs were produced after five months of feeding diets containing varying lipid levels and injection of Ovaprim. Interestingly, even FCE fish were unable to produce eggs after hormone injection. Although the same pattern was seen in the second feeding trial compared to Azuadi *et al.* (2013b), however the level are higher (0.03-0.75 ng ml^{-1}) compared to their study (0.02-0.26 ng ml^{-1}). It may be caused by the presence of post-vitellogenic oocyte which produced lower E2 levels (Matsuyama *et al.*, 1991) where in Azuadi *et al.* (2013b) research; they used broodstocks that has regularly been used for induced breeding. As mentioned before, the author was able to artificially bred *T. tambroides* in the hatchery. The size of females that successfully ovulated also varied with the smallest was 1.9 kg with ages around 4-5 years. However, the success of artificial breeding from these broodstocks was irregular. It was found that the success of breeding of *T. tambroides* in UMT was higher by using caught wild broodstocks (pers. comm). The longer the broodstocks were kept in captivity; the results start to decrease in term of successful breeding and even the color of eggs (from bright orange or yellow to pale

yellow). There is a possibility that female *T. tambroides* were affected with reproductive dysfunction in captivity.

Reproductive dysfunction of females in captivity can be generally characterized as failure of female broodstocks to go through final oocyte maturation (FOM) even with ovaries which has fully undergone vitellogenesis (Yaron, 1995; Mylonas and Zohar, 2001) like in carp (Yaron *et al.*, 2009), striped bass, *Morone saxatilis* (Mylonas *et al.*, 1998) and gilthed seabream, *Sparus aurata* (Zohar *et al.*, 1995). There are also fish that failed to undergo vittellogenesis, like seen in Japanese eel, *Anguila japonica* (Ohta *et al.*, 1997). This might be caused by unsuitable stimulants received in term of physical, chemical or environmental cues compared to their wild counterparts (Zohar and Mylonas, 2001). Female *T. tambroides* seems to be experiencing the first reproductive dysfunction, same as other cyprinide fish (Yaron, 1995). Recently, Akhtar *et al.* (2017) reports that, female golden mahseer, *Tor putitora* has the same reproductive dysfunction as *T. tambroides* in this current study. They found out that, wild *T. putitora* has higher E2, DHP and LH compared to captive females broodstocks. There are also evidence that captive female broodstocks has higher stress indicator compared to wild broodstocks which has then highlighted the endocrine failure of *T. putitora* broodstocks held in captivity.

Yaron (1995) stated that suboptimal conditions and lack of environmental cues is the reason for this reproductive dysfunction. This lead to the lack of endocrine surges needed for reproductive purposes. This theory was supported by studies done by Zohar *et al.* (1995) and (Mylonas *et al.*, 1997). They found out that the surge of LH in captive broodstocks was not as high as wild broodstocks. It was later found that the failure of LH secretion is the main reason of reproduction dysfunction in striped bass, *Morone saxatilis*, rather than problems in the LH synthesis in the pituitary (Steven, 1999).

Another possible factor that might cause reproductive dysfunction in this current feeding trial is water temperature. During the feeding trial, the water temperature recorded ranged between 28.95 to 30.95°C due to the equinox phenomenon ("Equinox Phenomenon Starts," 2015). Arctic charr, *Salvelinus*

alpinus,which were reared in different temperature (5 and 10°C) react differently to hormone induction (Gillet & Breton, 2009). At 10°C they found that sGnRHa alone is not enough to induce ovulation, compared to sGnRHa + pimozide, a dopamine inhibitor.

A study on grass carp, *Ctenopharyngodon idella*, later found that high temperature (28°C), induced dopamine inhibition (Glasser *et al.*, 2004). They reveal that the problem were more severe in the ovary itself. At higher temperature (above optimal), the ovary was unable to secrete DHP, despite normal LH levels. Although the LH and DHP levels were not evaluated in the second feeding trial, it can be hypothesized that the lack of LH surge, which later lead to lower or no DHP increase, caused the failure of female *T. tambroides* to undergo FOM even with the induction with Ovaprim injection. As for the second feeding trial, the possible hormonal cascade effect from the injection of Ovaprim was as shown in Figure 5.2. It might be possible that the lack of LH surge in addition with the increasing temperature above optimal level, led to the failure of *T. tambroides* females to undergo FOM.

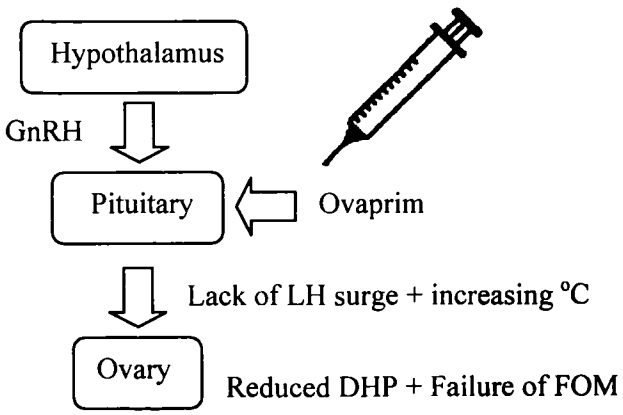


Figure 5.2: Possible hormonal cascade for hormonal induction on female *Tor tambroides* fed with diet containing varying lipid levels.

Finally, for the second feeding trial, it had shown that varying lipid levels effect the gonad development, fatty acid compositions (in the muscle, liver and gonad), and E2 levels in blood plasma of female *T. tambroides* broodstock. Fish fed with diet containing 82 g kg^{-1} of lipid has the numerically higher GSI percentages value with the only oocyte development up to stage 6 oocytes. Based on the fatty acid concentrations, results might suggest that both fish fed with diet containing 60 and 82 g kg^{-1} have fully undergone vitellogenesis but failed to go through with FOM although have been induced with ovaprim injection. The hormonal injection stimulates E2 concentration in all treated fish where fish fed with diet containing 82 g kg^{-1} of lipid has significantly higher E2 level compared to other treated fish which correlates with the GSI percentage. However, further studies need to be done, to see much deeper mechanism of hormonal induction in female *T. tambroides* broodstock. Readings of LH, DHP and to some extent cortisol and/ or glucose (as a stress indicator) are needed to be analyzed to see the bigger picture on hormonal induction on *T. tambroides.*

5.3 Effects of Varying Lipid Levels on Male *Tor tambroides* Broodstocks

5.3.1 Effects on the Gonad Development

In continuation from the second feeding trial on female T. tambroides, the third feeding trial was intended to investigate the effects of varying lipid levels on the gonad development of male *T.tambroides* broodstocks. The GSI values were affected by increasing level of lipid in the diet where the GSI of fish fed with diet containing 82 g kg^{-1} of lipid was the highest and significantly different compared to other treated fish. However, no significant differences were found in the HSI values between dietary treatments (Table 4.10). The male liver play important roles in digestion, nutrient metabolism, energy storage and detoxification process (Nunes *et al.*, 2011). Based on Wibowo and Kaban (2014) classification of testis maturation, all treated fish were determined at Stage 4 males with well-developed testis.

Observation on the gonad histology shows that male *T. tambroides* has a cystic type of spermatogenesis compared to Senagalese sole, *Solea senagalensis* (García-López *et al.*, 2006) which has a semi-cystic type of spermatogenesis. The difference between these two types of spermatogenesis, is that in cystic spermatogenesis, large number of spermatozoa were released simultaneously in the lumen (Mylonas *et al.*, 2017; Shahi *et al.*, 2015) whereas in semi-cystic spermatogenesis, spermatid were released in the lumen, where they complete spermatogenesis into spermatozoa (García-López *et al.*, 2006 and Mylonas *et al.*, 2017). In the third feeding trial, all stages of spermatogenesis were seen from the histological observation study. The anterior and lumen area were dominated by spermatozoa, ready to be released, achievable by gentle pressure on the abdomen. The posterior part showed other spermatogenic stages in form of spermatogonia, spermatocyte, spermatid and spermatozoa. This is in agreement with studies done by Ismail *et al.* (2011) and Wibowo and Kaban (2014) on the same species. This differs from results found in male golden mahseer, *Tor putitora* (Shahi *et al.*, 2015). They found that *T. putitora* exhibit a synchronous spermatocyte development. This is due to the fact that *T. putitora* is a seasonal spawner due to its temperate condition

(Nautiyal, 1984) while the tropical climate *Tor tambroides* is a non-seasonal spawner (Ismail *et al.*, 2011).

Studies of lipid nutrition on male fish were much less compared to studies on its effect for female fish. Most of the studies were conducted to investigate the effects of diets given on the sperm quality of the fish. Asturiano *et al.* (2001) found that male European sea bass, *Dicentrarchus labrax* L. performed best with diets enriched with fish oil and tuna orbital oil compared with wet diet (trash fish and squid). The PUFA enriched diet helps increased sperm count and spermatocrit values compared to the wet diet which is also supported by studies done by Nandi *et al.* (2007) on carp, *Catla catla*. Diet with n-3 deficient values has a negative effect on the reproductive performance of rainbow trout, *Oncorhynchus mykiss* (Vassallo-Agius *et al.*, 2001). The n-3 deficient diet gives lower sperm motility which led to lower fertilization and hatching rates. Studies by Henrotte *et al.* (2010) conclude that different ratio of n-3 to n-6 (0.2 and 7.0) affects the FA composition of semen but not sperm quality in Eurasian perch, *Perca fluviatilis*. Wassef *et al.* (2012) declare that diet with fish oil was better than linseed and soybean oil. They state that diets supplied with vegetables oil delayed maturation and was not enough for gonad maturation of male gilthead seabream, *Sparus aurata*, and a finishing diet with 100% fish oil was suggested to restore any problem by using vegetables oil. This was also confirm by Bogevik *et al.* (2014) that vegetable oil supplementation delayed maturation in male sea bass, *D. labrax*. A study on mahseer barb, *Neolissochillus stracheyi* (Duangjai *et al.*, 2017) founds that increasing level of n-3 inclusion through krill oil led to better milt volume and total sperm count while reducing abnormal sperm.

