A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Fish oil replacement with tallow and emulsifier in diet for olive flounder, *Paralichthys olivaceus*

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JEJU NATIONAL UNIVERSITY

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ABSTRACT

In Chapter I, the supplementary effect of an emulsifier mixture (polyoxyethylene (20) sorbitan monolaurate and distilled monoglycerides) was investigated on feed utilization, growth performance and immunity of olive flounder (*Paralichthys olivaceus*), replacing fish oil with beef tallow. A fish oil containing diet was considered as a positive control (PC) and a diet containing beef tallow replacing fish oil was considered as a negative control (TW). Two other diets (EM01 and EM02) were prepared by adding 0.01% and 0.02% emulsifier into TW diet. After eight weeks of a feeding trial, growth performance and feed utilization were significantly higher in fish fed EM01, EM02 or PC diets compared to those fed TW diet. Dry matter digestibility was higher in EM01 and EM02 diets than that in TW diet and comparable to PC diet. Lipid digestibility was significantly higher in PC diet compared to that in TW diet. Liver EPA and DHA levels were lower, and oleic acid level was higher in fish fed diets containing tallow compared to PC group. Significantly increased aspartate aminotransferase and triglyceride levels were observed in TW group. Dietary emulsifier restored the reduced performance of olive flounder fed diets containing tallow instead of fish oil. The optimum inclusion level of the emulsifier could be approximately 0.02% when beef tallow was included in diets.

In chapter II, effects of graded levels of arachidonic acid (ARA) in a low fish meal diet were evaluated on growth, feed utilization and immunity of olive flounder at suboptimal temperature (15.2±1.2 ˚C) season. Six low fish meal diets (30% fish meal and 30% plant protein blend) were formulated with graded levels of ARA. A control diet was formulated incorporating 3% fish oil and 5.1% soybean oil without ARA (ARA0.0). Five different levels ARA were added replacing soybean oil to contain 0.3, 0.6, 0.9, 1.2 and 1.5% ARA (ARA0.3, ARA0.6, ARA0.9, ARA1.2 and ARA1.5) respectively. Triplicate groups of fish (88.24 ±0.06 g) were distributed into 18

polyvinyl circular tanks (215 L) with 24 fish per tank and fed one of the experimental diets for nine weeks. Significantly improved growth performance was shown in ARA0.6 group. Liver C18:2n-6 was significantly reduced and liver ARA content significantly increased with its increase in diets. Nitroblue tetrazolium activity was significantly higher in ARA0.6 and the highest super oxide dismutase (SOD) and myeloperoxidase (MPO) levels were observed in ARA0.9 treatment. Immunoglobulin and lysozyme activity were significantly improved in ARA0.3 group. Antiprotease activity was increased with ARA0.6 and ARA0.9 diets. ARA0.3 diet fed fish showed lower liver lipid content compared to ARA0.0 group. These results indicates that the supplementation of 0.3-0.9% ARA might improve the growth, immune and hematological parameters of olive flounder.

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CHAPTER I

Effect of total dietary replacement of fish oil with tallow and emulsifier on growth, feed utilization and immunity of olive flounder

1.1. Introduction

Fish oil (FO) is an important raw material in aquafeed industry due to its beneficial effects including balanced fatty acid profile for marine species (Turchini et al. 2009). However, Nasopolou and Zebatakis (2012) reported that FO production reached its maximum level and it would not meet the demand for the increased aquafeed production. In addition, fish meal and FO prices have dramatically been increased over the years (FAO 2018). Therefore, the research communities must bear a collective responsibility on the identification of alternatives for fish meal and FO and increase their efficiency for the sustainable aquaculture.

Tallow is economical and widely available rendered fat obtained from fatty tissues of bovine or sheep (Turchini et al. 2009). It is a rich source of saturated fatty acids (55.4%) and monounsaturated fatty acids (34.2%) but not in poly unsaturated fatty acids (PUFA) (2.7%) (Hwang 2009). In the past few decades, tallow has been used to replace FO partially or totally in diets for several fish species. Red drum (*Sciaenops ocellatus*) fed diets containing coconut oil and tallow showed a significantly higher growth performance than a control (FO) diet fed fish (Craig and Gatlin 1995). Japanese sea bass (*Lateolabrax japonicus*) fed diets in which FO was replaced with six alternative fat sources including beef tallow did not show any significant difference in growth parameters (Xue et al. 2006). Bureau et al. (2008) suggested that saturated fat sources like tallow can be used to partially replace FO without affecting digestibility and

growth performance in rainbow trout (*Oncorhynchus mykiss*). Growth and nutrient digestibility of Atlantic salmon (*Salmo salar*) were not significantly affected by feeding a 50% of FO replaced diet with tallow (Emery et al. 2013). Growth performance of gilthead sea breams (*Sparus aurata*) fed a diet containing a blend of beef tallow and FO did not show any significant difference compared to a FO group (Pérez et al. 2014). Growth performance and whole-body composition of juvenile olive flounder (*Paralichthys olivaceus*) were not significantly affected by the FO replacement with beef tallow (Lee et al. 2019). However, Silvery-black porgy (*Sparidentex hasta*) fed diet containing 100% or 50% tallow in the expense of FO showed significantly lower growth performance compared to 100% FO incorporated diet (Mozanzadeh et al. 2015).

Polysorbates and distilled monoglycerides are widely used emulsifiers that bind fat molecules with the water phase. Polyoxyethylene (20) sorbitan monolaurate (Tween 20, Polysorbate 20) is a widely applicable emulsifier for animal feeds on the purpose of increasing lipid digestion and utilization. Distilled monoglycerides are also food-grade emulsifier with a low hydrophile-lipophile balance number.

Emulsifiers facilitate the breaking down of large fat globules into microscopic droplets and form a hydrophilic environment increasing the active surface of fats where the lipase activity takes place (Al-Marzooqi and Leeson 1999). Then, the digestion activity of lipase is facilitated by reaching fat molecules that are deep in the large lipid globules (Maldonado-Valderrama 2011). Ostos (1993) revealed that lipid digestion varies under different temperatures due to the varying gastrointestinal transition time and the lipase efficiency. As a solution, emulsifiers have been used in aquafeeds to overcome such constraints and improve the lipid digestion (Adhami et al. 2017). Yamamoto et al. (2007) observed that the addition of bile salts in the diet increased

digestion and lipid deposition in salmonids. Jiang (2017) observed a significant increasement of growth by dietary bile acids in juvenile Nile tilapia (*Oreochromis niloticus*). However, Bergman and Trushenski (2018) observed significantly impaired growth and survival in juvenile yellowtail (*Seriola dorsalis*) fed diet mixed with bile salts.

