THE EFFECT OF ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID ON GROWTH, IMMUNE RESPONSE AND TISSUE FATTY ACID COMPOSITION OF MALABAR RED SNAPPER (*Lutjanus malabaricus*)

by

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KESAN ASIK ARAKIDONIK DAN ASIK DOCOSAHEXAENOIC TERHADAP PERTUMBUHAN, TINDAK BALAS IMUN DAN KOMPOSISI ASIK LEMAK TISU PADA IKAN MERAH (*Lutjanus malabaricus*)

oleh

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Tesis yang diserahkan untuk
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LIST OF ABBREVIATIONS

ALA	Linolenic acid
ANOVA	One way analysis of variances
AOS	Antioxidative defense system
ARA	Arachidonic acid
ASC	Antibody-secreting cells
ATP	Adenosine triphosphate
CAT	Catalase
DGLA	Dihomo-gama-linolenic acid
DHA	Docosahexaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid
EPA	Eicosapetaenoic acid
FAME	Fatty acid methyl esters
FID	Flame ionization detector
GALT	Gut-associated lymphoid tissue
GIALT	Gill-associated lymphoid tissue
GR	Glutathione reductase
GSH-Px	Glutathione peroxidase
GST	Glutathione-S-transferase
HDL	High density lipoproteins
HSI	Hepatosomatic index
IPF	Intraperitoneal fat
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acid
LDL	Low density lipoproteins
MALT	Mucosa-associated lymphoid tissue
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
OA	Oleic acid
OD	Optical density
PGE_2	Prostaglandin E_2
PMA	Phorbol 12-myristate 13-acetate
PMS	Phosphate buffer saline
PPAR	Peroxisome proliferator-activated receptor
PVC	Polyvinyl chloride
ROS	Reactive oxygen species
SALT	Skin-associated lymphoid tissue
SOD	Superoxide dismutase
SREBP-1c	Sterol regulatory element binding protein-1c
VLDL	Very low density lipoproteins
VSI	Viscerosomatic index

LIST OF SYMBOLS

µl	microliter
µm	micrometer
BF_3	boron trifluoride
g	gram
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
kcal	kilocalorie
kg	kilogram
kPa	kilopascal
l	liter
Ln	natural logarithm
m	meter
M	moles
min	minutes
ml	milliliter
mM	millimole
mm	millimeter
nm	nanometer
O_2	oxygen
O_2^-	superoxide anion
ºC	Celcius
ppt	parts per thousand
S.E.	standard error
U	unit
v/v	volume/volume
kJ	kilojoule

KESAN ASIK ARAKIDONIK DAN ASIK DOCOSAHEXAENOIC TERHADAP PERTUMBUHAN, TINDAK BALAS IMUN DAN KOMPOSISI ASIK LEMAK TISU PADA IKAN MERAH (*Lutjanus malabaricus)*

ABSTRAK

Satu kajian mengenai kesan asik arakidonik (ARA) dan asik docosahexaenoic (DHA) terhadap pertumbuhan, tindak balas imun dan komposisi asik lemak tisu pada ikan merah, *Lutjanus malabaricu*s telah dijalankan di makmal nutrisi ikan, USM. Dalam eksperimen pertama, empat jenis diet dibekalkan mempunyai paras protein, lemak dan kalori yang sama manakala kandungan minyak ARA ditentukan dalam jumlah diet sebanyak 0.05, 0.26, 0.47 dan 0.65% masing-masing. Setiap jenis diet dibahagikan kepada 3 replikat dan ikan (5.92 ± 0.07 g) diberi makanan sehingga cukup dua kali setiap hari selama 42 hari. Secara ringkas, hasil kajian ini menunjukkan bahawa tiada signifikasi perubahan dalam tumbesaran ikan. Dari segi tindak balas imun, aktiviti superoxide dismutase (SOD) meningkat secara ketara (P<0.10). Selain itu, aktiviti respiratory burst (NBT), lysozyme dan catalase (CAT) ikan juga menunjukkan trend peningkatan dengan penambahan ARA dalam diet. Dalam eksperimen kedua sepanjang 53 hari, DHA ditambahkan dalam diet untuk mengkaji impak DHA dan ARA terhadap tumbesaran ikan. Kandungan ARA/DHA (% jumlah diet) ditentukan sebanyak 0.03/0.7, 0.46/1.4, 0.92/1.4 dan 1.82/1.4. Setiap jenis diet dibahagikan kepada 3 replikat dan ikan (15.82 ± 0.86 g) dan ikan diberi makanan sebanyak 3 kali setiap hari. Tumbesaran ikan meningkat secara ketara (P<0.05) dengan penambahan DHA dan ARA dalam diet. Aktiviti lysozyme dan SOD meningkat secara ketara (P<0.10) dengan peningkatan ARA dan DHA dalam diet. Kedua-dua eksperimen menunjukkan bahawa komposisi asik lemak diet dipaparkan dalam komposisi asik lemak tisu ikan masing-masing.

Sementelahan, perbezaan antara kandungan lemak dalam ikan tidak ketara. Di samping itu, jumlah MUFA, PUFA, nisbah ARA/EPA dan ARA/DHA antara ikan menunjukan perbezaan yang ketara (P<0.05). Kandungan EPA dalam lemak otot dan insang berkurang secara ketara dengan penambahan ARA dalam diet ikan. Selain itu, kandungan DHA dalam lemak hati meningkat secara ketara dengan kandungan DHA yang sama dalam diet. Secara keseluruhanya, kajian ini mengesyorkan bahawa penambahan ARA (tidak melebihi 0.6% dalam diet) dan DHA sebanyak 1.4% dalam diet akan meningkatkan tumbesaran, tindak balas imun dan komposisi asik lemak jenahak merah.