In the third feeding trial, after hormone induction, only three out of 12 fish were able to express measurable milt, two from the fish fed with diet containing 82 g kg^{-1} of lipid, and one from the fish fed with diet containing 128 g kg^{-1} of lipid. Sperm concentrations were found higher in fish fed with diet containing 82 g kg^{-1} of lipid compared to fish fed with diet containing 128 g kg^{-1} of lipid (Table 4.11). These concentrations were higher than reported in golden mahseer, *Tor putitora* (Basavaraja and Hegde, 2005). They found that fish injected with Ovaprim have

lower sperm concentration compared to uninjected fish. The same condition was also found in dace, *Leuciscus leuciscus* L. (Cejko *et al.*, 2012) and chub, *Leuciscus cephalus* L. (Cejko and Krejszeff, 2016).

Results from the third feeding trial shows that male *T. tambroides* broodstocks required lipid level of 82 g kg^{-1} (2.5% lipid inclusion) which gives better results in terms of GSI percentages. This is in agreement with results found in second feeding trial. This shows that both female and males broodstocks required the same lipid level in their diets. Therefore, the author would suggest the possibility of using lower fishmeal amount in the diet compared to present study (500g kg^{-1} of diet) for economical aspect.

5.3.2 Effects on the Fatty Acid Concentration

For the third feeding trial, significantly different FA concentration in the diet did not reflect in the FA concentration in the muscle of treated fish. However different pattern were shown in the FA concentration in the liver. As stated before, liver and adipose tissue is the main site for lipogenesis in most animals. The increasing total FA concentration in the diet (Table 4.5) was mirrored with the increasing concentration of total FA in the liver (Table 4.13). The ranges differs greatly with higher concentration in the liver (35.94-47.12 mg g^{-1}) compared to the diet given (11.55-33.64 mg g^{-1}). Meanwhile, in the gonad, the concentrations of total FA were higher in fish fed with diet containing 60, 82 and 128 g kg^{-1} of lipid compared to diet given except for fish fed with diet containing 105 g kg^{-1} of lipid (Table 4.15). The DHA values in the gonad increases as the dietary lipid level increases. Ramezani-Fard *et al.* (2012a) found that DHA level increases in the liver of *T. tambroides* fingerlings as the dietary lipid level increases. This may be caused by the importance of DHA in the membrane phospholipid and helps the quantity and quality of sperm (Henrotte *et al.*, 2010). This is shown from the feeding trial that, even though the DHA level was decreasing in the diets, the DHA level was increasing in the gonad. This confirms the metabolism to produce DHA from its FA precursor to counter the lesser concentration in the diets given.

Much of the researches of fatty acids on male fish were concentrated on the effects of ARA on the male reproductive performances. Lower ARA levels in captive broodstocks were reported in black sea bream, *Spondyliosoma cantharus* (Rodríguez *et al.*, 2004), European sea bass, *Dicentrarchus labrax* (Bell *et al.*, 1996), Senegalese sole, *Solea senegalensis* (Norambuena *et al.*, 2012a), greater amberjack, *Seriola dumerili* (Rodríguez-Barreto *et al.*, 2012) and common snook, *Centropomus undecimalis* (Hauville *et al.*, 2015) compared to wild broodstocks. This lower level of ARA has caused negative reproduction effects in Senegalese sole, *S. senegalensis* (Norambuena *et al.*, 2012b) and common snook, *C. undecimalis* (Hauville *et al.*, 2015). ARA is a precursor of prostaglandins which involves in stimulating later stages of gametogenesis, which forms 2-series prostaglandins, while EPA produced 3-series prostaglandins with antagonistic effects (Sargent *et al.*, 2002; Tocher, 2003).

In the third feeding trial, ARA levels were higher in the muscle and liver of fish fed with diet containing 82 g kg^{-1} of lipid, although it is not significantly different between treatments. Asturiano *et al.* (2001) stated that ARA helps in stimulating testicular T production by converting into prostaglandin. Increasing level of ARA supplementation in Senegalese sole, *Solea senegalensis* resulting in the increased of T and 11 keto testosterone (11 KT) (Norambuena *et al.*, 2013a). Baeza *et al.* (2015) also stated that the consumption of ARA led to higher sperm velocity in European eel, *Anguila anguila* L. In this current study, the concentration of ARA in the gonad of fish fed with diet containing 82 g kg^{-1} of lipid was lower compared to fish fed with diet containing 60, 105 and 128 g kg^{-1} of lipid, if the concentration was expressed in percentage of total FA. Its value was 3.51 ± 0.70 compared to 5.21 ± 0.10, 5.37 ± 0.48 and 5.40 ± 0.52 % respectively (Appendix 1). It can be hypothesized that lower percentages of ARA in fish fed with diet containing 82 g kg^{-1} of lipid was contributed by the assumptions that ARA were converted into prostaglandin, hence increasing sperm quality and volume. This was supported by the fact that two fish from fish fed with diet containing 82 g kg^{-1} of lipid expressed sperm.

The accumulation of ARA is also seen to be affected by gender. Compared to the second feeding trial, the ARA levels was higher in the third feeding trial in flesh

and liver samples. Positive correlation of ARA and the treatment can be seen in this feeding trial (Figure 4.17) compared to the second feeding trial (Figure 4.11). This gender specific ARA deposition was also seen in Senegalese sole, *Solea senagalensis* (Norambuena *et al.*, 2012a) and tongue sole, *Cynoglossus semilaevis* (Xu *et al.*, 2017). They found out that ARA supplementation induces ARA accumulation in the testis more, compared to ovary. A study on *S. senagalensis* indicate that ARA supplementation was between 2.3 and 3.2 % from tested diets (ranged 0.7-6.0%) to maintain the ARA levels in tissues (Norambuena *et al.*, 2013b).

It is suggested that further studies on the effect of different n-3 to n-6 ratio (~1.0) should also be conducted for male *T. tambroides* broodstocks, as suggested for females broodstocks (subchapter 5.2.2). However, the effects of ARA level should be emphasized for male *T. tambroides* on the gonad development and sperm quality parameters.

5.3.3 Effects on the Steroid Plasma Concentration

In the third feeding trial, the patterns of testosterone (T) in the plasma were monitored between dietary groups. At the end of the experimental duration, the broodstock were injected with Ovaprim to induced spermiation. The E2 concentrations were monitored at 0, 12 and 24h post hormonal injection of Ovaprim. The concentrations of T were elevated from 0h before reaching its peak at 12h and subsequently decrease at 24h PI. The pattern was the same in all diet treated fish. This pattern were also seen in another cyprinid fish, grass carp, *Ctenopharyngodon idellus* (Metwally and Fouad, 2008; Mousavi and Yousefian, 2012). The decreased of T after 12h post injection might be caused by a shift in the steroidogenic enzymes by the activity of 20β hydroxysteroid dehydrogenase, inducing 17α-hydroxyprogesterone synthesis, resulting in the production of the maturation inducing steroid (MIH), 17α,20β-dihydroxy-4-pregnen-3-one (DHP) (Cejco *et al.*, 2012 and Cejco and Krejszeff, 2016). Therefore, it can be assumed that this decrease of T levels was caused by the steroidogenic shift in the testis.

In the third feeding trial, fish fed with diet containing 82 g kg^{-1} of lipid exhibit the highest T level in all monitored hours compared to the other treatments. As discussed in sub-topic 5.3.1, fish fed with diet containing 82 g kg^{-1} of lipid has the highest GSI values. Weltzien *et al.* (2002) conclude that the androgen levels were correlated with GSI level in Atlantic halibut, *Hippoglossus hippoglossus* L. They found that higher T and 11 keto testosterone values in males with higher GSI. As mention before, only three fish out of 12 males were able to express measureable sperm at the end of the feeding treatment. This may be affected by the influence of temperature.

As discussed in subtopic 5.2.3, during the feeding trial, the water temperature recorded was between 28.95 to 30.95°C. It should also put into consideration, that the tolerance of male *T. tambroides* to increased temperature is the same with female *T. tambroides*. Therefore, it can be hypothesized that, the increased temperature also induced dopamine inhibition (Gillet and Breton, 2009) and/ or affects the hypothalamus-pituitary- gonad axis through negative effects on the DHP production (Glasser *et al.*, 2004) as in female fish. However, this should be confirmed by further studies on the effects of temperature on the hormonal cascade in male *T. tambroides*. As for the third feeding trial, the possible hormonal cascade effect from the injection of Ovaprim was as shown in Figure 5.3. It might be possible that the lack of LH surge in addition with the increasing temperature above optimal level, decrease the secretion of DHP, hence led to the failure of *T. tambroides* male broodstocks undergo spermiation.

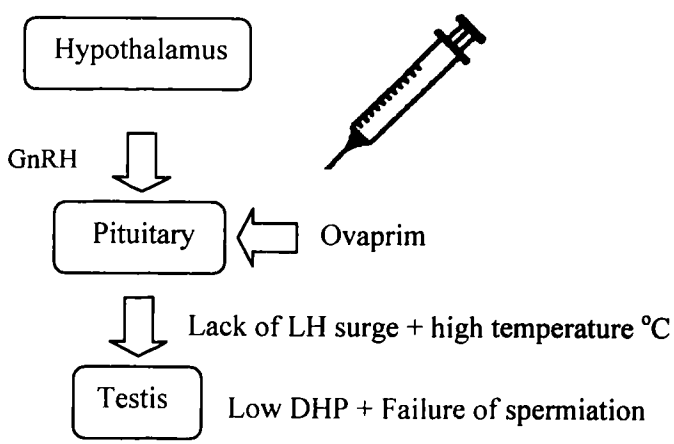


Figure 5.3: Possible hormonal cascade for hormonal induction on male *Tor tambroides* fed with diet containing varying lipid levels.