Olive flounder is a marine demersal flat fish widely cultured in East Asian countries. According to FAO (2018), the aquaculture production of olive flounder in South Korea is 41,207 mt and this represent a significant proportion of total fish production (Hamidogili et al., 2019). Therefore, it is important to identify new trends and innovations on this section. This study was designed to investigate the supplemental effects of an emulsifier on feed utilization, growth performance and health status of olive flounder when total FO in diets was replaced with tallow.

1.2. Materials and Methods

1.2.1. Experimental Diets

Dietary formulation and proximate composition of five experimental diets are shown in the Table 1-1. The positive control (PC) diet was prepared containing FO as main lipid source and the negative control (TW) diet was prepared by replacing the total FO content with beef tallow. Two other diets were prepared by adding 0.05% or 0.1% of an emulsifier (included polyoxyethylene (20) sorbitan monolaurate and distilled monoglycerides at a ratio of 8.5%: 1.5%, silica: 8.5%, starch: 57.6% and calcium carbonate: 23.9%) to the negative control diet (named as EM01 and EM02 respectively). All the dry ingredients were thoroughly mixed and extruded by a pelletizer machine (SP-50, Gum Gang Engineering, Daegu, Korea) in a proper size. The pellets were air-dried with an electric drier machine (SI-2400, Shinil General Drier Co., Ltd, Daegu, Korea) at 25 ˚C for 12 hours and stored at -4 ˚C until use.

Figure 1.1. Diet preparation, feeding trial, digestibility trial, sample preparation and lipase activity analyzing.

Table 1.1. Dietary formulation and proximate composition of the experimental diets for olive flounder (*Paralichthys olivaceus*).

Ingredients	PC	TW	EM01	EM02
Sardine fish meal[1]	50.00	50.00	50.00	50.00
Wheat flour	19.50	19.50	19.40	19.40
Tankage meal	5.00	5.00	5.00	5.00
Soybean meal	12.00	12.00	12.00	12.00
Wheat gluten	5.00	5.00	5.00	5.00
Fish oil[2]	6.00	0.00	0.00	0.00
Tallow[3]	0.00	6.00	6.00	6.00
Mono calcium phosphate	0.50	0.50	0.50	0.50
Mineral Mixture[4]	1.00	1.00	1.00	1.00
Vitamin Mixture[5]	1.00	1.00	1.00	1.00
Emulsifier (10%)[6]	0.00	0.00	0.10	0.20
Proximate composition (% of dry matter)				
Moisture	6.50	6.48	7.34	6.05
Crude protein	53.8	51.8	52.8	53.0
Crude lipid	14.4	14.2	14.5	14.7
Crude ash	10.1	9.9	10.0	10.1

Abbreviations: PC, No replacement of fish oil with tallow; TW, 100% fish oil replacement with tallow; EM01, 0.01% emulsifier addition to TW diet; EM02, 0.02% emulsifier addition to TW diet.

[1]Orizon S.A, Chile.

[2]Corp. E-wha oil & fat Ind, Korea.

[3]SNH Biotech Co., Ltd., Daejeon 34008, Republic of Korea

[4]Mineral premix (g kg^{-1} of mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3.6H2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[5]Vitamin premix (g kg1 of mixture): L-ascorbic acid, 121.2; DL-a tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

[6]Polysorbate (20) based emulsifier, containing 10% emulsifier.

Table 1.2. Fatty acids composition of the experimental diets for olive flounder (*Paralichthys olivaceus*).

	PC	TW	EM01	EM02
Fatty acids (% of fatty acid)				
C14:0	5.78	5.03	5.02	4.99
C16:0	21.9	24.6	24.6	24.5
C16:1	8.10	6.04	6.03	5.99
C17:1	1.56	1.60	1.61	1.61
C18:0	5.08	9.24	9.30	9.36
C18:1 n-9	17.2	23.0	23.0	23.2
C18:2 n-6	8.67	8.18	8.15	8.21
C18:3 n-3	0.97	0.82	0.81	0.83
C20:1	5.08	3.02	3.02	3.02
C20:2	0.21	0.15	0.14	0.14
C20:4 n-6	0.42	0.37	0.34	0.34
C22:1 n-9	0.86	0.50	0.49	0.50
C24:1	0.85	0.61	0.62	0.62
Fatty acids (% of fatty acid)				
C20:5 n-3 (EPA)	13.5	9.72	9.68	9.57
C22:6 n-3 (DHA)	9.80	7.19	7.17	7.10
EPA+DHA	23.3	16.9	16.8	16.7

Abbreviations: PC, No replacement of fish oil with tallow; TW, 100% fish oil replacement with tallow; EM01, 0.01% emulsifier addition to TW diet; EM02, 0.02% emulsifier addition to TW diet.

1.2.2. Fish and feeding trial

The feeding trial was conducted at the Marine and Environmental Research Institute of Jeju National University (Jeju, South Korea). Olive flounder yearlings were transported from a private hatchery (Jeju, South Korea). All fish were acclimatized to experimental conditions and facilities for two weeks while feeding a commercial diet. The initial weight of fish was 100 ± 0.26 g at the end of acclimatization. A set of 216 fish were randomly selected and introduced into 12 polyvinyl circular tanks (215L) as 18 fish per tank to be triplicated each four treatments. Tanks were supplied with continuously filtered seawater at a flow rate of 3 $Lmin^{-1}$ and aerated with an air-stone to maintain sufficient dissolved oxygen. Average water temperature was 22.1±1.4 ˚C while conducting this feeding trial. Fish were hand-fed with one of test diets to apparent satiation every day at 08:30h and 18:00 h for 8 weeks. Water was changed 30 min after feeding and remaining diets in tanks were collected and weighed to calculate feed intake. Growth performance was measured at the end of 8^{th} week. Feeding was stopped 24 hours before the weighing and the sample collection.

1.2.3. Sample collection and analysis

At the end of the feeding trial, all the fish in each tank were counted and individual fish weight were measured. The body length of each fish was measured for calculation of condition factor. Ten fish were selected randomly from each tank and anesthetized with 2-phenoxyethanol ($200mgl^{-1}$) for blood sampling. Blood samples were taken from three fish with heparinized syringes and plasma was separated from the remaining blood samples by centrifugation at 5000 × g for 10 min using a high-speed refrigerated microcentrifuge (Micro 17 TR; HanilBioMed Inc., Gwangju, Korea) after the determination of hemoglobin level and hematocrit level. Plasma

samples were stored at -70 ˚C for the determination of total immunoglobulin (Ig) level and blood biochemical parameters. Hematocrit was determined by the microhematocrit technique (Brown,1980). Hemoglobin and plasma levels of glucose, total protein, total cholesterol, triglyceride, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels by an automated blood analyzer (SLMI; SEAC Inc, Florence, Italy.).