THE EFFECT OF ARACHIDONIC ACID AND DOCOSAHEXAENOIC ACID ON GROWTH, IMMUNE RESPONSE AND TISSUE FATTY ACID COMPOSITION OF MALABAR RED SNAPPER (*Lutjanus malabaricus)*

ABSTRACT

This study was conducted to investigate the effect of arachidonic acid (ARA) and docosahexaenoic acid (DHA) on growth, immune response and tissue fatty acid composition of Malabar red snapper fingerlings, *Lutjanus malabaricus*. In the first experiment, four isonitrogenous, isolipidic and isocaloric experimental diets were formulated and ARA was added to contain 0.05, 0.26, 0.47 or 0.65% of total diet, respectively. Forty two days feeding trial was conducted and triplicate groups of fingerlings (5.92 ± 0.07 g) were fed to apparent satiation twice daily. No significant (P>0.05) differences were found in the growth and survival of fish. For immune response, superoxide dismutase (SOD) activity was significantly (P<0.10) increased with increasing dietary ARA levels. Similarly, an increasing trend was detected in respiratory burst activity, lysozyme and catalase (CAT) activity in fish fed with increasing dietary levels of ARA. In second experiment, DHA was supplemented to determine its impact on growth with increasing dietary ARA levels. Four isonitrogenous, isolipidic and isocaloric experimental diets were formulated and consist ARA/DHA (% of diet) at 0.03/0.7, 0.46/1.4, 0.92/1.4, 1.82/1.4. A 53-day feeding trial was carried out on triplicate groups of fingerlings (15.82 ± 0.86 g) and they were fed three times daily. Growth was significantly (P<0.05) increased with increasing dietary ARA and DHA levels. Survival was found independent of dietary ARA and DHA. Lysozyme and SOD activity were significantly (P<0.10) different with dietary ARA and DHA levels. Both experiments showed that the fatty acid composition of experimental diets were reflected in the fatty acid composition of

fish tissue lipids. Total MUFA, PUFA, ratio of ARA/EPA and ARA/DHA increased significantly ($P<0.05$) in fish fed higher dietary ARA levels. Content of EPA showed significant reduction in muscle and gill lipids of fish fed increasing dietary ARA level. DHA significantly increased in liver lipid of fish fed constant dietary DHA. This study recommended that dietary 1.4% DHA and 0.6% ARA is an optimal diet for growth and immune response of Malabar red snapper fingerlings.

CHAPTER 1

GENERAL INTRODUCTION

Aquaculture industry has a prominent role in providing protein source to the growing human population. Extensive studies have been conducted to ensure the sustainability of aquaculture. In this case, aquaculture nutrition is crucial to influence physiology of aquatic animals such as growth, digestion, reproduction, osmoregulation and behavior.

Nowadays, nutrition requirement to maintain growth and health of most commonly cultured aquatic species are well established (NRC, 2011). Recent studies showed significant improvement on growth performance and health of fish fed diet with higher dietary nutrient than basic requirement level.

Fatty acids especially long-chained polyunsaturated fatty acids (LC-PUFA) such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) are known as essential fatty acids (EFA). EFA were extensively studied on their impacts on metabolic pathway, composition of cell membranes, reproduction, immune response and regulation of ion balance (Sargent et al., 1999). Compared to freshwater fish, marine fish are known to have less ability to biosynthesize LC-PUFA with linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6) due to the deficiency of elongase and desaturase enzyme (Bell & Sargent, 2003). Therefore, supplying LC-PUFA directly to dietary cultured marine fish is more appropriate and beneficial than inclusion of LA and ALA as precursor in diet (Glencross, 2009).

EFA studies of different life stage of marine fish have been carried out. Effect of EFA on growth and survival were studied on fish larvae such as red drum (Brinkmeyer & Holt, 1998), gilthead seabream (Koven et al., 1989; Rodríguez et al., 1998; Villalta et al., 2005), striped trumpeter (Bransden et al., 2005*a*), common sole (Lund et al., 2007), California halibut (Vizcaíno-Ochoa et al., 2010) and Senegalese sole post larvae (Navarro-Guillén et al., 2014).

Effect of LC-PUFA on growth performance and tissue fatty acid composition were examined on juvenile fish such as starry flounder (Lee et al., 2003), European sea bass (Skalli & Robin, 2004), Atlantic salmon (Hatlen et al., 2012) and barramundi (Morton et al., 2014). For broodstock, impact of dietary LC-PUFA on reproduction and egg quality were investigated on Japanese flounder (Furuita et al., 2002 & 2003), Atlantic halibut (Mazorra et al., 2003), crescent sweetlips (Li et al. 2005), Japanese eel (Furuita et al., 2007), yellowfin sea bream (Zakeri et al., 2011), Senegalese sole (Norambuena et al., 2013; Morais et al., 2014) and Siberian sturgeon (Luo et al., 2015).

Fatty acids portray vital role in immunological and inflammatory process (Zurier, 1993). Eicosanoids are derivatives of LC-PUFA. Eicosanoids include prostaglandin (PGE), thromboxanes (TX) and leukotrienes (LT) are essential in the immune response and inflammatory process (Kiron, 2012). Dietary ARA was reported to show elevation of PGE_2 in striped trumpeter larvae (Bransden et al., 2004), Atlantic cod larvae (Bransden et al., 2005), juvenile Atlantic salmon (Berge et al., 2009) and Senegalese sole (Boglino et al., 2014). Besides, higher PGE_2 was observed in isolated head kidney leukocytes of Atlantic cod with increasing ARA supplemented *in vitro* (Furne et al., 2013). PGE_2 of isolated head kidney macrophage was elevated in large yellow croaker with addition of EPA and DHA (Li et al., 2013).