Finally, for the third feeding trial, it had shown that varying lipid levels affect the gonad development, fatty acid compositions (in the muscle, liver and gonad), and T levels in blood plasma of male *T. tambroides* broodstocks. Fish fed with diet containing 82 g kg^{-1} of lipid has significantly higher GSI percentages value compared to other treated fish. The ARA level affect the ability of male *T. tambroides* to express sperm after hormone injection and T concentration was significantly higher in fish fed with diet containing 82 g kg^{-1} of lipid. Therefore, it is concluded that diet containing 82 g kg^{-1} of lipid where fish gives the best results in term of the above criteria. However, further studies need to be done on the effect of ARA on male reproductive quality and to see much deeper mechanism of hormonal induction in male *T. tambroides* broodstocks.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATION

The study on lipid nutrition on gonad development of Malaysian mahseer, *Tor tambroides* was completed. The main objective to investigate the effects of different lipid source and lipid inclusion percentages was achieved. This was achieved by analyzing the GSI and HSI values, gonadal histology observations, oocyte diameters, sperm quality, fatty acid compositions, and the level of steroid after hormonal induction using Ovaprim. First, three experimental diet with different fish oil to corn oil ratios were tested. Results confirmed that *T. tambroides*, just like other cyprinid fish, prefer diets with supplementation from both n-3 and n-6 oil source. Only fish fed with diet containing fish oil to corn oil ratio of 1:1 exhibit numerically higher GSI percentages, farthest development of oocytes stages, with numerically higher E2 levels. However, this conclusion need to be treat with extreme caution as it was based on qualitative aspects. Consequently, four experimental diets with varying lipid levels were tested on both females and males *T. tambroides* broodstocks. The highest GSI value for the second and third feeding trial was displayed by fish fed with diet containing 82 g kg^{-1} of lipid (2.5% inclusion) with value of n-3 to n-6 ratio of 1.25. This value is much lower than other lipid level reported to be used in diets for *T. tambroides* broodstocks. Results from FA composition shows that *Tor tambroides* broodstocks were able to elongate LA and ALA to HUFA. ARA levels in male were higher compared to female in all tissue samples. Consumption of ARA in fish fed with diet containing 82 g kg^{-1} of lipid led to higher T levels and also the ability to express sperm. Besides normal reproductive dysfunctions, both female and males *T. tambroides* broodstock might also be affected by high temperature in terms of failure on FOM and ability to express sperm. It is possible that the high temperature induced dopamine inhibition and disrupted both male and females hormonal HPG-axis.

Future research may focus on formulating a broodstocks specific diet for *T. tambroides* by adapting from the results of this study. It should be focused on ARA, EPA and DHA levels in the diets for female broodstock, while for male broodstock, the effects of ARA levels should be emphasized. Further studies on molecular levels are suggested for the knowledge of exact and precise mechanism of lipid nutrition and hormonal manipulation on *T. tambroides* broodstocks at optimal condition.

REFERENCES

Abdulkadir, S., & Tsuchiya, M. (2008). One-step method for quantitative and qualitative analysis of fatty acids in marine animal samples. Journal of Experimental Marine Biology and Ecology, 354(1), 1-8.

Acharjee, A., Chaube, R., & Joy, K. P. (2016). Ovaprim, a commercial spawning inducer, stimulates gonadotropin subunit gene transcriptional activity: A study correlated with plasma steroid profile, ovulation and fertilization in the catfish *Heteropneustes fossilis*. *General and Comparative Endocrinology*.

Akhtar, M. S., Ciji, A., Sarma, D., Rajesh, M., Kamalam, B. S., Sharma, P., & Singh, A. K. (2017). Reproductive dysfunction in females of endangered golden mahseer (*Tor putitora*) in captivity. *Animal Reproduction Science*, *182*, 95–103.

Almansa, E., Martian, M. V., Cejas, J. R., Badi, P., Jerez, S., & Lorenzo, A. (2001). Lipid and fatty acid composition of female gilthead seabream during their reproductive cycle: effects of a diet lacking n- 3 HUFA. Journal of fish biology, 59(2), 267-286.

Ambak, M. A., Ashraf, A. H., & Budin, S. 2007. Conservation of the Malaysian mahseer in Nenggiri basin through community action. In S. Siti Shapor, A. Christianus, C. K. Ng, & S. . De Silva (Eds.), *Mahseer: The Biology, Culture and Conservation* (Vol 14, pp. 217-228). Kuala Lumpur: Malaysian Fisheries Society Occasional Publication.

Ando, H., Swanson, P., Kitani, T., Koide, N., Okada, H., Ueda, H., & Urano, A. (2004). Synergistic effects of salmon gonadotropin-releasing hormone and estradiol-17β on gonadotropin subunit gene expression and release in masu salmon pituitary cells in vitro. *General and Comparative Endocrinology*, *137*(1), 109–121.

AOAC (Association of Official Analytical Chemists). 2006. Official methods of analysis of AOAC International, 18 ed. AOAC International, Maryland, USA.

Asaduzzaman, M., Kader, M. A., Bulbul, M., Abol-Munafi, A. B., Ghaffer, M. A., & Verdegem, M. (2016). Biochemical composition and growth performances of Malaysian Mahseer *Tor tambroides* larvae fed with live and formulated feeds in indoor nursery rearing system. *Aquaculture Reports*, *4*, 156–163.

Asturiano, J. F., Sorbera, L. A., Ramos, J., Kime, D. E., Carrilo, M., & Zanuy, S. (2000). Hormonal regulation of the European sea bass reproductive cycle: an individualized female approach. Journal of Fish Biology, 56(5), 1155-1172.

Asturiano, J. F., Sorbera, L. A., Carrillo, M., Zanuy, S., Ramos, J., Navarro, J. C., & Bromage, N. (2001). Reproductive performance in male European sea bass (*Dicentrarchus labrax, L.*) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. *Aquaculture*, *194*(1–2), 173–190.

Ayub,M., Ahmad, I. and Pervaiz, K. (2007). Breeding of *Tor putitora* with and without hypophysation at Hattian Nursery Unit District Attock, Punjab-Pakistan. n S. Siti Shapor, A. Christianus, C. K. Ng, & S. . De Silva (Eds.), *Mahseer: The Biology, Culture and Conservation* (Vol 14, pp. 99-106). Kuala Lumpur: Malaysian Fisheries Society Occasional Publication.

Azuadi, N. M., Siraj, S. S., Daud, S. K., Christianus, A., Harmin, S. A., Sungan, S., & Britin, R. (2011). Enhancing ovulation of Malaysian mahseer (*Tor tambroides*) in captivity by removal of dopaminergic inhibition. *Journal of Fisheries and Aquatic Science*, *6*(7), 740–750.

Azuadi, N. M., Siraj, S. S., Daud, S. K., Christianus, A., Harmin, S. A., Sungan, S., & Britin, R. (2013a). Induction of Ovulation in F1 Malaysia Mahseer, *Tor tambroides* (Bleeker, 1854) by using synthetic and Non-synthetic hormones. *Asian Journal of Animal and Veterinary Advances*, *8*(4), 582– 592.

Azuadi, N. M., Siraj, S. S., Daud, S. K., Christianus, A., Harmin, S. A., Sungan, S.,

& Britin, R. (2013b). Sex steroid levels and breeding performance of f1 generation malaysian mahseer, *Tor tambroides* (Bleeker, 1854) by removal of dopaminergic inhibition. *Asian Fisheries Science*, *8*(2), 257–265.

Baeza, R., Mazzeo, I., Vílchez, M. C., Gallego, V., Peñaranda, D. S., Pérez, L., & Asturiano, J. F. (2015). Relationship between sperm quality parameters and the fatty acid composition of the muscle, liver and testis of European eel. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, *181*, 79–86.

Bami, M. L., Kamarudin, M. S., Saad, C. R., Ebrahimi, M., Hafid, S. R. A., Chakravarthi, S., & Radhakrishnan, A. K. (2017a). Effects of palm oil products on growth performance, body composition and fatty acid profile of juvenile Malaysian mahseer (*Tor tambroides*). *Journal of Oil Palm Research*, *29*(3), 387-400.

Bami, M. L., Kamarudin, M. S., Saad, C. R., Arshad, A., & Ebrahimi, M. (2017b). Effects of canarium fruit (*Canarium odontophyllum*) oil as a dietary lipid source for juvenile mahseer (*Tor tambroides*) performance. *Aquaculture Reports*, *6*, 8–20.

Basavaraja, N., & Hegde, S. N. (2005). Some characteristics and short-term preservation of spermatozoa of Deccan mahseer, *Tor khudree* (Sykes). *Aquaculture Research*, *36*(5), 422–430.

Bazaz, M. M., & Keshavanath, P. (1993). Effect of feeding different levels of sardine oil on growth, muscle composition and digestive enzyme activities of mahseer, Tor khudree. Aquaculture, 115(1-2), 111-119.

Bell, G., Farndale, B. M., Bruce, M. P., Navas, J. M., Carillo, M., Bell, J. G., Carillo, M. (1997). Effects of broodstock dietary lipid on fatty acid compositions of eggs from sea bass (*Dicentrarchus labrax*). *Aquaculture*, *149*(1–2), 107–119.

Bell, J. G., Castell, J. D., Tocher, D. R., MacDonald, F. M., & Sargent, J. R. (1995). Effects of different dietary arachidonic acid: docosahexaenoic acid ratios on

phospholipid fatty acid compositions and prostaglandin production in juvenile turbot (*Scophthalmus maximus*). *Fish Physiology and Biochemistry*, *14*(2), 139–151.

Bell, M. V., Dick, J. R., Thrush, M., & Navarro, J. C. (1996). Decreased ratio in sperm from cultured sea bass, *Dicentrarchus labrax*, broodstock compared with wild fish. *Aquaculture*, *144*(1–3), 189–199.

Bell, J.G., McEvoy, J., Tocher, D.R., McGhee, F., Campbell, P.J., Sargent, J.R., 2001. Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (Salmo salar) affects tissue lipid compositions and hepatocyte fatty acid metabolism. Journal of. Nutrition. 131, 1535-1543.

Bell, J. G., Tocher, D. R., Henderson, R. J., Dick, J. R., & Crampton, V. O. (2003). Altered fatty acid compositions in Atlantic salmon (Salmo salar) fed diets containing linseed and rapeseed oils can be partially restored by a subsequent fish oil finishing diet. The Journal of nutrition, 133(9), 2793-2801.

Bell, M. V, & Tocher, D. R. (2009). Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: general pathways and new directions. In *Lipids in aquatic ecosystems* (pp. 211–236). Springer.

Bogevik, A. S., Rathore, R. M., Arjona, Y., Atack, T., Treasurer, J., Rønnestad, I., & Kousoulaki, K. (2014). Dietary plant oils delay early sexual maturation compared with marine fish oil in male European seabass (*Dicentrarchus labrax*) - Effects on testis histology and key reproductive hormones. *Aquaculture*, *431*, 73–84.