Another set of blood samples was taken from three fish from each tank, allowed to clot at room temperature for 30 min and serum was separated by centrifugation at 5000 × g for 10 min using high-speed refrigerated microcentrifuge (Micro 17 TR; HanilBioMed Inc., Gwangju, Korea). Serum samples were stored at -70 ˚C. These serum samples were used for the determination of lysozyme activity and myeloperoxidase (MPO) activity as non-specific immune parameters. Visceral and liver samples were dissected from the fish that used to collect blood samples and weighed to calculate the vicserosomatic index (VSI) and hepatosomatic index (HSI).

Remaining four anesthetized fish were stored at -20 ˚C for analysis of whole-body proximate composition. Moisture and ash content were analyzed according to the AOAC (1995). Crude protein was analyzed by an automatic Kjeltec Analyzer Unit 2300 (FOSS, Sweden) and crude lipid was determined according to Folch et al. (1957). Plasma immunoglobulin (Ig) levels were measured by Siwicki and Anderson (1993). A turbidimetric method was used to measure the level of serum lysozyme (Hultmark 1980) with a slight adjustment. Myeloperoxidase activity was measured using the method described by Quade and Roth. (1997).

1.2.4. Lipase activity

Three fish were randomly selected after the feeding trial, middle intestine samples (2 cm) were incised and homogenized 5 ml chilled distilled water. Homogenate was centrifuged at 15000 rpm for 30 min at 0 ˚C and supernatant was used for crude enzyme assay. Lipase activity was assayed based on measurements of fatty acid release due to enzymatic hydrolysis of triglycerides in a stabilized standard emulsion of olive oil (Borlongan, 1990). Briefly, crude enzyme mixture (1 ml) was added to 1.5 ml of stabilized olive oil substrate in 1 ml of 0.1 M Tris–HCl buffer at pH 8.0 and incubated for 6 h at 37 ˚C. Reaction was stopped adding 3 ml of 95% ethyl alcohol and titrated with 0.01 N NaOH using 0.9% (w/v) thymolphthalein in ethanol as the indicator.

1.2.5. Determination of apparent digestibility of diets

The control diet and other four experimental diets were formulated with adding 1% chromic oxide (Cr_2O_3) (Sigma-Aldrich, St. Louis, USA), inert indicator, to the diet. All the dry ingredients were thoroughly mixed and pelletized through a pelletizer machine (SP-50, Gum Gang Engineering, Daegu, Korea) to proper size. Drying, sieving and storing were done following the same procedure followed in experiment diet preparation. Hundred and twenty olive flounders (initial Body weight 100±26g) were distributed into 300L Guelph system (Fecal collection system) tanks at a density of 30 fish per tank. The five tanks were supplied with a cartridge-filtered seawater at a flowrate of $3Lmin^{-1}$ and aerated with air stones. Fish were hand fed until apparently satiate and excess feed were removed after one hour later. Fecal matters collected into a feces collection tube, fixed to the bottom of the tanks, were filtered through a Whatman filter paper, separated and stored at -40 ˚C. This process was carried out for 3 weeks

inter-changing diets between tanks to be triplicate the treatments. After collecting enough fecal matter, samples were freeze-dried for 24 h and stored at -20 ˚C until analyzed. Chromium oxide content of the experimental diets and freeze-dried feces samples were analyzed according to Divakaran et al. (2002). Apparent digestibility coefficient of dry matter, protein and lipid were calculated according to following formulas.

1.2.6. Statistical analysis

All experimental diet groups were subjected to a complete randomized design. Data were analyzed by one-way ANOVA using the SPSS (version 18.0) program. When ANOVA was identified differences among groups, the differences in mean values were compared using Duncan's multiple range test ($P \leq 0.05$). Data were expressed as mean ± standard deviation (mean ± SD). Percentage data were arcsine transformed before analysis.

1.3. Results

1.3.1. Growth performance and feed utilization

Growth performance, feed utilization and biometric indexes of olive flounder are shown in Table 1-3. Final body weight (FBW), feed conversion ratio (FCR) and protein efficiency ratio (PER) were significantly improved in fish fed PC or EM02 diet compared to those of fish fed TW or EM01 diet. Compared to TW diet, EM01 diet resulted in significantly higher FBW, FCR and PER values. Weight gain (WG) and specific growth rate (SGR) of EM02 diet group were comparable to those of PC diet group. However, WG and SGR were impaired in fish fed TW diet. Significantly higher feed intake (FI) and survival were recorded in fish fed PC, EM01 and EM02 treatments compared to the TW group.

1.3.2. Biometric parameters

Hepatosomatic index (HSI) was significantly improved in fish fed the PC diet in comparison to fish fed the TW diet. The condition factor (CF) and vicserosomatic index (VSI) were not significantly differenced among dietary treatments.

1.3.3. Hematological Parameters

Results of blood parameters are shown in Table 1-7. Significantly lower plasma aspartate aminotransferase (AST) level was shown in fish fed the PC diet in comparison to that of fish fed the TW diet. Significantly lower plasma triglyceride levels were observed in PC and EM02 groups compared to those of fish in the TW group. Hematocrit, hemoglobin, total cholesterol,

total proteins, and alanine aminotransferase levels did not show any significant differences among diet groups.

1.3.4. Non-specific immune parameters

Myeloperoxidase activity (MPO), lysozyme activity and Ig level were not significantly changed among treatments although the lower values were observed in the TW diet group.

1.3.5. Whole-body proximate composition and liver fatty acids composition

The results of whole-body proximate composition showed no significant difference among dietary treatments (Table 1-7). However, crude lipid content of fish fed diets containing emulsifier were showed higher values than the other diets. The liver oleic acid (C18:1 n-9) levels of fish fed diets containing tallow were higher than that of fish fed the PC diet (Table 1-4). Comparatively higher eicosenoic acid (C20:1), eicosadienoic acid (C20:2), arachidonic acid (C20:4 n-6), erucic acid (C22:1 n-9) and nervonic acid (C24:1) were observed in the liver of fish fed the PC diet compared to other diets. Similarly, higher levels of eicosapentaenoic acid (EPA, C20: 5n-3) and docosahexaenoic acid (DHA, C22: 6n-3) were also observed in fish fed PC diet compared to those of fish fed the TW diet.