Besides, dietary fatty acids improved non-specific immune response of fish. For example, respiratory burst activity was improved in juvenile grouper fed dietary ALA and LA (Wu & Chen, 2012). Dietary ALA improved respiratory burst activity and anti-inflammatory response of juvenile Nile tilapia (Chen et al., 2016).

Quick expanding of aquaculture industry in the past few decades rapidly increased demand of fish oil as oil source for fish feed. Hence, alternative oil sources such as vegetable oils are greatly encouraged to reduce high consumption of fish oil without compromising fish growth. Abundant researches of fish oil replacement using vegetable oil have been carried out (Turchini et al., 2009; Turchini et al., 2011). For examples, vegetable oil such as soybean oil, canola oil, olive oil, sunflower oil, flaxseed oil, palm oil and rapeseed oil were used to partially substitute fish oil. However, some studies reported that entirely substitution of fish oil with vegetable oil compromise fish growth and health due to deficiency of LC-PUFA in vegetable oil (Regost et al., 2003; Peng et al., 2008). Subsequently, algal oil rich in LC-PUFA are supplemented into diet to balance the deficiency of LC-PUFA in vegetable oil. DHA-rich algal oil is suggested as an alternative to fish oil (Miller et al., 2007; Ganuza et al., 2008; Ryan & Symington, 2014). Significant increase was detected in growth of channel catfish fed diet with supplementation of DHA-rich algal meal (Li et al., 2009). Study of Sprague et al. (2015) reported that substitution of fish oil with DHA-rich algal meal significantly decreased persistent organic pollutant levels in flesh of Atlantic salmon. Supplementation of LC-PUFA rich algal oil is encouraged to reduce high consumption of fish oil in aquaculture industry. In this study, increasing dietary ARA and DHA levels are designated by supplementation of ARA-rich fungal oil and DHA-rich algal meal.

Malabar red snapper, *Lutjanus malabaricus*, a widespread Indo-Pacific species is an important commercial food fish in Southeast Asia. It commands a favorable market price similar to groupers and seabass (Wells et al., 2008). Malabar red snapper has been widely accepted by worldwide consumers as an excellent food fish and with a high market value has caused over exploitation of wild fisheries stock (Miller et al., 2005). Lately, studies such as toughness and texture of Malabar red snapper are being investigated in order to improve the food fish quality (Forrest et al., 2012). On the other hand, cultured fish health often threatened by various factors and maintaining fish health is important to ensure sustainable culture practice. Due to all the reasons above, this study is designed to determine the effect of ARA and DHA on growth, immune response and tissue fatty acid composition of Malabar red snapper fingerlings. Despite being a high-value marine fish of commercial importance, the nutritional requirements of this fish species is currently mostly not known. In this case, nutrition plays an important role to maintain fish health and reduce the chances of disease infection. Therefore, the objectives of this study are

a. To evaluate the effect of ARA and DHA on growth, survival and tissue fatty acid composition of Malabar red snapper fingerlings,

b. To determine optimal level of dietary ARA and DHA for growth and immune response of Malabar red snapper fingerlings,

CHAPTER 2

LITERATURE REVIEW

2.1 Global aquaculture industry

The global total production of fishery has grown remarkably over the past decades and reached 86.6 million tons of wild fish captures in 2012. At the same time, aquaculture production is also showing a remarkable increment. According to FAO (2014), the total aquaculture production was 36.8 million tons in 2002 and increased to 66.6 million tons in 2012 with an average increasing rate of 6.1% annually. Besides, China is the biggest producer country in the fishing industry, followed by Indonesia, USA, India and Peru. In addition, China also acts as the main producer in aquaculture with 41.1 million tons of production in 2012, followed by India, Vietnam, Indonesia, Bangladesh, Norway, Thailand, Chile, Egypt and Myanmar. Meanwhile, 66% of the world food fish aquaculture production was contributed by finfish culture in 2012, followed by mollusks and crustaceans. Besides, expanding human population worldwide resulted in the increase of fish consumption. For example, 10kg per capita fish in 1960s was increased to 19kg per capita fish for human consumption in 2012 (FAO, 2014). Hence, it is believed that improving fish health through their diet is important to ensure a promising protein source and provide good quality of food fish.

2.1.1 Tropical marine culture

Tropical marine food fish culture practices have been expanding rapidly over decades. Marine fish show higher growth rate and provide high quality of protein source compared to freshwater fish and highly preferred by fish farmers and consumers. Some of the popular cultured food fish with high commercial value such as groupers, barramundi, snappers and pompanos are widely cultured in tropical waters. Finfish culture production contributed 63.5% of total world aquaculture production in 2012 (FAO, 2014). Most of the grow-outs are cage-cultured in high stocking density which lead to stress and resulted in disease outbreak (Leong, 1997). Besides, rough handling during grading, poor water quality and sanitation could lead to rapid infection of parasite and disease in cultured fish. In order to prevent production loss due to disease infection, maintaining fish health is essential during culture practice. Apart from maintaining water quality, grading fish carefully and avoid overstocking fish, fish health can be improved through supplementation of diet with fatty acids (Zurier, 1993; Calder, 1999). Fish oil enriched diet contains EFA and their derivatives play important roles in immune response (*refer* Section 2.3.5).

Despite expanding of marine culture in tropical zone, there is lack of nutrient requirement information on tropical marine food fish. Trash fish have been utilized as feed for marine culture. However, the source of trash fish and nutrient information are unknown. Therefore, the use of trash fish potentially facilitates the introduction of parasites to cultured species. Besides, deficiency of certain fatty acids compromise survival and health of cultured species. In order to maintain sustainability in production, promising survival and growth, it is a consensus to determine nutrient requirement for tropical marine food fish.