Bromage, N., Hardiman, P., Jones, J., Springate, J., & Bye, V. (1990). Fecundity, egg size and total egg volume differences in 12 stocks of rainbow trout, *Oncorhynchus mykiss* Richardson. *Aquaculture Research*, *21*(3), 269–284.

Brooks, S., Tyler, C. R., & Sumpter, J. P. (1997). Egg quality in fish: what makes a good egg?. *Reviews in Fish Biology and fisheries*, *7*(4), 387-416.

Butts, I. A. E., Baeza, R., Støttrup, J. G., Krüger-Johnsen, M., Jacobsen, C., Pérez, L., Tomkiewicz, J. (2015). Impact of dietary fatty acids on muscle composition, liver lipids, milt composition and sperm performance in European eel. *Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology*, *183*, 87–96.

Buzzi, M., Henderson, R. J., & Sargent, J. R. (1996). The desaturation and elongation of linolenic acid and eicosapentaenoic acid by hepatocytes and live microsomes from rainbow trout (*Oncorhynchus mykiss*) feeds diets containing fish oil or olive oil. *Biochimica et Biophysica Acta*, *1299*, 235–244.

Buzzi, M., Henderson, R. J., & Sargent, J. R. (1997). Biosynthesis of docosahexaenoic acid in trout hepatocytes proceeds via 24-carbon intermediates. *Comparative Biochemistry and Physiology B*, *116*(2), 263–267.

Caballero, M.J., Obach, A., Rosenlund, G., Montero, D., Gisvold, M., Izquierdo, M.S., 2002. Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, Oncorhynchus mykiss. Aquaculture. 214, 253-271.

Castro, L. F. C., Tocher, D. R., & Monroig, O. (2016). Long-chain polyunsaturated fatty acid biosynthesis in chordates: Insights into the evolution of Fads and Elovl gene repertoire. *Progress in Lipid Research*, *62*, 25–40.

Cejko, B. I., & Krejszeff, S. (2016). Sperm characteristics of chub *Leuciscus cephalus* (L.) collected in artificial condition after Ovopel and Ovaprim treatment. *Aquaculture Research*, *47*(3), 847–856.

Cejko, B. I., Targońska, K., Kowalski, R. K., Zarski, D., Sarosiek, B., Kucharczyk, D., & Glogowski, J. (2012). The effectiveness of hormonal preparations (Ovopel, Ovaprim, LHRHa, hCG and CPE) in stimulating spermiation in dace *Leuciscus leuciscus* (L.). *Journal of Applied Ichthyology*, *28*(6), 873–877.

Cerdá, J., Carrillo, M., Zanuy, S., Ramos, J., & de la Higuera, M. (1994). Influence of nutritional composition of diet on sea bass, Dicentrarchus labrax L., reproductive performance and egg and larval quality. Aquaculture, 128(3-4), 345-361.

Chaube, R., Singh, R. K., & Joy, K. P. (2014). Effects of ovaprim, a commercial spawning inducer, on vasotocin and steroid hormone profiles in the catfish *Heteropneustes fossilis*: In vivo and in vitro studies. *General and Comparative Endocrinology*, *195*, 190–200.

Chew, P. C., Abd- Rashid, Z., Hassan, R., Asmuni, M., & Chuah, H. P. (2010). Semen cryo- bank of the Malaysian Mahseer (Tor tambroides and T. douronensis). Journal of Applied Ichthyology, 26(5), 726-731.

Chowdhury, A. J. K., Zakaria, N. H., Abidin, Z. A. Z., & Rahman, M. M. (2016). Phototrophic purple bacteria as feed supplement on the growth, feed utilization and body compositions of Malaysian Mahseer, *Tor tambroides* juveniles. *Sains Malaysiana*, *45*(1), 135–140.

Chyb, J., Mikolajczyk, T., & Breton, B. (1999). Post-ovulatory secretion of pituitary gonadotropins GtH I and GtH II in the rainbow trout (*Oncorhynchus mykiss*): Regulation by steroids and possible role of non-steroidal gonadal factors. *Journal of Endocrinology*, *163*(1), 87–97.

Coldebella, I. J., Emanuelli, T., Veiverberg, C. A., Pretto, A., Rossato, S., Ferreira, D., ... Neto, J. R. (2013). Effect of different dietary lipid levels on the reproduction of *Rhamdia quelen* (Quoy and Gaimard, 1824). *Aquaculture Nutrition*, *19*(5), 751–764.

Coward, K., & Bromage, N. R. (2000). Reproductive physiology of female tilapia broodstock. Reviews in Fish Biology and Fisheries, 10(1), 1-25.

da Silva, F. F. G., Støttrup, J. G., Kjørsvik, E., Tveiten, H., & Tomkiewicz, J. (2016). Interactive effects of dietary composition and hormonal treatment on

reproductive development of cultured female European eel, *Anguilla anguilla*. *Animal Reproduction Science*, *171*, 17–26.

Dahle, R., Taranger, G. L., Karlsen, O., Kjesbu, O. S., & Norberg, B. (2003). Gonadal development and associated changes in liver size and sexual steroids during the reproductive cycle of captive male and female Atlantic cod (*Gadus morhua* L.). *Comp Biochem Physiol A Mol Integr Physiol*, *136*(3), 641–653.

Desai, V.R. 2003. Synopsis of Biological data on The Tor Mahseer *Tor tor* (Hamilton, 1822). FAO Fisheries Synopsis No. 158 Rome FAO: 36p.

De Silva, S. S., Gunasekera, R. M., & Ingram, B. A. (2004). Performance of intensively farmed Murray cod *Maccullochella peelii peelii* (Mitchell) fed newly formulated vs. currently used commercial diets, and a comparison of fillet composition of farmed and wild fish. *Aquaculture Research*, *35*(11), 1039–1052.

De Silva, S. S., Ingram, B., Sungan, S., Tinggi, D., & Sim, S. Y. (2004). Artificial propagation of the indigenous Tor species, empurau (*T. tambroides*) and semah (*T . douronensis*), Sarawak , East Malaysia. In S. Wilkinson (Ed.), *Aquaculture Asia* (Vol. IX, pp. 15–20). Network of Aquaculture Centres in Asia-Pacific.

Department of Fisheries. (2016). SIARAN MEDIA.

Desai, V. R. 1973. Studies on the fishery and biology of Tor tor (Hamilton) from River Namada. II. Maturity, fecundity and larval development. Proceeding of the Indian National Science Acad. Vol. 39(2), 228-248.

DOF (2015). Annual Fisheries Statistics 2014. Department of Fisheries, Malaysia.

Duangjai, E., Punya-in, S., A-ta, A., Arin, S., Sangkamruang, A., & Sresutham, P. (2017). Effects of omaga-3 fatty acids (from krill oil) on sperm quality of

mahseer barb , *Neolissochilus stracheyi* , brooders reared in captivity. *SNRU Journal of Science and Technology*, *9*(2), 474-482.

El-Sayed, A. F. M., Mansour, C. R., & Ezzat, A. A. (2005). Effects of dietary lipid source on spawning performance of Nile tilapia (Oreochromis niloticus) broodstock reared at different water salinities. Aquaculture, 248(1-4), 187-196.

Emery, J. A., Norambuena, F., Trushenski, J., & Turchini, G. M. (2016). Uncoupling EPA and DHA in fish nutrition: Dietary demand is limited in Atlantic Salmon and effectively met by DHA alone. *Lipids*, *51*(4), 399–412.

Esa, Y. B., Siraj, S., Daud, K., Rovie, J., & Japning, R. (2006). Mitochondrial DNA Diversity of *Tor douronensis* Valenciennes (Cyprinidae) in Malaysian Borneo. *Pertanika Journal of Tropical Agricultural Science*, *29*, 47–55.

Esa, Y. B., Siraj, S. S., Daud, S. K., Rahim, K. A. A., Japning, J. R. R., & Tan, S. G. (2008). Mitochondrial DNA diversity of Tor tambroides Valenciennes (Cyprinidae) from five natural populations in Malaysia. Zoological Studies, 47(3), 360-367.

Equinox Phenomenon starts today afternoon, (2015, March 21). *Mynewshub*. Retrieve from http://eng.mynewshub.cc/equinox-phenomenon-starts-today-afternoon/

Fernandes, C. A. F., Oliveira, P. G. V., Oliveira, C. H. B., Hazin, F. H. V., & Travassos, P. (2016). Oocyte development and fecundity type of the Brazilian Snapper *Lutjanus alexandrei* Moura & Lindeman, 2007 (Perciformes: Lutjanidae). *Brazilian Journal of Biology*, *76*(1), 126–135.

Fernández-Palacios, H., Izquierdo, M. S., Robaina, L., Valencia, A., Salhi, M., & Vergara, J. (1995). Effect of n − 3 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata* L.). *Aquaculture*, *132*(3–4), 325–337.

Fonseca-Madrigal, J., Navarro, J. C., Hontoria, F., Tocher, D. R., Martínez-Palacios, C. A., & Monroig, Ó. (2014). Diversification of substrate specificities in teleostei Fads2: characterization of Δ4 and Δ6Δ5 desaturases of Chirostoma estor. *Journal of Lipid Research*, *55*(7), 1408–1419.

Furuita, H., Hori, K., Suzuki, Sugita, T., & Yamamoto, T. (2007). Effect of n-3 and n-6 fatty acids in broodstock diet on reproduction and fatty acid composition of broodstock and eggs in the Japanese eel *Anguilla japonica*. *Aquaculture*, *267*(1–4), 55–61.

Furuita, H., Tanaka, H., Yamamoto, T., Shiraishi, M., & Takeuchi, T. (2000). Effects of n y 3 HUFA levels in broodstock diet on the reproductive performance and egg and larval quality of the Japanese flounder , *Paralichthys olivaceus*. *Aquaculture*, *210*(3–4), 387–398.

Furuita, H., Tanaka, H., Yamamoto, T., Suzuki, N., & Takeuchi, T. (2002). Effects of high levels of n− 3 HUFA in broodstock diet on egg quality and egg fatty acid composition of Japanese flounder, *Paralichthys olivaceus*. *Aquaculture*, *210*(1), 323–333.