1.3.6. Apparent digestibility coefficient (ADC)

A significantly higher dry matter digestibility was observed in the EM01 group than that of the TW group (Table 1-5). Significantly higher lipid digestibility of the PC diet was

determined than that of the TW diet while the results of EM01 and EM02 diets were comparable to both PC and TW diets. ADC of protein was not affected by dietary tallow and/or emulsifier inclusion instead of FO in the diet.

1.3.7. Lipase activity

The lipase activity of olive flounder was not shown any significant difference among dietary treatments although the lowest lipase activity was observed in fish fed TW diet (Table 1-6).

Table 1.3. Growth performance and biometric indexes of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 8 weeks.

	PC	TW	EM01	EM02
FBW[1]	277±2[a]	209±2 [c]	255±9[b]	272±7[a]
WG[2]	177±2[a]	110±2[c]	155±10[b]	171±8[ab]
SGR[3]	1.82±0.01[a]	1.32±0.02[c]	1.67±0.07[b]	1.78±0.06[ab]
FI[4]	183±4[a]	169±0[b]	186±1[a]	181±4[a]
FCR[5]	1.03±0.03[a]	1.54±0.03[c]	1.21±0.06[b]	1.06±0.02[a]
PER[6]	1.68±0.05[a]	1.17±0.02[c]	1.46±0.08[b]	1.68±0.04[a]
Survival	94.4±5.6[a]	81.5±3.2[b]	98.1±3.2[a]	100±0[a]
CF[7]	1.05±0.07	0.99±0.04	1.01±0.01	1.03±0.06
HSI[8]	2.05±0.29[a]	1.59±0.08[b]	1.89±0.13[ab]	1.93±0.30[ab]
VSI[9]	4.67±0.12	4.21±0.14	4.42±0.10	4.55±0.52

Abbreviations: PC, No replacement of fish oil with tallow; TW, 100% fish oil replacement with tallow; EM01, 0.01% emulsifier addition to TW diet; EM02, 0.02% emulsifier addition to TW diet.

Values are means form triplicate groups of fish where the values in each row with the different superscripts are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Final body weight(g); [2]Weight gain(%) = [(FBW−initial body weight)/initial body weight] × 100; [3]Specific growth rate(%) = {[ln (FBW) – ln (initial body weight)] / days of feeding} × 100; [4]Feed intake(g/fish) = dry feed consumed(g)/fish number; [5]Feed conversion ratio = (wet weight gain/dry feed intake) × 100; [6]Protein efficiency ratio = wet weight gain/protein intake; [7]Condition factor = (Fish weight/Fish length^3) × 100; [8]Hepatosomatic index(%) = (Liver weight/Fish weight) × 100; [9]Viscerosomatic index(%) = (Visceral weight/Fish weight) × 100.

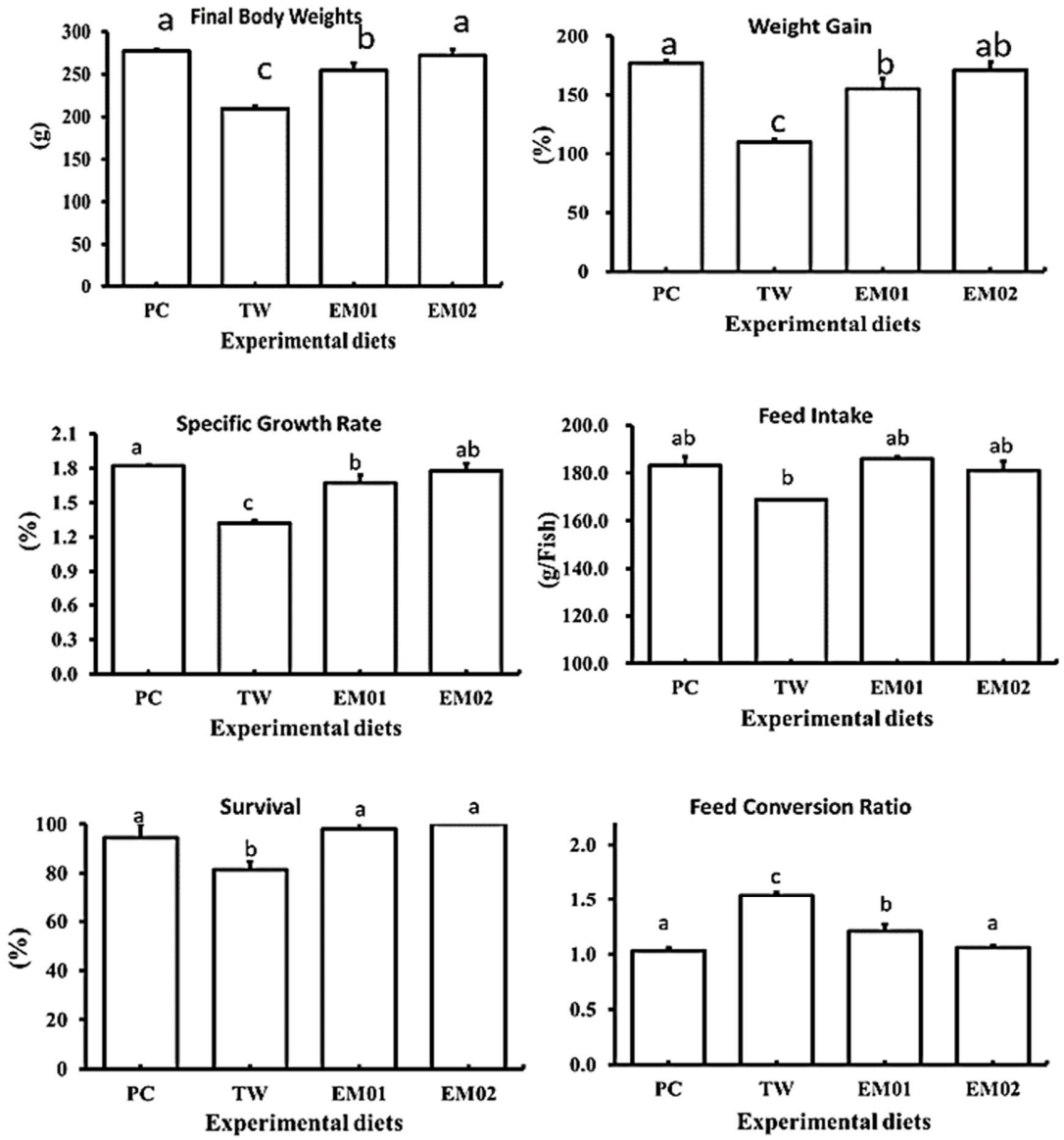


Figure 1.2. Growth performance and feed utilization of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 8 weeks.