2.2 Malabar red snapper

2.2.1 Scientific classification of Malabar red snapper

Snappers belongs to the family Lutjanidae (Salini et al., 2006). Snappers are arranged in the subfamily Lutjaninae with 73 species, the largest subfamily of family Lutjanidae. In the present study, Malabar red snapper is named as *Lutjanus malabaricus* in scientific term with other common names such as larged-mouthed Nannygai, Malabar blood snapper, saddle tail sea perch or scarlet sea perch.

Kingdom:	Animalia
Phylum:	Chordata
Class:	Actinoterygii
Order:	Perciformes
Family:	Lutjanidae
Genus:	Lutjanus
Species:	*Lutjanus malabaricus* (Bloch & Schneider, 1801)

2.2.2 Snapper morphology

Lutjanidae possesses 10-12 spines and 10-17 soft rays in dorsal fin while 3 spines and 7-11 soft rays in anal fin. The pelvic fins are originated just behind pectoral base. Besides, the fish jaws contain enlarged canine teeth and small palatine teeth. The maxilla is covered by preorbital with closed mouth. They have 7 branchiostegal rays, total 24 gill rakers at which 10 at upper and 14 at lower.

According to Kim et al. (2012), the entire head and body of Malabar red snapper are uniformly reddish in colour and their fins are bordered with black line. It has a compressed body, oblong in shape and covered with oblique rows of etenoid scales horizontally. Eye is oval in shape. Snout is round and projecting. Mouth is

large and oblique. Its caudal fin is truncate. Interestingly, Malabar red snapper is identified with a dark mark located on the upper side of the caudal peduncle.

In study of Kim et al. (2012), Malabar red snapper is identified with 11 spines and 13 soft rays in dorsal fin, 16 soft rays in pectoral fin, 1 spine and 5 soft rays in pelvic fin, 3 spines and 9 soft rays in anal fin and gill rakers with 4 at upper and 12 at lower.

Plate 2.1 Adult Malabar red snapper

2.2.3 Snapper biology

Snappers lives in marine and estuaries. They are found in tropical and subtropical ocean such as Atlantic, Pacific and Indian Oceans. Most of Lutjanidae are predators. They are carnivorous, feed on crustaceans, molluscs, invertebrate and smaller fishes.

Snapper egg size is ranged from 0.6 to 1mm in diameter, wrapped with oil droplet to allow buoyancy. The fertilized eggs are hatched into larvae after approximately 17 to 36 hours. The newly hatched larvae is usually 2mm in total length, with unpigmented eyes, no mouth and incapable to swim. In 3 to 4 days, the large yolk sac is consumed, eye pigmented and the mouth begins to function (Lythgoe et al., 1994). After 1 week, the larvae develop spines on the head, dorsal

and pelvic fins on the body. The larval stage takes 25-47 days to develop into juvenile. Juvenile snappers stay on nursery grounds for 2 to 4 years to achieve maturity. Adult snappers live offshore and stay in a group (Cocheret de la Morinière et al., 2003). Snappers reach their maturity at nearly 50% of the maximum total length. The size at maturity acts as an important parameter to predict and evaluate the spawning stock biomass for a conservative and sustainable exploitation in fishery.

In terms of reproductive biology, snappers are dioecious and gonochoristic. In other words, they have separate distinct sexes and the sex of an individual remains during its lifetime. They migrate from inshore to offshore during spawning season. The aggregation of fish during spawning season is remarked as target by the commercial fishery (Erisman et al., 2010; Granados-Dieseldorff et al., 2013). Most of the preferable aggregation sites are located on the edge of a steep drop off and the spawning usually occurs during twilight. On the other hand, snappers spawn several times in a year (Mikulas & Rooker, 2008). Fish size is highly correlated with fecundity. It is reported that a 12.5kg female red snapper yield same amount of eggs with nearly 200 individuals of 1.1kg female red snapper (Pauly et al., 2002).

2.2.4 Importance of snappers in fishery and marine culture

One of the notable Lutjanidae is snappers which served as important food fish with high market value. Lutjanidae constitutes a high commercial value in fisheries sector. A study was conducted on the length-weight relationship of several Lutjanidae such as *L. agennes*, *L. bohar* and *L. fulviflamma* at Kenyan coastal (Mbaru et al., 2010). Besides, previous studies on the growth and age determination of vulnerable Lutjanidae such as *L. carponotatus* and *L. vitta*, *L. erythropterus*, *L.*

sebae and Malabar red snapper using otolith to regulate a conservative harvest strategy (Newman et al., 2000*a*; Newman et al., 2000*b*; Newman, 2002; Newman & Dunk, 2002). In spite of length-weight relationship, to date, otolith weight and shape was used to determine the growth rate of fish populations and identify different fish stocks such as John's snapper, *L. johnii* (Sadighzadeh et al., 2014).

On the other hand, the high demand of snapper as food fish was satisfied through marine culture. Reliable reproduction technique and feeding method are essential to ensure the sustainability of marine fish culture. A few millions of Russell snapper, *L. russell* fry were artificially cultured annually in China (Hong & Zhang, 2003). Meanwhile, hormone-induced method was used to increase the spawning rate of wild Pacific red snapper broodstock , *L. peru* (Dumas et al., 2004). Besides, a study on practical diet for mangrove red snapper, *L. argentimaculatus* broodstock was conducted to maintain the egg quality and ensure the consistency of culture activity (Emata & Borlongan, 2003). Several studies have been carried out to improve the commercial production of snappers. Fish meal substitution by soybean meal was experimented on growth rate and digestibility of Australian snapper, *P. auratus* (Quartararo et al., 1998), growth rate and survival of red snapper *L. campechanus* (Davis et al., 2005), growth rate, feed efficiency, body composition and blood chemistry of juvenile spotted rose snapper, *L. guttatus* (Silva-Carrillo et al., 2012). At the same time, the effect of fish meal replacement by poultry by-product meal in diets on growth performance and digestibility was analyzed on juvenile spotted rose snapper (Hernández et al., 2014).