Furuita, H., Yamamoto, T., Shima, T., Suzuki, N., & Takeuchi, T. (2003). Effect of arachidonic acid levels in broodstock diet on larval and egg quality of Japanese flounder *Paralichthys olivaceus*. *Aquaculture*, *220*(1–4), 725–735.

García-López, Á., Fernández-Pasquier, V., Couto, E., Canario, A. V. M. V. M., Sarasquete, C., & Martínez-Rodríguez, G. (2006). Testicular development and plasma sex steroid levels in cultured male Senegalese sole *Solea senegalensis* Kaup. *General and Comparative Endocrinology*, *147*(3), 343–351.

Gauthier-Clerc, S., Pellerin, J., Amiard, J.C., 2006. Estradiol-17 and testosterone concentrations in male and female Mya arenaria during the reproductive cycle. General and Comparative Endocrinology 145, 133–139.

Gillet, C., & Breton, B. (2009). LH secretion and ovulation following exposure of

Arctic charr to different temperature and photoperiod regimes: Responsiveness of females to a gonadotropin-releasing hormone analogue and a dopamine antagonist. *General and Comparative Endocrinology*, *162*(2), 210–218.

Ghaedi, A., Kabir, M. A., & Hashim, R. (2016). Effect of lipid levels on the reproductive performance of Snakehead murrel, Channa striatus. Aquaculture research, 47(3), 983-991.

Glasser, F., Mikolajczyk, T., Jalabert, B., Baroiller, J.-F., & Breton, B. (2004). Temperature effects along the reproductive axis during spawning induction of grass carp (*Ctenopharyngodon idella*). *General and Comparative Endocrinology*, *136*(2), 171–179.

Glencross, B. D. (2009). Exploring the nutritional demand for essential fatty acids by aquaculture species. Reviews in Aquaculture, 1(2), 71-124.

Grande, M., Murua, H., Zudaire, I., & Korta, M. (2012). Oocyte development and fecundity type of the skipjack, *Katsuwonus pelamis*, in the Western Indian Ocean. *Journal of Sea Research*, *73*, 117–125.

Haryono, A. H. T. (2006). Morphological Study for Identification Improvement of Tambra Fish (Tor spp: Cyprinidae) from Indonesia. *Biodiversitas*, *7*(1), 59–62.

Hauville, M. R., Rhody, N. R., Resley, M. J., Bell, J. G., Main, K. L., & Migaud, H. (2015). Comparative study of lipids and fatty acids in the liver, muscle, and eggs of wild and captive common snook broodstock. *Aquaculture*, *446*, 227–235.

Henrotte, E., Kaspar, V., Rodina, M., Psenicka, M., Linhart, O., & Kestemont, P. (2010). Dietary n-3/n-6 ratio affects the biochemical composition of Eurasian perch (*Perca fluviatilis*) semen but not indicators of sperm quality. *Aquaculture Research*, *41*(9), 31–38.

Hiramatsu, N., Todo, T., Sullivan, C. V., Schilling, J., Reading, B. J., Matsubara, T.,

... & Wu, M. (2015). Ovarian yolk formation in fishes: molecular mechanisms underlying formation of lipid droplets and vitellogenin-derived yolk proteins. General and comparative endocrinology, 221, 9-15.

Hismayasari, I. B., Pramana, A., & Marhendra, W. (2015). Gonadosomatic index (GSI), Hepatosomatic index (HSI) and proportion of oocytes stadia as an indicator of rainbowfish *Melanotaenia boesemani* spawning season. *International Journal of Fisheries and Aquatic Studies*, *2*(5), 359-362.

Ingram, B., Sungan, S., Gooley, G., Sih, Y. S., Tinggi, D., & De Silva, S. S. (2005). Induced spawning, larval development and rearing of two indigenous Malaysian mahseer, *Tor tambroides* and *T. douronensis*. *Aquaculture Research*, *36*(10), 1001–1014.

Ingram, B. A., Sungan, S., Tinggi, D., Sim, S. Y., Gooley, G. J., & De Silva, S. S. (2007a). Observations on the growth of cage- and pond-reared *Tor tambroides* and *T. douronensis* in Sarawak, Malaysia. In: Siti Shapor, A. Christianus, C. K. Ng, & S. . De Silva (Eds.), *Mahseer: The Biology, Culture and Conservation* (Vol 14, pp. 145–159). Kuala Lumpur: Malaysian Fisheries Society Occasional Publication.

Ingram, B., Sungan, S., Tinggi, D., Sim, S. Y., & De Silva, S. S. (2007b). Breeding performance of Malaysian mahseer, *Tor tambroides* and *T. douronensis* broodfish in captivity. *Aquaculture Research*, *38*(8), 809–818.

Ingram., B., Sungan, S., Tinggi, D., Sim, S.Y., Gooley, G.J. and De Silva, S.S. (2007c). Developments in the spawning of *Tor tambroides* and *T. douronensis* in captivity. In: Siti Shapor, A. Christianus, C. K. Ng, & S. . De Silva (Eds.), *Mahseer: The Biology, Culture and Conservation* (Vol 14, pp. 123-128). Kuala Lumpur: Malaysian Fisheries Society Occasional Publication.

Ishak, S D; Kamarudin, M S; Ramezani-Fard, E; Saad, C R And Yusof, Y A (2016). Effects of varying dietary carbohydrate levels on growth performance, body composition and liver histology of Malaysian mahseer

fingerlings (Tor tambroides). J. Env. Biol., 37: 755-764.

Islam, M. S., & Tanaka, M. (2004). Optimization of dietary protein requirement for pond-reared mahseer *Tor putitora* Hamilton (Cypriniformes: Cyprinidae). *Aquaculture Research*, *35*(13), 1270–1276.

Ismail, M. F. S., Siraj, S. S., Daud, S. K., & Harmin, S. A. (2011). Association of annual hormonal profile with gonad maturity of mahseer (*Tor tambroides*) in captivity. *General and Comparative Endocrinology*, *170*(1), 125–130.

Izquierdo, M. S., Fernandez-Palacios, H., & Tacon, a G. J. (2001). Effect of broodtsock nutrition on reproductive performance of fish. *Aquaculture*, *197*, 25–42.

Izquierdo, M.S., Montero, D., Robaina, L., Caballero, M.J., Rosenlund, G., Gines, R., 2005. Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. Aquaculture 250, 431-444.

Izquierdo, M. S., Turkmen, S., Montero, D., Zamorano, M. J., Afonso, J. M., Karalazos, V., & Fernández-Palacios, H. (2015). Nutritional programming through broodstock diets to improve utilization of very low fishmeal and fish oil diets in gilthead sea bream. Aquaculture, 449, 18-26.

Jalal, A.C. K., Ambak, M. A., Abol-Munafi, A. B., Hassan, T. H., & Zahangir Alam, M. (2005). Effect of Feed Additives on the Development of Proteolytic Enzymes of the Tropical Sport Fish Malaysian Mahseer (*Tor tambroides* - Bleeker) Fry. *American Journal of Biochemistry and Biotechnology*, *1*(3), 132–134.

Jerez, S., Rodríguez, C., Cejas, J. R., Bolaños, A., & Lorenzo, A. (2006). Lipid dynamics and plasma level changes of 17β-estradiol and testosterone during the spawning season of gilthead seabream (*Sparus aurata*) females of different ages. *Comparative Biochemistry and Physiology - B Biochemistry and*

Molecular Biology, *143*(2), 180–189.

Johnson, R. B. (2009). Lipid Deposition in Oocytes of Teleost Fish During Secondary Oocyte Growth. *Reviews in Fisheries Science*, *17*(1), 78–89.

Kamarudin, M. S. S., Ramezani-fard, E., Saad, C. R. R., & Harmin, S. A. (2012). Effects of dietary fish oil replacement by various vegetable oils on growth performance, body composition and fatty acid profile of juvenile Malaysian mahseer, *Tor tambroides*. *Aquaculture Nutrition*, *18*(5), 532–543.

Kamarudin, M. S., Bami, M. L., Arshad, A., Saad, C. R., & Ebrahimi, M. (2018). Preliminary study of the performance of crude illipe oil (*Shorea macrophylla*) as a dietary lipid source for riverine cyprinid *Tor tambroides*. *Fisheries Science*, 1-13.

Kamler, E. (2005). Parent-egg-progeny relationships in teleost fishes: An energetics perspective. *Reviews in Fish Biology and Fisheries*, *15*(4), 399–421.

Khan, K. U., Zuberi, A., Nazir, S., Ullah, I., Jamil, Z., & Sarwar, H. (2017). Synergistic effects of dietary nano selenium and vitamin C on growth, feeding, and physiological parameters of mahseer fish (Tor putitora). Aquaculture Reports, 5, 70-75.

Kottelat, M. 1998. Fishes of the Nam Theun and Xe Bangfai basins, Laos, with diagnoses of twenty-two new species (Teleostei: Cyprinidae, Balitoridae, Cobitidae, Coiidae and Odontobutidae). Ichthyol. Explor. Freshwater. 9: 1–128.

Kuah, M. K., Jaya-Ram, A., & Shu-Chien, A. C. (2015). The capacity for long-chain polyunsaturated fatty acid synthesis in a carnivorous vertebrate: Functional characterisation and nutritional regulation of a Fads2 fatty acyl desaturase with Δ4 activity and an Elovl5 elongase in striped snakehead (*Channa striata*). *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1851*(3), 248–260.

Kunlapapuk, S., & Kulabtong, S. 2011. Breeding, Nursing and Biology of Thai Mahseer (*Tor tamboides*) in Malaysia: An Overview. *Journal of Agricultural Science and Technology A*, 1214.

Lane, R. L., & Kohler, C. C. (2006). Effects of Dietary Lipid and Fatty Acids on White Bass Reproductive Performance, Egg Hatchability, and Overall Quality of Progeny. *North American Journal of Aquaculture*, *68*(2), 141–150.

Liang, M. Q., Lu, Q. K., Qian, C., Zheng, K. K., & Wang, X. X. (2014). Effects of dietary n-3 to n-6 fatty acid ratios on spawning performance and larval quality in tongue sole *Cynoglossus semilaevis*. *Aquaculture Nutrition*, *20*(1), 79–89.