Table 1.4. Fatty acids composition of liver in olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 8 weeks.

	PC	TW	EM01	EM02
Fatty acids (% of fatty acid)				
C14:0	4.79	4.15	4.28	3.98
C16:0	20.8	21.7	21.2	20.1
C16:1	7.96	6.47	6.23	6.12
C17:1	2.69	2.25	1.81	1.85
C18:0	2.86	3.68	4.02	3.70
C18:1 n-9	27.9	36.0	38.6	38.9
C18:2 n-6	7.68	8.39	6.55	7.74
C18:3 n-3	0.54	0.51	0.37	0.44
C20:1	5.31	3.64	3.97	3.92
C20:2	0.95	0.81	0.73	0.83
C20:4 n-6	0.40	0.34	0.28	0.29
C22:1 n-9	1.79	1.20	1.29	1.25
C24:1	1.38	1.03	0.97	0.90
Fatty acids (% of fatty acid)				
C20:5 n-3 (EPA)	6.90	4.81	4.31	4.63
C22:6 n-3 (DHA)	7.64	4.63	5.04	4.93
EPA+DHA	14.5	9.45	9.35	9.56

Abbreviations: PC, No replacement of fish oil with tallow; TW, 100% fish oil replacement with tallow; EM01, 0.01% emulsifier addition to TW diet; EM02, 0.02% emulsifier addition to TW diet.

Table 1.5. Apparent digestibility coefficient (%) of dry matter, crude protein and crude lipid in olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 8 weeks.

Treatment	PC	TW	EM01	EM02
Dry matter	74.6±4.01^{ab}	70.3±8.06^{b}	81.2±2.46^{a}	79.3±3.24^{ab}
Crude protein	91.9±1.27	90.6±2.55	92.6±0.96	93.5±1.02
Crude lipid	95.5±0.7^{a}	93.0±1.9^{b}	94.8±0.7^{ab}	93.3±1.0^{ab}

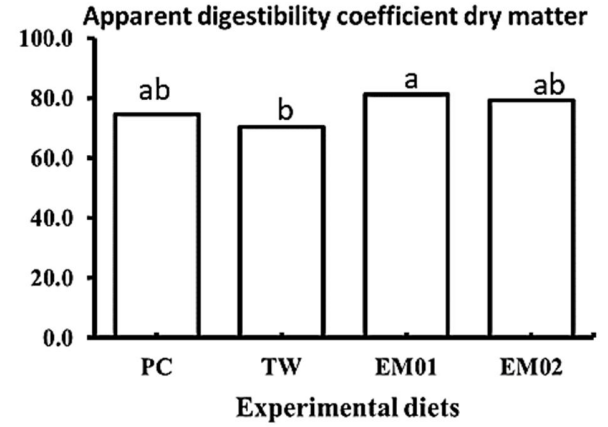
Abbreviations: PC, No replacement of fish oil with tallow; TW, 100% fish oil replacement with tallow; EM01, 0.01% emulsifier addition to TW diet; EM02, 0.02% emulsifier addition to TW diet.

Values are means form triplicate groups of fish where the values in each row the different superscripts are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

ADC of DM (%) = 100 - 100 × (% Cr2O3 in diet/ %Cr2O3 in feces) × (% dry matter in feces/ %dry matter in diet).

ADC of protein (%) = 100 - 100 × (Cr2O3 in diet/ %Cr2O3 in feces) × (%protein in feces/ %protein in diet).

ADC of Lipid (%) = 100 – 100 × (Cr2O3 in diet/ Cr2O3 in feces) × (%lipid in feces/ %lipid in diet).



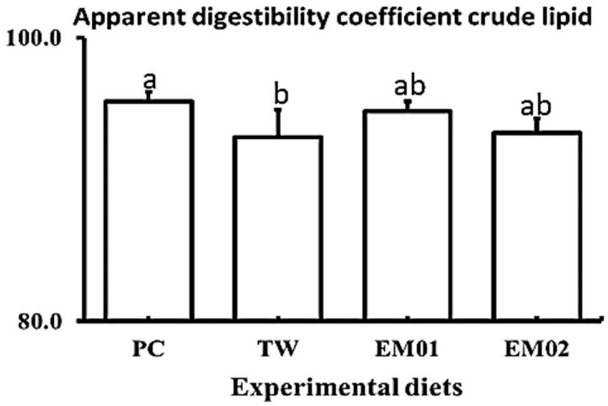


Figure 1.3. Apparent digestibility coefficient (%) of dry matter and crude lipid in olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 8 weeks.

Table 1.6. Intestinal lipase activity of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 8 weeks.

Treatment	PC	TW	EM01	EM02
Lipase activity (units/mg protein)	0.26±0.03	0.20±0.04	0.24±0.03	0.25±0.05

Abbreviations: PC, No replacement of fish oil with tallow; TW, 100% fish oil replacement with tallow; EM01, 0.01% emulsifier addition to TW diet; EM02, 0.02% emulsifier addition to TW diet.

Values are means form triplicate groups of fish.

Table 1.7. Hematological parameter of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 8 weeks.

Treatment	PC	TW	EM01	EM02
Hb[1]	3.48±0.94	3.64±0.22	3.18±0.70	3.15±0.98
Ht[2]	29.1±2.9	26.3±2.1	26.1±0.8	27.6±1.1
AST[3]	9.8±2.6[b]	14.2±3.1[a]	11.7±1.1[ab]	12.3±0.5[ab]
ALT[4]	6.73±0.29	7.22±1.73	7.43±1.28	7.52±0.37
TP[5]	3.76±0.50	3.33±0.26	3.46±0.27	3.65±0.44
TG[6]	273±60[b]	389±15[a]	362±42[ab]	278±63[b]
CHOL[7]	259±17	204±24	214±15	243±27

Abbreviations: PC, No replacement of fish oil with tallow; TW, 100% fish oil replacement with tallow; EM01, 0.01% emulsifier addition to TW diet; EM02, 0.02% emulsifier addition to TW diet.

Values are means form triplicate groups of fish where the values in each row the different superscripts are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Hemoglobin (g/dL); [2]Hematocrit (%); [3]Aspartate aminotransferase (U/L); [4]Alanine aminotransferase (U/L); [5]Total protein (g/dL); [6]Triglyceride (mg/dL); [7]Cholesterol (mg/dL).