2.2.5 Snapper culture status in Malaysia

Snappers are high valued commercial food fish that widely cultured in Malaysia and other parts of Asia. Mangrove snapper, *L. argentimaculatus*, golden snapper, *L. johni*, Russelli's snapper, *L. russelli*, red snapper, *L. sanguineus* and *L. malabaricus* are known as the popular snapper culture species in Malaysia. Snapper culture are commonly carried out at marine floating cage culture and brackish water pond. The marine culture areas are located at coastal waters off Penang island, Bukit Tambun, Sungai Udang, Pangkor Island, Kuala Seputang and Gula at North; Pulau Ketam at Selangor in central region and Kukup, Johor in South of Peninsular Malaysia (Khoo & Merican, 2009). Marine culture often encounters problems with fish parasites due to open and accessible environment such as sea cage. For example, *Neobenedenia sp.* (skin fluke), *Haliotrema spp.* (gill fluke), *Cryptocaryon irritans* (white spot) and *Caligus sp.* (sea lice) are some of the parasites that found in marine cultured fish in Asia-Pacific region (Leong et al., 2006). These parasites can cause serious disease outbreak and eventually lead to fish mortality. However, it is impossible to eliminate free living parasites at open sea cage. Hence, it is important to ensure immunity of cultured fish besides implementing systematic farm management.

2.3 Immune response of fish

2.3.1 Specific immune response and innate immune response

Specific immune response, also known as adaptive immune response is defined as a body mechanism respond specifically to pathogen invasion or viral infection and followed by development of immunological memory. However, the

scarce memory limits the ability of specific immune response in fish. Therefore, nonspecific immune response, as known as innate immune response is playing a key role on fish health.

Innate immune response parameters are considered as indicators to evaluate fish health status (Uribe et al., 2011). Innate immune response functions as the primary defense of immune system and prevents microbial invasion promptly. In fish, innate immune response is built up by the physical, cellular and humoral components. Physical components serve as the first line of defense system against the pathogens. Organs involved in physical barriers are consisted of fish skin, gill and mucus (Uribe et al., 2011). Initially, mucus serves as primary defense against pathogens. Mucosa-associated lymphoid tissue (MALT) plays a key role in fish immune system. MALT is divided into gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT) and gill-associated lymphoid tissue (GIALT) (Lazado & Caipang, 2014). GALT is discovered to compose several immune molecules such as lymphocytes, plasma cells, granulocytes and macrophages in the fish epithelium (Rauta et al. 2012). Fish skin is directly exposed to external environment and therefore it is highly susceptible to various types of pathogens and stressors. SALT is enriched by T cells while the epidermal layer contains high concentration of immune cells. For example, epithelial cells, mucus cells, club cells, goblet cells are aggregated to form a protective skin layer. Gill acts as a multilayered filter that allows osmotic balance regulation, waste excretion and ion exchange. Hence, gill has similar susceptibility to external environment as fish skin. GIALT is consisted of lymphocytes, macrophages, eosinophilic granulocytes, neutrophils and antibody-secreting cells (ASC) (Lazado & Caipang, 2014).

Kidney is considered as a well innervated organ and acts as an important endocrine organ especially anterior kidney. It contains abundant of developing B lymphoid cells and small amount of antibody-secreting cells which is also major site for antibody production (Tort et al. 2003). Secondly, spleen, a major immune organ that plays a vital role in antigen trapping. Spleen size is used to examine parasite infections level in fish. Thymus plays an essential role in phagocytosis stimulation, allograft rejection and antibody production.

Phagocytosis is known as an important mechanism for bacteria elimination. For instances, there are respiratory burst activity occurs in neutrophils and macrophages. Also, bacteria damaged by the process of halogenation in which, myeloperoxidase secreted by neutrophils destructs bacteria cell wall by converting amino acids into aldehydes that allow antimicrobial activity (Nayak, 2010; Uribe et al., 2011). Complement system functions as the central immune responses by three pathways which are the classical pathway, complement system is activated by binding of antibody to cell surface; alternative pathway, in spite of antibody, the complement system is induced by foreign microorganism and lectin pathway, complement system is activated by binding of lectins contain sugar in bacteria cells (Rauta et al., 2012). Lectins, a complex protein with at least two binding sites for sugar, function as activators in the complement system and act as opsonin during phagocytosis process (Tort et al., 2003). Opsonin is a molecule that assists the process of phagocytosis by acting as a binding enhancer for antibodies (Rauta et al., 2012).

2.3.2 Respiratory burst activity

Respiratory burst plays an important role in the immune system and defined as a process of sharp increase of oxygen uptake for reactive oxygen species (ROS) production in phagocytes such as macrophages and neutrophils. Initially, reduction of oxygen (O_2) was catalysed by NADPH oxidase to produce O_2^-, whereas most of the O_2^- undergoes dismutation itself and generated O_2 and hydrogen peroxide (H_2O_2). Due to the harmless for microbial killing, O_2^- and H_2O_2 act as precursors of microbicidal oxidants in phagocytes. Myeloperoxidase-catalyzed reaction of Cl^- to H_2O_2 is aimed to produce hypochlorite (OCl^-), a substantial microbicidal agent and also the precursor of the chloramines which is a group of microbicidal oxidized halogens (Babior, 1984). On the other hand, metal-catalyzed reaction (Haber-Weiss reaction) of O_2^- and H_2O_2 produced an oxidizing radical as known as hydroxyl radical ($OH^{\cdot}$) (Babior, 1984). Oxidized halogens and oxidizing radical were both known to be exerting damage on bacteria promptly.