Ling, S., Hashim, R., Kolkovski, S., & Chong Shu-Chien, A. (2006). Effect of varying dietary lipid and protein levels on growth and reproductive performance of female swordtails *Xiphophorus helleri* (Poeciliidae). *Aquaculture Research*, *37*(13), 1267–1275.

Lubzens, E., Young, G., Bobe, J., & Cerdà, J. (2010). Oogenesis in teleosts: how fish eggs are formed. *General and Comparative Endocrinology*, *165*(3), 367–389.

Lubzens, E., Bobe, J., Young, G., & Sullivan, C. V. (2017). Maternal investment in fish oocytes and eggs: the molecular cargo and its contributions to fertility and early development. Aquaculture, 472, 107-143.

Lund, I., Steenfeldt, S. J., Suhr, K. I., & Hansen, B. W. (2008). A comparison of fatty acid composition and quality aspects of eggs and larvae from cultured and wild broodstock of common sole (*Solea solea* L.). *Aquaculture Nutrition*, *14*(6), 544–555.

Luo, L., Ai, L., Li, T., Xue, M., Wang, J., Li, W., & Liang, X. (2015). The impact of dietary DHA/EPA ratio on spawning performance, egg and offspring quality in Siberian sturgeon (*Acipenser baeri*). *Aquaculture*, *437*, 140–145.

Luquet, P., & Watanabe, T. (1986). Interaction "nutrition-reproduction" in fish. Fish

Physiology and Biochemistry, 2(1-4), 121-129.

Maina, J. G., Beames, R. M., Higgs, D., Mbugua, P. N., Iwama, G., & Kisia, S. M. (2003). Partial replacement of fishmeal with sunflower cake and corn oil in diets for tilapia Oreochromis niloticus (Linn): effect on whole body fatty acids. Aquaculture Research, 34(8), 601-608.

Manissery JK, Krishnamurthy D, Gangadhara B, Nandeesha MC (2001) Effect of varied levels of dietary protein on the breeding performance of common carp Cyprinus carpio. Asian Fish Sci 14:317–322

Matsuyama, M., Adachi, S., Nagahama, Y., Kitajima, C., & Matsuura, S. (1991). Annual reproductive cycle of the captive female Japanese sardine *Sardinops melanostictus*: Relationship to ovarian development and serum levels of gonadal steroid hormones. *Marine Biology*, *108*(1), 21–29.

Metwally, M. A. A., & Fouad, I. M. (2008). Some biochemical changes associated with injection of Grass Carp (*Ctenopharyngodon idellus*) with Oviaprim and Pregnyl for induction of artificial spawning. *Global Veterinaria*, *2*(6), 320–326.

Misieng, J. D., Salleh Kamarudin, M., Musa, M., Kamarudin, M. S., & Musa, M. (2011). Optimum Dietary Protein Requirement of Malaysian Mahseer (*Tor tambroides*) Fingerling. *Pakistan Journal of Biological Sciences*. Pakistan Journal of Biological Sciences.

Mohsin, A.K., and Ambak, M. A. 1983. Fresh water fishes of Peninsular Malaysia. Penerbit Universiti Pertanian Malaysia.

Mohsin, A.K., dan A.M. Ambak, 1992. Ikan Air Tawar di Semenanjung Malaysia. Cetakan Kedua. Dewan Bahasa dan Pustaka. Kementerian Pendidikan Malaysia, Kuala Lumpur. pp. 98-100.

Mousavi, S. E., & Yousefian, M. (2012). Effects of exogenous hormones on plasma cortisol , sex steroid hormone and glucose levels in male and female grass carp ,

Ctenopharyngodon idellus , during the spawning induction. *African Journal of Biotechnology*, *11*(36), 8920–8927.

Muchlisin, Z. A. (2005). Factors affect gonadal development and egg quality of female broodfish: a review. Berkala Ilmiah Biologi, 4(2005).

Muchlisin, Z. A., Hashim, R., & Chong Shu Chien, A. (2006). Influence of dietary protein levels on growth and egg quality in broodstock female bagrid catfish (Mystus nemurus Cuv. & Val.). Aquaculture Research, 37(4), 416-418.

Mylonas, C. C., Duncan, N. J., & Asturiano, J. F. (2017). Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. *Aquaculture*, *472*, 21–44.

Mylonas, C. C., Fostier, A., & Zanuy, S. (2010). Broodstock management and hormonal manipulations of fish reproduction. *General and Comparative Endocrinology*, *165*(3), 516–534.

Mylonas, C. C., Scott, a P., & Zohar, Y. (1997). Plasma gonadotropin II, sex steroids, and thyroid hormones in wild striped bass (*Morone saxatilis*) during spermiation and final oocyte maturation. *General and Comparative Endocrinology*, *108*(2), 223–236.

Mylonas, C. C., Woods III, L. C., Thomas, P., & Zohar, Y. (1998). Endocrine Profiles of Female Striped Bass (*Morone saxatilis*) in Captivity, during Postvitellogenesis and Induction of Final Oocyte Maturation via Controlled-Release GnRHa-Delivery Systems. *General and Comparative Endocrinology*, *110*(3), 276–289.

Mylonas, C. & Robles R., 2014.Enhancing the European aquaculture production by removing production bottlenecks of emerging species, producing new products and accessing new market. Aquaculture Europe, Vol. 39, pp. 1-15

Mylonas, C. C., & Zohar, Y. (2001). Use of GnRHa-delivery systems for the control

of reproduction in fish. *Reviews in Fish Biology and Fisheries*, *10*, 463–491.

Nagahama, Y., & Yamashita, M. (2008). Regulation of Oocyte Growth and Maturation in Fish. *Development, Growth & Differentiation, 50*, 195–219.

Nandi, S., Chattopadhyay, D. N., Verma, J. P., Sarkar, S. K., & Mukhopadhyay, P. K. (2001). Effect of dietary supplementation of fatty acids and vitamins on the breeding performance of the carp *Catla catla*. *Reproduction, Nutrition, Development*, *41*, 365–375.

Nandi, S., Routray, P., Gupta, S. D., Rath, S. C., Dasgupta, S., Meher, P. K., & Mukhopadhyay, P. K. (2007). Reproductive performance of carp, *Catla catla* (Ham.), reared on a formulated diet with PUFA supplementation. *Journal of Applied Ichthyology*, *23*(6), 684–691.

Nanton, D. A., Vegusdal, A., Rørå, A. M. B., Ruyter, B., Baeverfjord, G., & Torstensen, B. E. (2007). Muscle lipid storage pattern, composition, and adipocyte distribution in different parts of Atlantic salmon (*Salmo salar*) fed fish oil and vegetable oil. *Aquaculture*, *265*(1), 230–243.

Nautiyal, P. (1984). Natural History of the Garhwal himalaysan Mahseer *Tor Putitora* (Hamilton) II. Breeding biology. *Proceedings: Animal Sciences, 93(2), 97-106.*

Navas, J. M., Thrush, M., Zanuy, S., Ramos, J., Bromage, N., & Carrillo, M. (2001). Total lipid in the broodstock diet did not affect fatty acid composition and quality of eggs from sea bass (*Dicentrarchus labrax* L .) *Scientia Marina*, *65*(1), 11-19.

Navas, J. M., Bruce, M., Thrush, M., Farndale, B. M., Bromage, N., Zanuy, S., ... & Ramos, J. (1997). The impact of seasonal alteration in the lipid composition of broodstock diets on egg quality in the European sea bass. Journal of Fish Biology, 51(4), 760-773.

Navas, J. M., Mananos, E., Thrush, M., Ramos, J., Zanuy, S., Carrillo, M.,Zohar, Y. & Bromage, N. (1998). Effect of dietary lipid composition on vitellogenin, 17β-estradiol and gonadotropin plasma levels and spawning performance in captive sea bass (Dicentrarchus labrax L.). Aquaculture, 165(1-2), 65-79.

Ng, C. K. 2004. Kings of the Rivers: Mahseer in Malaysia and the Region. Inter Sea Fishery

Ng, W.K., Sungan, S. and De Silva, S.S. (2007). Changes in the morphometry, protein, lipid, and fatty acid composition of developing eggs and larvae from the induced spawns of the Malaysian Mahseer, *Tor tambroides* (Bleeker). In: Siti Shapor, A. Christianus, C. K. Ng, & S. . De Silva (Eds.), *Mahseer: The Biology, Culture and Conservation* (Vol 14, pp. 67-78). Kuala Lumpur: Malaysian Fisheries Society Occasional Publication.

Ng, W. K., Abdullah, N., & De Silva, S. S. (2008). The dietary protein requirement of the Malaysian mahseer, *Tor tambroides* (Bleeker), and the lack of protein-sparing action by dietary lipid. *Aquaculture*, *284*(1–4), 201–206.

Ng, W. K., & Andin, V. C. (2011). The Malaysian mahseer, *Tor tambroides* (Bleeker), requires low dietary lipid levels with a preference for lipid sources with high omega-6 and low omega-3 polyunsaturated fatty acids. *Aquaculture*, *322–323*, 82–90.

Ng, W. K., & Wang, Y. (2011). Inclusion of crude palm oil in the broodstock diets of female Nile tilapia, *Oreochromis niloticus*, resulted in enhanced reproductive performance compared to broodfish fed diets with added fish oil or linseed oil. *Aquaculture*, *314*(1–4), 122–131.

Norambuena, F., Estevez, A., Bell, G., Carazo, I., & Duncan, N. (2012a). Proximate and fatty acid compositions in muscle, liver and gonads of wild versus cultured broodstock of Senegalese sole (*Solea senegalensis*). *Aquaculture*, *356–357*, 176–185.

Norambuena, F., Estévez, A., Sánchez-Vázquez, F. J., Carazo, I., & Duncan, N. (2012b). Self-selection of diets with different contents of arachidonic acid by Senegalese sole (*Solea senegalensis*) broodstock. *Aquaculture*, *364–365*, 198–205.

Norambuena, F., Estévez, A., Mañanós, E., Bell, J. G., Carazo, I., & Duncan, N. (2013a). Effects of graded levels of arachidonic acid on the reproductive physiology of Senegalese sole (*Solea senegalensis*): Fatty acid composition, prostaglandins and steroid levels in the blood of broodstock bred in captivity. *General and Comparative Endocrinology*, *191*, 92–101.