Table 1.8. Non-specific immune parameters of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 8 weeks.

Treatment	PC	TW	EM01	EM02
MPO[1]	1.41±0.15	1.27±0.08	1.33±0.13	1.39±0.10
Ig[2]	13.6±1.0	12.8±1.4	13.4±1.1	14.7±1.1
Lysozyme[3]	10.7±1.9	9.7±1.8	11.0±2.9	11.3±1.0

Abbreviations: PC, No replacement of fish oil with tallow; TW, 100% fish oil replacement with tallow; EM01, 0.01% emulsifier addition to TW diet; EM02, 0.02% emulsifier addition to TW diet.

Values are means form triplicate groups of fish.

[1]Myeloperoxidase (absorbance); [2]Immunoglobulin (mg/mL); [3]Lysozyme (μg/mL).

Table 1.9. Whole-body composition (%) of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 8 weeks.

Treatment	PC	TW	EM01	EM02
Moisture	71.0±0.7	71.7±0.4	71.3±0.4	71.0±1.5
Crude protein	21.3±0.5	19.5±1.2	19.6±2.4	20.4±1.1
Crude lipid	5.00±0.14	4.96±0.76	5.33±0.66	5.64±0.47
Crude ash	2.90±0.05	3.08±0.28	2.78±0.45	2.67±0.24

Abbreviations: PC, No replacement of fish oil with tallow; TW, 100% fish oil replacement with tallow; EM01, 0.01% emulsifier addition to TW diet; EM02, 0.02% emulsifier addition to TW diet.

Values are means form triplicate groups of fish.

1.4. Discussion

The total replacement of FO by tallow led to a significant reduction in growth performance (Table 1-3). However, these reduced performances were recovered by the inclusion of 0.02% of an emulsifier in the diet containing tallow (EM02) instead of FO. The decreased growth performance associated with the TW diet was not restored by the EM01 diets, although the growth results were significantly higher than in the TW group. In line with this study, the total or partial replacement of FO with beef tallow was reported to reduce the growth performance and feed efficiency of pollock (*Salmo gairdneri*) (Takeuchi, Watanabe & Ogino, 1978; Bieber□Wlaschny & Pfeffer, 1987), channel catfish (*Ictalurus punctatus*) (Fracalossi & Lovell, 1994b) and coho salmon (*Oncorhynchus kisutch*) (Yu & Sinnhuber, 1981). Furthermore, Yu and Sinnhuber (1981) suggested that the growth performance of coho salmon fed a diet containing 8% of salmon oil and 8% tallow was similar to that of a diet containing 16% salmon oil, thereby reducing production costs. However, Lee et al. (2019) observed no significant difference in the growth performance of olive flounder diets containing tallow as an FO replacement. In the present study, the growth performance of fish was significantly reduced by the inclusion of dietary tallow compared to the PC diet. Therefore, the discrepancies between the results of the two aforementioned studies might be due to lower FI, differences in protein sources and their proportions, as well as water temperature. Additionally, Lee et al. (2019) incorporated 5% krill meal to test diets. Krill meal is an essential fatty acid (EFA)-rich source, which has been reported to improve the growth performance and FI of olive flounder (Tharaka et al., 2020). Therefore, krill meal might supply EFAs to compensate the fatty acid deficiencies in diets containing tallow while improving FI. In this study, the reduced growth parameters and feed utilization of juvenile olive flounder fed with tallow containing diets were restored by adding an emulsifier to the diets. The addition of monolaurin and an emulsifier was recommended to restore the growth performance of yellowtail fed with a diet containing hydrogenated soybean oil instead of FO (Bergman, Trushenski & Drawbridge, 2018). In contrast, Adhami et al. (2017) observed that the growth performance or feed utilization of rainbow trout was not affected by FO replacement with a fat powder when diets were supplemented with an emulsifier. The authors suggested

that an improved energy retention resulted from higher fat digestibility and absorption and therefore growth performance was not adversely affected. Lipase activity was not improved by emulsifier supplementation in the present study suggesting that lipid digestion was not accelerated due to increased lipase level. Therefore, compared to the PC and TW groups, the addition of an emulsifier might improve the emulsification of chyme and absorption of lipids, which might be further utilized as energy. A significant reduction in the feed intake of fish fed with the TW diet was observed in the present study. Feed palatability is a key driver of feed intake (Kasumyan, 2014). Smell-releasing agents in feeds such as amino acids in fish meal, water-soluble compounds dissolved in fish oil, are responsible for the feed searching behavior of fish (Gaber, 2010; Wei et al., 2019). Dietary deficiencies of EFAs also cause reduced feed intake (Glencross, 2009). Tallow lacks EPA and DHA. Therefore, to increase the intake of tallow-containing, an emulsifier additive must improve feed attraction by increasing the fat solubility. The survival of fish in the TW group was significantly lower than that of other dietary groups. Improved blood AST levels in the fish fed with the PC diet might also be related to their survival. A significantly higher AST value indicates that the TW group had been subjected to chronic stress conditions. Glencross (2009) reported that dietary deficiencies of EFAs can result in reduced feed intake, poor growth rates, mortality and other adverse effects. Accordingly, it might be assumed that the significantly lower survival of the TW group was due to a lack of EFAs and energy. The survivals of other groups were significantly increased compared to the TW group. The improved utilization of EFAs in fish meal supplemented with an emulsifier might have led to the increased survival of the EM groups. Overall, in the present study, fish growth performance and feed efficiency might be improved by dietary emulsifier in several ways such as improved digestion, absorption, lipid digestibility, EFA availability and feed intake.

VSI and HSI are important indicators of fat deposition around the viscera and liver of fish in response to ingestion, digestion and absorption of fats and carbohydrates (Grigorakis, 2015). In the present study, the lowest HSI and VSI values were observed in fish fed with the TW diet. Particularly, the HSI increased when diets contained more emulsifier. The liver size varies according to the nutritional

status and growth rate of fish, as they store excess energy in the liver and other tissues as glycogen and triacylglycerols (Chellappa et al., 1995). Therefore, the fat metabolism and nutrient absorption of fish might have been enhanced by the dietary emulsifier used in the present study, thereby increasing HSI and VSI. This assumption was further confirmed by the increased growth, feed utilization and diet digestibility of fish fed with emulsifier supplemented diets.