2.3.3 Lysozyme activity

Lysozyme as known as N-acetylmuramide glycan hydrolase, is one of the ubiquitous enzyme in the immunity mechanism of fish (Jollès & Jollès, 1984; Saurabh & Sahoo, 2008; Uribe et al., 2011). Generally, lysozyme exhibits lytic activity against Gram-positive and Gram-negative bacteria by hydrolyzing the peptidoglycan of bacteria cell walls. Besides, lysozyme is found to stimulate opsonin in order to catalyze phagocytosis activity by leukocytes (Uribe et al., 2011; Kiron, 2012). In fish, lysozyme often used to quantify the nonspecific immune activity (Saurabh & Sahoo, 2008). Lysozyme released by leukocyte and mainly distributed

in fish skin mucus, head kidney, gill, gastrointestinal tract and eggs where the fish stands a high risk of bacterial attack, thus this shows the importance of lysozyme in immune system of fish (Trichet, 2010).

2.3.4 Catalase activity and superoxide dismutase activity

Excessive of ROS production and oxygen free radical can lead to oxidative stress (Schrader & Fahimi, 2006; Radovanović et al., 2010). Hence, the antioxidative defense system (AOS) takes place to neutralize the excessive ROS. AOS composed of enzymatic and non-enzymatic components. Enzymatic ROS scavenger consist of SOD, catlase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and biotransformation phase II enzyme glutathione-S-transferase (GST) (Schrader & Fahimi, 2006; Radovanović et al., 2010).

CAT and SOD are often used as indicator to show level of pollutant or oxidative stress in aquatic organisms (Janssens et al., 2000; Velkova-Jordanoska et al. 2008; Radovanović et al., 2010; Vasylkiv et al., 2011). CAT is involved in detoxification of H_2O_2 and several toxic such as ethanol, methanol, phenol and nitrites by peroxidatic activity (Schrader & Fahimi, 2006). On the other hand, SOD is known as an oxido-reductase, catalyzes the dismutation of the O_2^- into molecular oxygen and H_2O_2, which can be detoxified into water and oxygen by CAT or peroxidases (Janssens et al., 2000). In fish, CAT and SOD are served as primary defense system against free radicals. However, there are several factors that affect the levels of enzyme CAT and SOD such as dissolved oxygen concentration, light intensity, swimming activity and food supply (Radovanović et al., 2010).

2.3.5 Dietary EFA studies on immune response of fish

Previous studies have been focused on the effect of dietary EFA on non-specific immune response of fish. In study of Puangkew et al. (2004), alternative complement activity, total immunoglobulin, phagocytosis and cytotoxicity increased in rainbow trout *Oncorhynchus mykiss* fed diet supplemented with vitamin E and n-3 LC-PUFA. Besides, there was an increase of eicosanoid production such as prostaglandin in plasma of gilthead seabream fed diet inclusion of vegetable oil rich in oleic acid (OA, 18:1n-9), LA and ALA (Ganga et al., 2005). Dietary ALA and LA at 2% of diet in ratio of 3:1 increased on head-kidney leucocyte phagocytic and respiratory burst activities of juvenile grouper (Wu & Chen, 2012). At the same time, large yellow croaker fed dietary n-3 LC-PUFA demonstrated higher resistance to parasites *Cryptocaryon irritans* (Zuo et al., 2012). Atlantic cod *Gadus morhua* fed diet containing camelina oil with higher n-3 PUFA and γ-tocopherol showed improvement on anti-viral immune response (Booman et al., 2014).

2.4 Fatty acid

2.4.1 Nomenclature of fatty acid

Fatty acid is known as a carboxylic acid attached with a long non-aromatic chain containing two or above double bonds (Wynn, 2011). Commonly, the n-nomenclature is often being used in the naming of PUFA (Warren & Vineyard, 2013). For instance, ARA, n-nomenclature as 20:4n-6, indicates a 20 carbon chain that contains 4 double bonds and the first double bond is located 6 carbon atoms from the methyl end. Similarly, DHA, n-nomenclature as 22:6n-3, defines a 22

carbon chain that comprises 6 double bonds with the first double bond situated 3 carbon atoms from the methyl end.

Figure 2.1 ARA, 20:4n-6 in n-nomenclature of fatty acids

Figure. 2.2 DHA, 22:6n-3 in n-nomenclature of fatty acids

2.4.2 Functions of fatty acid in body metabolism

Most of the fatty acids are utilized for energy source, phospholipid membrane formation, lipid transport and pathway signaling (Shahidi & Senanayake, 2008). For example, 1 g of fatty acids provides nearly 9kcal of energy while 4kcal of energy yield from 1 g of carbohydrates. In addition, catabolism of fatty acids, also known as β-oxidation, supplies substantial amount of energy source in fish (Watkins, 2013). The activated fatty acids undergo a series of cyclic reactions catalyzed by various enzymes to produce acetyl-CoA and NADH. Then, acetyl-CoA is utilized to generate more NADH via the tricarboxylic cycle, followed by oxidative phosphorylation that allows NADH to provide metabolic energy in the form of ATP (Shahidi & Senanayake, 2008). On the other hand, fatty acids are essential to cell membrane formation (Bass, 1988). The cell membrane contains phospholipids which are the major type of lipid, followed by glycolipids and sterols. PUFA derived

from phospholipids possesses kinks that avoid the fatty acids pack tightly and maintain the membrane fluidity (Murphy, 1990).

Besides, the liver processes fatty acids by converting them to triglyceride and synthesized into several forms of lipoprotein such as very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) (Murphy, 1990). According to Shahidi & Senanayake (2008), VLDL serves as transporter for lipids such as triglycerides, phospholipids and cholesterol from liver to the body cells. At the same time, LDL delivers cholesterol around the body while HDL carries cholesterol and glycerol from the body back to the liver for decomposition and excretion. Furthermore, fatty acids appear as carriers for the fat soluble vitamins A, D, E and K and assist the vitamins absorption in the intestine (Shahidi & Senanayake, 2008).