Norambuena, F., Morais, S., Estevez, A., Bell, J. G., Tocher, D. R., Navarro, J. C., Duncan, N. (2013b). Dietary modulation of arachidonic acid metabolism in Senegalese sole (*Solea Sengalensis*) broodstock reared in captivity. *Aquaculture*, 80–88.

Norberg, B., Kleppe, L., Andersson, E., Thorsen, A., Rosenlund, G., & Hamre, K. (2017). Effects of dietary arachidonic acid on the reproductive physiology of female Atlantic cod (*Gadus morhua* L.). *General and Comparative Endocrinology*, *250*, 21–35.

Nunes, C., Silva, A., Soares, E., & Ganias, K. (2011). The use of hepatic and somatic indices and histological information to characterize the reproductive dynamics of Atlantic sardine *Sardina pilchardus* from the Portuguese coast. *Marine and Coastal Fisheries*, *3*(1), 127–144.

Oguntuase, B. G., & Adebayo, O. T. (2014). Sperm Quality and reproductive performance of male *Clarias Gariepinus* induced with synthetic hormones (Ovatide and Ovaprim). *International Journal of Fisheries and Aquaculture*, *6*(1), 9–15.

Ogale, S. N. 2002. Mahseer breeding and conservation and possibilities of commercial culture.The Indian experience.*FAO Fisheries Technical Paper*, 193-212

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Iinuma, N., & Hirose, K. (1997). Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. *Fish Physiology & Biochemistry*, *17*(1–6), 163–169.

Pathani SS. 1978. A note on secondary sexual characters in Kumaun mah- seers Tor tor and Tor putitora. Ind J Anim Sci. 48:773–775.

Patterson, J. T., & Green, C. C. (2015). Physiological and reproductive response to varying quantitative lipid inclusion in diets for Gulf killifish *Fundulus grandis* Baird and Girard. *Aquaculture Research*, *46*(9), 2236–2247.

Podhorec, P., Socha, M., Ammar, I. Ben, Sokolowska-Mikolajczyk, M., Brzuska, E., Milla, S., Kouril, J. (2016). The effects of GnRHa with and without dopamine antagonist on reproductive hormone levels and ovum viability in tench *Tinca tinca*. *Aquaculture*, *465*, 158–163.

Qiu, H., Jin, M., Li, Y., Lu, Y., Hou, Y., & Zhou, Q. (2017). Dietary lipid sources influence fatty acid composition in tissue of large yellow croaker (*Larmichthys crocea*) by regulating triacylglycerol synthesis and catabolism at the transcriptional level. *PLoS ONE*, *12*(1), 1–19.

Quinitio E.T., Gabasa P.G., Sunaf F.P., Reyes E.P. & Delapena D.T. (1984) Prawn Hatchery Design and Operation.Exten- sion Manual No. 9, SEAFDEC Aquaculture Department, Tigbauan, Iloilo, Philippines.

Ramezani-fard, E., Kamarudin, M. S., Saad, C. R., & Harmin, S. A. (2011). Changes over time in muscle fatty acid composition of Malaysian mahseer, *Tor tambroides*, fed different dietary lipid percentage. *African Journal of Biotechnology*, *10*(79), 18256–18265.

Ramezani-Fard, E., Kamarudin, M. S., Saad, C. R., Harmin, S. A., & Meng, G. Y. (2012a). Dietary Lipid Levels Affect Growth and Fatty Acid Profiles of Malaysian Mahseer *Tor tambroides*. *North American Journal of Aquaculture*,

74(4), 530–536.

Ramezani Fard, E., Kamarudin, M. S., & Harmin, S. A. (2012b). Endocrine Control of Oogenesis in Teleosts. *Asian Journal of Animal and Veterinary Advances*, 1–11.

Ramezani Fard, E., Salleh Kamarudin, M., Ehteshami, F., Shakiba Zadeh, S., Roos Saad, C., & Zokaeifar, H. (2014). Effect of dietary linolenic acid (18:3n–3)/linoleic acid (18:2n–6) ratio on growth performance, tissue fatty acid profile and histological alterations in the liver of juvenile *Tor tambroides*. *Iranian Journal of Fisheries Sciences*, *13*(1), 185–200.

Rangan, V. S., & Smith, S. (2002). Fatty acid synthesis in eukaryotes. In *New Comprehensive Biochemistry* (Vol. 36, pp. 151-179). Elsevier.

Razak, N. A. A., Madzin, H., Khalid, F., & Mustaffa, M. R. (2017). Gonad Ultrasonography Image Preprocessing for Mahseer (*Tor Tombroides*). *Journal of FisheriesSciences. Com*, *11*(3), 26–35.

Rodríguez-Barreto, D., Jerez, S., Cejas, J. R., Martin, M. V., Acosta, N. G., Bolaños, A., & Lorenzo, A. (2012). Comparative study of lipid and fatty acid composition in different tissues of wild and cultured female broodstock of greater amberjack (*Seriola dumerili*). *Aquaculture*, *360–361*, 1–9.

Rodríguez, C., Acosta, C., Badía, P., Cejas, J. R., Santamaría, F. J., & Lorenzo, A. (2004). Assessment of lipid and essential fatty acids requirements of black seabream (*Spondyliosoma cantharus*) by comparison of lipid composition in muscle and liver of wild and captive adult fish. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, *139*(4), 619–629.

Rueda-Jasso, R. A., Campos-Mendoza, A., Arreguín-Sánchez, F., Díaz-Pardo, E., & Martínez-Palacios, C. A. (2013). The biological and reproductive parameters of the invasive armored catfish *Pterygoplichthys disjunctivus* from Adolfo López Mateos El Infiernillo Reservoir, Michoacán-Guerrero, Mexico. *Revista*

Mexicana de Biodiversidad, *84*(1), 318–326.

Sangma, K. O. N., & Basavaraja, N. (2010). Induced Breeding , Embryology and Rearing of Fry of Deccan Mahseer , *Tor Khudree* (Sykes). *Journal of Aquaculture in the Tropics*, 25(1-4), 13.

Santiago, C. B., & Reyes, O. S. (1993). Effects of dietary lipid source on reproductive performance and tissue lipid levels of Nile tilapia *Oreochromis niloticus (Linnaeus)* broodstock. Journal of Applied Ichthyology, 9(1), 33-40.

Sargent, J., Bell, G., McEvoy, L., Tocher, D., & Estevez, A. (1999). Recent developments in the essential fatty acid nutrition of fish. *Aquaculture*, *177*(1–4), 191–199.

Sargent, J. R., Bell, J. G., Bell, M. V, Henderson, R. J., & Tocher, D. R. (1993). The metabolism of phospholipids and polyunsaturated fatty acids in fish. *Aquaculture: Fundamental and Applied Research*, 103–124.

Sargent, J. R., Bell, J. G., Bell, M. V, Henderson, R. J., & Tocher, D. R. (1995). Requirement criteria for essential fatty acids. *Journal of Applied Ichthyology*, *11*(3- 4), 183–198.

Sargent, J. R., McEvoy, L. A., & Bell, J. G. (1997). Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture*, *155*(1–4), 117–127.

Sargent, J. R., Tocher, D. R., & Bell, J. G. (2002). The lipids. *Fish Nutrition*, *3*, 181–257.

Schultz, S., Koussoroplis, A. M., Changizi-Magrhoor, Z., Watzke, J., & Kainz, M. J. (2015). Fish oil-based finishing diets strongly increase long-chain polyunsaturated fatty acid concentrations in farm-raised common carp (*Cyprinus carpio* L.). *Aquaculture Research*, *46*(9), 2174–2184.

Shahi, N., Mallik, S. K., Pande, J., Das, P., & Singh, A. K. (2015). Spermatogenesis and related plasma androgen and progestin level in wild male golden mahseer, *Tor putitora* (Hamilton, 1822), during the spawning season. *Fish Physiology and Biochemistry*, *41*(4), 909–920.

Shepherd, C. J., & Bromage, N. R. (1988). Intensive fish farming. Blackwell Scientific Publications Ltd.

Shrestha, T.K. 1997. The Mahseer in the rivers of Nepal disrupted by Dams and ranching strategis. Departement Zoology. Tribhuvan University. Kathmandu, Nepal. 259p.

Siddiqui, A.Q., Howlader, M.S. and Adam, A.A. 1988. Effects of dietary protein levels on growth, feed conversion and protein utilization in fry and young Nile tilapia, Oreochromis niloticus. Aquaculture 70, 63–73.

Siraj, S. S., Esa, Y. B., Keong, B. P., & Daud, S. K. (2007). Genetic characterization of the two colour-type of Kelah. Malaysian Applied Biology, 36(1), 23.

Smith, B. B., & Walker, K. F. (2004). Spawning dynamics of common carp in the River Murray, South Australia, shown by macroscopic and histological staging of gonads. *Journal of Fish Biology*, *64*(2), 336–354.

Sopha, L. 1999.Distribution and ecology of the Malaysian Mahseer (Genus: Tor) in Kenyir Lake, Malaysia (Doctoral dissertation, Fakulti Sains dan Teknologi

Steven, C. R. (1999). Studies on the GnRH/GtH System of Female Striped Bass (*Morone Saxatilis*): Effects of GnRH Agonist Therapy and Comparison of Reproductive Endocrine Parameters Between Wild and Captive Fish. (Doctoral dissertation, University of Maryland, College Park).

Tan, E.S.P. 1980. Some aspects of the biology of malaysian riverine cyprinids. Aquaculture, 20 : 281-289.

Tamaru, C. S., Kelley, C. D., Lee, C. S., Aida, K., Hanyu, I., & Goetz, F. (1991). Steroid profiles during maturation and induced spawning of the striped mullet, *Mugil cephalus* L. *Aquaculture*, *95*(1–2), 149–168.

Tessaro, L., de Toledo, C. P. R., Neumann, G., Krause, R. A., Meurer, F., Natali, M. R. M., & Bombardelli, R. A. (2012). Animal performance and reproductive aspects of female *Rhamdia quelen* fed on different levels of digestible energy. *Aquaculture Research*, 1–9.

Tocher, D. R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science*, *11*(2), 107–184.

Tocher, D. R. (2015). Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture*, *449*, 94–107.