The TW diet exhibited a significantly lower dry matter and lipid digestibility than other diets (Table 5). Therefore, lipid digestibility and/or absorption were inhibited by the inclusion of tallow in olive flounder diets. Morais et al. (2005) identified that the melting point of fat significantly affected lipid absorption. According to Francis et al. (2007), the level of unsaturation, aliphatic chain length and the melting points of each fat source fatty acid alter the lipid digestibility of a given diet. Tallow is a saturated fat type, with a melting point ranging from 40 to 48 ℃ (Patterson, 2011) and contains a high amount of saturated fats and medium-chain fatty acids. Furthermore, in the present experimental conditions (average water temperature 22.1±1.4˚C) tallow is in solid form and FO is a liquid. Therefore, the reduced lipid digestibility of TW diets might be attributed to the differences in fatty acid types and the higher melting point of tallow. Dry matter digestibility was significantly higher in the EM01 diet than that of the TW diet. Additionally, both dry matter and the lipid digestibility of the EM01 group were higher than that of the EM02 group, although the differences were not significant. Adhami et al. (2016) observed a significant reduction in the digestibility of diets supplied with 2% and 4% of an emulsifier (Tween 80). Roy et al. (2010) observed a similar trend in a broiler chicken feeding trial by supplementing the diet with glyceryl polyethylene glycol ricinolate as an emulsifier. Heugten and Odle (2000) observed a quadratic relationship between increasing exogenous emulsifier levels and lipid digestibility in weaning pigs. Saunders and Sillery (1976) observed a reduction in free fatty acid absorption with diets containing lecithin as an emulsifier. They assumed that increases bile salt-lecithin micelle sizes limit the diffusion towards the absorptive cell surface. Further, the authors suggested that the persistence of bile salt-lecithin micelles might alter the partition to be absorbed via the absorptive cell surface. Therefore, it can be

inferred those higher levels of exogenous emulsifiers can reduce fatty acid digestion. However, diet digestibility was not reduced compared to the PC upon the addition of emulsifier up to a .02% level, indicating that these emulsifier levels are optimum to restore diet digestibility when FO is replaced with tallow in olive flounder diets.

Blood triglyceride content was significantly increased in fish fed with the TW diet. Peres et al. (2014) suggested that there was a relationship between the plasma triglycerides, cholesterol levels and FI of fish. Gao, Shi & Ai (2005) and Baoshan et al. (2019) observed a significant increase in blood triglyceride levels in fish when the level of wheat germ oil was increased in their diets at the expense of dietary FO. The authors suggested that these observations might have been related to the fat reserves of fish. Furthermore, Klingel et al. (2019) found that DHA reduces serum triglyceride levels promoting triglyceride clearance by activating lipoprotein lipase in humans. Therefore, triglyceride level of fish might be affected by FI, plasma cholesterol level, type of lipid and fatty acid composition of diet according to previous studies. FO is richer in DHA than tallow. In the present study, DHA availability might have been increased by the incorporation of an emulsifier. Accordingly, the blood triglyceride levels might be significantly decreased in fish fed with the EM02 diet compared to the TW group. In the contrast, we observed that the triglyceride levels in fish fed with the EM01 diet were not significantly different to those of the TW, FO or EM02 groups. Therefore, we can assume that blood triglyceride levels were correlated with the emulsifier dose. However, further studies are required to elucidate this phenomenon.

Myeloperoxidase (MPO), which is released from neutrophils and monocytes through a degranulation process, reacts with the hydrogen peroxide produced by the respiratory burst and makes a complex that destroying pathogenic bacteria (Klebanoff, 1999). In this study, MPO activity was reduced by FO replacement with TW, but emulsifier incorporation increased the MPO level. Ig and lysozyme showed the same pattern. FO is rich in EFA, which is important in oxidative radical scavenging (Winston & Giulio, 1991). Therefore, it can be assumed that the emulsifier employed in this study increased the

absorption of the EFA present in the sardine meal used to formulate the diets. Similarly, Sun et al. (2011) found no significant difference in non-specific immune parameters of black carp (*Mylopharyngodon piceus*) fed a diet containing rapeseed oil instead of FO. Replacement of FO with tallow coupled with an emulsifier is likely to benefit fish without any significant immunity impairments.

The liver fatty acid compositions reflected the composition of each diet (Table 4). Similar results were observed in previous studies when fish were fed with diets containing beef tallow (Pérez et al., 2014; Baoshan et al., 2019; Lee et al., 2019). EPA and DHA contents are different in tallow-added diets and FO- added diet. Bureau et al. (2008) attributed the low EFA composition in fish fed with diets containing tallow to the absence of EPA and DHA in tallow itself. In the present study, the liver EPA and DHA levels were drastically reduced in fish fed TW, EM01 and EM02 diets. In contrast, we observed considerable levels of liver EPA and DHA in fish fed TW, EM01 and EM02 diets. Fish meal also contains EPA and DHA. Therefore, sardine meal might have served as an EPA and DHA source for fish fed TW, EM01 and EM02 diets. Moreover, we observed a high level of C18:1 n-9 fatty acid (oleic acid) in the livers of fish fed with tallow-containing diets, which was similar to the fatty acid composition of the diets (Table 2). Importantly, oleic acid is more highly abundant in beef tallow than in FO (Lee et al., 2019).

The results of the lipase activity assays clearly demonstrated that the lipase activity of fish fed with the TW diet was low, although this result was not significant (Table 6). Lipid digestion is mainly influenced by bile salts and lipase. Bile salt and lipase are released into the intestine upon stimulation with cholecystokinin hormone. This hormone is released by intestinal cells when food reaches the small intestine in the form of chyme. Ledeboer et al. (1999) revealed that long-chain triglycerides emulsified by low emulsifier doses play a major role in stimulating the cholecystokinin release. Low blood-cholesterol levels also indicate the low bile activity in the intestine (Barth, 1983; Crespo and Esteve- Garcia, 2003). However, our results did not show any significant effects of tallow on lipase activity, although blood cholesterol concentration was lower in the TW group. Therefore, further studies should be undertaken to

elucidate the effects of tallow and emulsifiers on the lipase activity of olive flounder. The total replacement of dietary FO by tallow can lead to impairments in growth, feed utilization, digestibility, innate immunity and survival of olive flounder. The emulsifier supplementation appeared to counteract the adverse effects of replacing FO for tallow in formulated fish diets. Future studies should be conducted to elucidate the optimum inclusion level of emulsifier in olive flounder diet though the minimum level was 0.02% of emulsifier in the diet.