2.4.3 Fatty acid conversion pathway in fish

In fish, dietary PUFA are essential for the LC-PUFA biosynthesis. EFA of the freshwater fish such as EPA, DHA and ARA are biosynthesized from the elongation and desaturation of ALA and LA. Briefly, EPA is synthesized by the Δ6 desaturation of ALA to 18:4n-3 or elongation to 20:3n-3, followed by the elongation or Δ8 desaturation to 20:4n-3 and Δ5 desaturation. DHA is synthesized by the elongation of EPA to 22:5n-3 and Δ4 desaturation. ARA is synthesized by Δ6 desaturation of LA to 18:3n-6 or elongation to 20:2n-6, followed by the elongation or Δ8 desaturation to DGLA and completed by Δ5 desaturation.

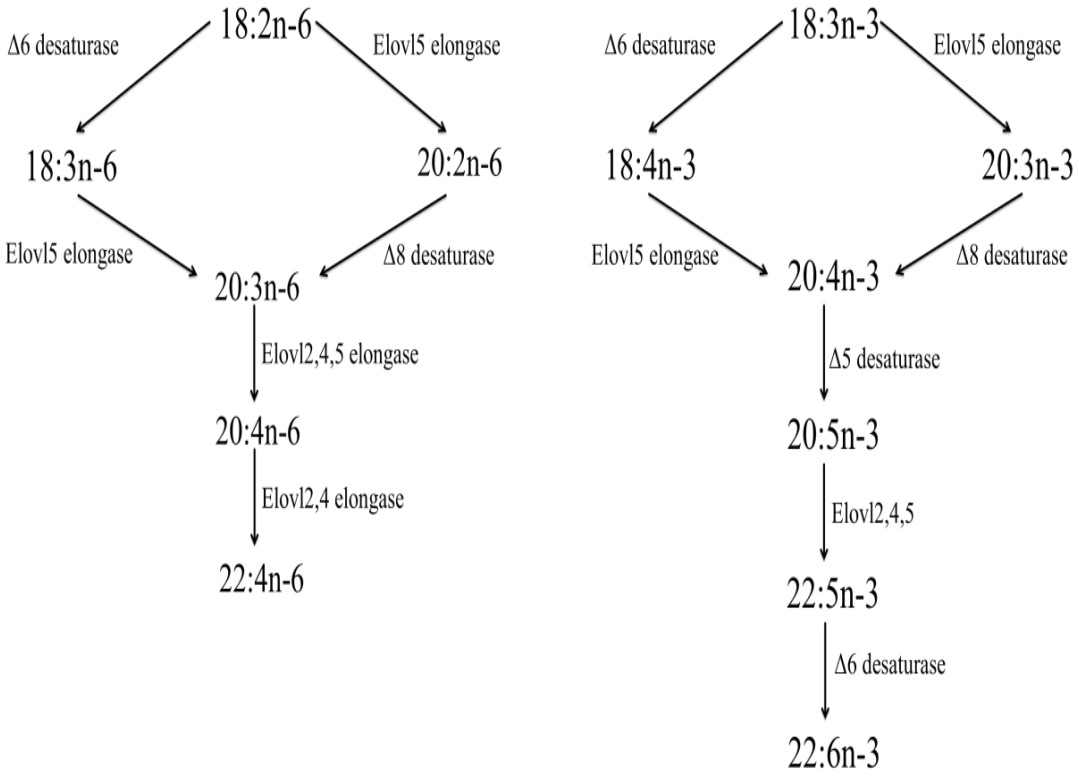

Figure 2.3 Biosynthesis pathway of ARA, EPA and DHA from LA and ALA

2.4.4 Impact of PUFA alteration in fish fatty acid composition

Generally, fish are unable to biosynthesis C18 PUFA such as ALA and LA which are substrates to produce LC-PUFA such as ARA, EPA and DHA. Therefore, LC-PUFA requirement of fish usually satisfied through dietary fish oil. Unfortunately, tremendous demand in fish oil has led to reduction in fish oil production and increasing price of fish oil. In order to ensure cost effective in aquaculture industry, replacement of fish oil with plant oil is introduced. According to Trushenski & Bowzer (2013), fish oil replacement with alternative dietary lipid source greatly altered fish tissue fatty acid composition. LC-PUFA in tissue of fish fed alternative lipid source showed different fatty acid composition compared to fish fed dietary fish oil. Fatty acid composition of n-6 PUFA-rich plant oil reflected on

fatty acid composition of fish tissues. Consequently, alteration of ratio n-3/n-6 PUFA in fish tissues cause differences in production of eicosanoids derived from n-3 PUFA and n-6 PUFA. Omega-6 PUFA derived eicosanoids are pro-inflammatory compared to eicosanoids derived from n-3 PUFA. Modification of ratio n-3/n-6 PUFA in fish tissues might affect immune mechanism of fish.

2.4.5 Role of PUFA in fish immune response

PUFA is playing a vital role as eicosanoid precursor (Zurier, 1993). Generally, eicosanoids are known as signaling molecules derived from the oxidation of n-3 and n-6 PUFA such as EPA, ARA or dihomo-gamma-linolenic acid (DGLA, 20:3n-6) by different pathways. Then, the eicosanoids productions are derived into prostaglandins, leukotrienes, thromboxanes and prostacyclin that function as inflammation regulator (Hornick, 2002). Leukotrienes induce aggregation and release of antioxidant enzyme such as superoxide in neutrophils. Prostaglandins influence the gene expression directly by activating peroxisome proliferator-activated receptors (PPAR). Thromboxanes stimulate platelet aggregation in order to reduce blood flow to the injury site. Prostacyclins inhibit platelet activation and serve as vasodilator. However, eicosanoid productions are inhibited by the competitive behavior of ARA, EPA and DGLA. For instance, competition activity of lipoxygenase enzyme between the PUFA precursors results in reduction of eicosanoid production.