Tocher, D. R., Dick, J. R., MacGlaughlin, P., & Bell, J. G. (2006). Effect of diets enriched in ?6 desaturated fatty acids (18:3n-6 and 18:4n-3), on growth, fatty acid composition and highly unsaturated fatty acid synthesis in two populations of Arctic charr (*Salvelinus alpinus* L.). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *44*(2), 245–253.

Trushenski, J., Schwarz, M., Bergman, A., Rombenso, A., & Delbos, B. (2012). DHA is essential, EPA appears largely expendable, in meeting the n-3 long-chain polyunsaturated fatty acid requirements of juvenile cobia *Rachycentron canadum*. *Aquaculture*, *326–329*, 81–89.

Vagner, M., & Santigosa, E. (2011). Characterization and modulation of gene expression and enzymatic activity of delta-6 desaturase in teleosts: A review. *Aquaculture*, *315*(1–2), 131–143.

Vassallo-Agius, R., Watanabe, T., Yoshizaki, G., Satoh, S., & Takeuchi, Y. (2001). Quality of eggs and spermatozoa of rainbow trout fed an n-3 essential fatty acid-deficient diet and its effects on the lipid and fatty acid components of eggs,

semen and livers. *Fisheries Science*, *67*(5), 818–827.

Wang, J. T., Liu, Y. J., Tian, L. X., Mai, K. Sen, Du, Z. Y., Wang, Y., & Yang, H. J. (2005). Effect of dietary lipid level on growth performance, lipid deposition, hepatic lipogenesis in juvenile cobia (*Rachycentron canadum*). *Aquaculture*, *249*(1–4), 439–447.

Wassef, E. A., Wahbi, O. M., & Shalaby, S. H. (2012). Effects of dietary vegetable oils on liver and gonad fatty acid metabolism and gonad maturation in gilthead seabream (*Sparus aurata*) males and females. *Aquaculture International*, *20*(2), 255–281.

Watanabe, T., Koizumi, T., Suzuki, H., Satoh, S.,Takeuchi, T., Yoshida, N., Kitada, T., & Tsukashima, Y. (1985). Improvement of quality of red sea bream eggs by feeding broodstock on a diet containing cuttlefish meal or on raw krill shortly before spawning. Buletin of the Japanese Society of Scientific Fisheries, 51(9), 1511-1521.

Watanabe, T., Lee, M. J., Mizutani, J., Yamada, T., Satoh, S., Takeuchi, T., Yoshida, N., Kitada, T. & Arakawa, T. (1991). Effective components in cuttlefish meal and raw krill for improvement of quality of red sea bream Pagrus major eggs. Nippon Suisan Gakkaishi, 57(4), 681-694.

Weltzien, F.-A., Taranger, G. L., Karlsen, Ø., & Norberg, B. (2002). Spermatogenesis and related plasma androgen levels in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, *132*(3), 567–575.

West, G. (1990). Methods of assessing ovarian development in fishes: a review. Marine and Freshwater Research, 41(2), 199-222.

Wibowo, A., & Kaban, S. (2014). Reproductive characteristics of Indonesia mahseer (*Tor tambroides* , Bleeker , 1854) , in two different rivers in Western Sumatera. *Indonesian Fisheries Research Journal*, *20*(2), 49–57.

Xu, H., Cao, L., Zhang, Y., Johnson, R. B., Wei, Y., Zheng, K., & Liang, M. (2017). Dietary arachidonic acid differentially regulates the gonadal steroidogenesis in the marine teleost, tongue sole (*Cynoglossus semilaevis*), depending on fish gender and maturation stage. *Aquaculture*, *468*, 378–385.

Xu, Y., Li, W., & Ding, Z. (2017). Polyunsaturated fatty acid supplements could considerably promote the breeding performance of carp. *European Journal of Lipid Science and Technology*, *119*(5).

Yaron, Z. (1995). Endocrine control of gametogenesis and spawning induction in the carp. *Aquaculture*, *129*(1–4), 49–73.

Yaron, Z., Bogomolnaya, A., Drori, S., Biton, I., Aizen, J., Kulikovsky, Z., & Levavi-Sivan, B. (2009). Spawning Induction in the Carp: Past Experience and Future Prospects - A Review. *The Israeli Journal of Aquaculture-Bamidgeh*, *61(1)*, 5–26.

Yaron, Z., & Levavi-Sivan, B. (2011). Hormonal control of reproduction and growth | Endocrine Regulation of Fish Reproduction. *Encyclopedia of Fish Physiology* (Vol. 1). Elsevier Inc.

Zakeri, M., Kochanian, P., Marammazi, J. G., Yavari, V., Savari, A., & Haghi, M. (2011). Effects of dietary n-3 HUFA concentrations on spawning performance and fatty acids composition of broodstock, eggs and larvae in yellowfin sea bream, *Acanthopagrus latus*. *Aquaculture*, *310*(3–4), 388–394.

Zakeri, M., Savari, A., Kochanian, P., & Haghi, M. (2010). Effects of Artificial Diets on Biological Performances of *Acanthopagrus latus* Broodstock in the Persian Gulf. *Journal of the Persian Gulf*, *1*(2).

Zheng, X., Torstensen, B. E., Tocher, D. R., Dick, J. R., Henderson, R. J., & Bell, J. G. (2005). Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and expression of fatty acyl desaturase and elongase genes in

liver of Atlantic salmon *(Salmo salar). Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* (Vol. 1734).

Zohar, Y., & Mylonas, C. C. (2001). Endocrine manipulation of spawning induction in cultured fish from hormone to gene. *Aquaculture International, 197*, 99–139.

Zohar, Y., Harel, M., Hassin, S., Tandler, A., 1995. Gilthead Sea bream (*Sparus aurata*). In: Bromage, N.R., Roberts, R.J. (Eds.), Broodstock Management and Egg and Larval Quality. Blackwell Science Ltd, Oxford, pp. 94–117.

Appendix 1

Fatty acid concentrations (% of total fatty acids± SEM) of the gonad of *Tor tambroides* males fed with different percentages of lipid inclusion diet for 5 months.

Fatty acid	Diet 1 (0%)	Diet 2 (2.5%)	Diet 3 (5.0%)	Diet 4 (7.5%)
C16:0	11.19±0.75^{a}	13.19±0.77^{b}	13.65±0.12^{b}	13.24±0.11^{b}
C18:0	19.39±1.40^{a}	18.21±2.39^{a}	17.74±0.93^{a}	16.45±0.40^{a}
C16:1	1.64±0.48^{a}	1.94±0.61^{a}	1.62±0.45^{a}	1.09±0.20^{a}
C18:1n9	14.55±2.52^{a}	16.11±3.47^{a}	13.65±2.22^{a}	13.02±0.08^{a}
C18:2n6	7.70±1.95^{a}	7.32±1.98^{a}	5.39±1.27^{a}	5.00±0.60^{a}
C18:3n3	0.13±0.07^{a}	0.28±0.15^{a}	0.30±0.17^{a}	0.09±0.05^{a}
C20:4n6	5.21±0.10^{a}	3.51±0.70^{b}	5.37±0.48^{a}	5.40±0.52^{a}
C20:5n3	0.46±0.27^{a}	0.47±0.24^{a}	0.59±0.28^{a}	0.76±0.28^{a}
C22:6n3	16.07±6.64^{a}	22.71±4.96^{a}	26.55±5.29^{a}	30.67±3.26^{a}
$\sum$ SFA	41.99±0.56^{a}	40.24±1.46^{ab}	39.54±1.07^{ab}	37.80±0.15^{b}
$\sum$ MUFA	26.23±4.09^{a}	24.29±2.45^{a}	21.40±3.12^{a}	19.14±1.48^{a}
$\sum$ PUFA	9.24±2.70^{a}	7.82±1.96^{a}	6.00±1.26^{a}	5.57±0.78^{a}
$\sum$ HUFA	22.55±6.24^{a}	27.65±5.80^{a}	33.06±5.46^{a}	37.50±2.41^{a}
$\sum$ n3	17.26±5.95^{a}	23.47±4.79^{a}	27.44±4.83^{a}	31.71±2.97^{a}
$\sum$ n6	14.52±2.42^{a}	11.92±1.16^{a}	11.61±0.64^{a}	11.35±1.34^{a}
n3:n6	1.41±0.67^{a}	2.09±0.65^{a}	2.42±0.55^{a}	2.93±0.62^{a}

Values are (mean + SE).
Different superscripts letter within a row indicates significant differences ($p < 0.05$)

Appendix 2

Water temperature records for the second and third feeding trial from February 2015 until June 2015.

Month	Temperature Morning (°C)	Temperature Evening (°C)
Feb-15	28.38 ± 0.47	28.98 ± 0.45
Mac-15	28.73 ± 0.19	29.40 ± 0.18
Apr-15	30.00 ± 0.60	30.80 ± 0.60
May-15	30.20 ± 0.66	30.95 ± 0.59
Jun-15	28.85 ± 0.45	29.51 ± 0.39

PRESENTED PAPERS

Abduh, M.Y., Noordiyana, M.N. and Abol-Munafi A.B. 2012. Histological observation on the gonad of male mahseer, *Tor tambroides* in captivity. **International Fisheries Symposium,6-8 December, Can Tho, Vietnam. (Poster).**

Abduh, M.Y., Noordiyana, M.N. and Abol-Munafi A.B. 2015. Impact of dietary lipid sources and ratio on gonad development and maturation in captive Malaysian mahseer (*Tor tambroides*).
International Fisheries Symposium, 1-4 December, Penang, Malaysia. (Poster).

Abduh, M.Y., Noordiyana, M.N. and Abol-Munafi A.B. 2016. Effects of different lipid levels on gonad development and maturation of female Malaysian mahseer (*Tor tambroides*) in captivity.
International Fisheries Symposium, IFS 2016, Phu Quoc Island, Vietnam, October 31-November 2. (Poster).

Abduh, M.Y., Noordiyana, M.N. and Abol-Munafi A.B. 2016. Effects of dietary lipid ratio and sources on gonad development and maturation of Malaysian mahseer (*Tor tambroides*) in captive condition.
Universiti Malaysia Terengganu International Annual Symposium on Sustainability Science and Management. 13-15 December, Terengganu, Malaysia (Oral).

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