Apart from eicosanoids production, oxidation of PUFA generates ROS by lipoxygenase. ROS plays a key role in microbicidal killing. According to Sessler & Ntambi (1998), PUFA are also involved in modulation of gene transcription, mRNA stability and cellular differentiation. PUFA modulates gene transcription through

effects on receptor and intracellular signal transduction (Trichet, 2010). Briefly, PUFA regulate gene expression in metabolism of lipid and energy. Sterol regulatory element binding protein-1c (SREBP-1c) and PPAR are the two transcription factors act as key mediators in gene modulation of PUFA. SREBP-1c stimulates formation of lipogenic enzymes but PUFA inhibit the induction of lipogenic genes by suppressing the SREBP-1c expression.

At the same time, PPAR, a transcription factor induces genes for PUFA oxidation in mitochondrial and peroxisomal (Nakamura et al., 2004). There are three isotypes of PPAR which are α, β and γ. PPARα mRNA is found highly expressed in liver, heart and gastrointestinal mucosa. For instance, PPARα is required for gene induction for mitochondrial and peroxisomal β-oxidation especially during fasting. Notably, dietary LC-PUFA that contains 20 carbons of chain length can acts as PPARα ligand, thus induces β-oxidation enzymes in mitochondria and peroxisomes. PPARγ is expressed in adipose and lymphoid tissue while PPARβ is expressed widespread (Nakamura et al., 2004).

2.4.6 Role of ARA in aquaculture

Recently, the importance of ARA, a n-6 LC-PUFA, has been highlighted in fish nutrition (Bell & Sargent, 2003). Previous studies showed that increasing dietary ARA improve growth of marine larval fish such as gilthead sea bream, *Sparus aurata* larvae (Bessonart et al., 1999) and striped bass, *Morone saxatilis* larvae (Harel et al., 2001). Cortisol is secreted by the interrenal tissue at head kidney of fish under stress conditions, and it is being used as a fish stress indicator (Martinez-Porchas et al., 2009). It was reported that ARA-enriched rotifers improved the survival and reduce cortisol levels of gilthead seabream larvae

encountering handling stress (Koven et al., 2001, 2003). Besides, dietary ARA resulted in significant down regulation of cortisol in gilthead seabream larvae after air exposure e (Van Anholt et al., 2004). Study of Lund et al. (2007) indicated that malpigmentation on common sole *Solea solea* larvae was attributed to ARA-enriched Artemia.

Previous studies showed importance of ARA in fish reproduction. Study of Norambuena et al. (2013*a*) detected that ARA content highly deposited in testis or ovary, followed by liver and muscle of senegalese sole, *Solea senegalensis* broodstock. Steroid levels of male Senegalese sole broodstock increased significantly with increasing dietary ARA levels compared to female broodstock (Norambuena et al., 2013*b*). Besides, diets supplemented with higher level of ARA (0.6% of diet) showed better quality of egg production in Japanese flounder *Paralichthys olivaceus* broodstock but excessive ARA supplementation (1.2% of diet) resulted in the lowest egg production among all the dietary treatments (Furuita et al., 2003).

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Fish and experimental condition

Malabar red snapper, *Lutjanus malabaricus* fingerlings were purchased from a local fish farm (Sungai Petani, Kedah, Malaysia). The fish were stocked in a 1000-l circular fiberglass tank for 1 week and fed with commercial marine fish pellet (Cargill, USA). Prior to feeding trial, 20 fish were randomly distributed into each 500-l fiberglass tank attached to a recirculating aquaculture system and acclimatized to the control diet for two weeks. At the initiation of the feeding trial, groups of 20 fish were randomly selected, reweighed and restocked into the 500-l fibreglass tank. Fish from each tank were bulk-weighed weekly. During the experimental period, nitrite, ammonia and phosphate of water were measured weekly.

3.2 Recirculating aquaculture system (RAS)

Triplicate groups of fish were reared within a RAS system at the Fish Nutrition Laboratory, Universiti Sains Malaysia, Penang. The RAS composed of 12 units of 500-l fiberglass tanks, protein skimmer, high performance fiberglass filter and biological filtration system connected by polyvinyl chloride (PVC) pipes. Briefly, artificial seawater was prepared by mixing dechlorinated freshwater with sea salt (Instant Ocean, USA) at salinity of 25ppt. The overflow water from each tank was flowed to the sedimentation tank on the floor by gravity through the PVC pipe. The protein skimmer connected with the sedimentation tank was used to skim

out the organic waste of the water using a submersible pump and the water circulated back to the tank. Simultaneously, water from the sedimentation tank was transported to a high performance fiberglass filter by a self-priming pump (Techno Takatsuki, Japan) in order to remove particulate matter in the water, followed by biological filter. The bio-filter bed substrate contained bio-ball and coral chip that allowed bacteria to grow and undergo nitrification process. Eventually, the water flowed back to the experimental tanks by gravity.

Plate 3.1 Tanks of RAS in the experiment

3.3 Sample collection

At the end of the experiment, all fish were starved for 24 hours to empty the digestive tract before sampling. The fish were anesthetized and individually weighed. Blood samples were collected by severing the caudal peduncle of fish. Plasma was prepared by centrifugation of heparinized blood at 1000 ×g, 4ºC for 10 minutes and the supernatant was frozen at -80ºC until use. For serum, unheparinized blood was allowed to clot for 30 minutes at room temperature prior to centrifugation at 2000×g, 4ºC for 15 minutes and the supernatant was frozen at -80ºC until use. Liver, viscera and fat were excised and weighed to determine hepatosomatic index (HSI), viscerosomatic index (VSI) and intraperitoneal fat (IPF), respectively. Liver