CHAPTER – II

Effects of dietary arachidonic acid supplementation in high plant protein diets on growth, feed utilization and immunity of olive flounder during suboptimal temperature season

2.1. Introduction

Arachidonic acid (ARA), eicosatetraenoic acid, is an omega 6 fatty acid that contains four cis double bonds which give a hairpin structure. These double bonds are the key to its physical properties and biochemical activities including interaction with oxygen and proteins (Rich, 1993; Brash, 2001; Hanna et al., 2018). ARA remains liquid at subzero temperatures due to its structure. Phospholipids including ARA helps to maintain proper biological membrane fluidity and flexibility in animals at suboptimal temperatures (Brash, 2001; Tocher, 2003; Parrish, 2013; Tallima and Ridi, 2018). The biological membrane is the reservoir to store ARA in the form of phospholipid and phospholipase A2, cyclooxygenase, lipoxygenases and cytochrome P450 enzymes stimulate the production of important inflammatory mediators such as prostanoids, lipoxins and leukotrienes from ARA (Smith, 1992; Brash, 2001; Davies, 2008). This fatty acid plays a key role in the activation of several signal transduction processes such as enhancing the activation of potassium channels that are essential for normal pathophysiological processes (Fink et al., 1998; Davies, 2008) and synaptic activation of glutamate receptors (Zerangue et al., 1995). Further, compared to freshwater and euryhaline fish, marine fish are unable to bio-convert α-linolenic and linoleic acids into essential fatty acids including EPA and DHA in adequate quantities due to lack of an enzyme in the conversion pathway (Ghioni et al. 1999; Bell and Sargent, 2003; Trushenski and Rombenso, 2020). Dietary deficiencies of essential fatty acids in marine fish species can lead to express deficiency signs, poor growth and mortality of juvenile

fish (Izquierdo, 1996; Sargent et al., 2003). It is important to give attention to the dietary ARA requirement because its importance as a precursor of eicosanoids and stimulate several signaling pathways in fish. Further, dietary inclusion of optimum level of ARA is a necessity for the fishes farmed in intensive culture systems and fishes feeding on a low fish meal diet to maintain the optimum growth and health (Bell and Sargent, 2003). Previous studies showed that intestinal inflammations and absorptive tissue damages were the detrimental effects from the utilization of low-fish meal diets in aquaculture. Wound healing and inflammation regulating properties of ARA could be a sustainable solution (Tallima and Ridi, 2018). Further, ARA-derived endocannabinoids are important for brain reward signaling and stress responses that can positively affect feed intake and stress tolerance in fish (Tallima and Ridi, 2018). Aguilar et al. (2012) observed dietary ARA could minimize the stress response Pacific white shrimp (*Litopenaeus vannamei*) cultured at high densities. Therefore, this study in this chapter was conducted to determine the effects of ARA supplementation in diets on growth performance, feed utilization and innate immunity for olive flounder.

2.2. Material And Methods

2.2.1. Experimental diets and design

Six low fish meal diets (300g/kg fish meal and plant protein blend) were formulated with graded levels of ARA to be isonitrogenous (495g/kg) and iso lipidic (137g/kg). ARA was purchased from Salus Natura Inc, Xi'an City, China. The control diet was formulated incorporating 30g/kg fish oil and 51g/kg soybean oil without ARA (ARA0.0). Five different levels of 40% ARA-enriched oil as 7.5g/kg, 15.0g/kg, 22.5g/kg, 30.0g/kg and 37.5g/kg were added replacing soybean oil to formulate five diets to contain 0.3, 0.6, 0.9, 1.2 and 1.5% ARA respectively. The diets were named ARA0.3, ARA0.6, ARA0.9, ARA1.2 and ARA1.5 according to their ARA level. The formulation of diets, proximate composition and fatty acid profiles are shown in tables 3-1 and 3-2. All the dry ingredients were mixed and extruded by a pelletizer machine in a suitable size for juvenile olive flounder.

Table 2.1. Dietary formulation and proximate composition of the experimental diets for olive flounder (*Paralichthys olivaceus*).

Ingredients	ARA0.0	ARA0.3	ARA0.6	ARA0.9	ARA1.2	ARA1.5
Fish meal[1]	30.00	30.00	30.00	30.00	30.00	30.00
Wheat gluten	5.10	5.10	5.10	5.10	5.10	5.10
Soybean meal	19.00	19.00	19.00	19.00	19.00	19.00
Corn gluten meal	10.00	10.00	10.00	10.00	10.00	10.00
Wheat flour	20.00	20.00	20.00	20.00	20.00	20.00
Fish oil[2]	3.00	3.00	3.00	3.00	3.00	3.00
Arachidonic acid (40%)[3]	0.00	0.75	1.50	2.25	3.00	3.75
Soybean oil	5.10	4.40	3.56	2.86	2.10	1.40
Mineral Mix[4]	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin Mix[5]	1.00	1.00	1.00	1.00	1.00	1.00
Starch	1.60	1.55	1.64	1.59	1.60	1.55
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50
L-Lysine	1.00	1.00	1.00	1.00	1.00	1.00
L-Methionine	0.20	0.20	0.20	0.20	0.20	0.20
Taurine	1.00	1.00	1.00	1.00	1.00	1.00
Mono-calcium phosphate	1.50	1.50	1.50	1.50	1.50	1.50
Proximate composition (% of dry matter)						
Moisture	9.32	9.62	10.8	11.2	12.0	11.7
Crude protein	49.6	49.5	49.5	49.0	49.1	48.6
Crude lipid	13.8	13.4	13.9	13.4	14.0	14.0
Crude ash	8.36	8.46	8.45	8.30	8.31	8.39
Arachidonic acid (g/kg identified fatty acids)	0.00	2.95	5.76	8.51	11.4	14.8

Abbreviations: ARA0.0, low fish meal diet without arachidonic acid; ARA0.3, ARA0.6, ARA0.9, ARA1.2 and ARA1.5, low fish meal diets added graded levels of arachidonic acid as 0.3, 0.6, 0.9, 1.2 and 1.5%.

[1]Blumar, Chile.

[2]Cod liver oil, Corp. E-wha oil & fat Ind, Korea.

[3]Arachidonic acid level is 410 g/kg of total fatty acids, Salus Natura Inc, Xi'an City, China.

[4]Mineral premix (g kg^{-1} of mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3.6H2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[5]Vitamin premix (g kg1 of mixture): L-ascorbic acid, 121.2; DL-a tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

Table 2.2. Fatty acids composition of the experimental diets for olive flounder (*Paralichthys olivaceus*).

Fatty acids	ARA0.0	ARA0.3	ARA0.6	ARA0.9	ARA1.2	ARA1.5
Fatty acids (% of fatty acid)						
C14:0	2.46	2.44	2.58	2.64	2.69	2.63
C16:0	17.5	17.5	16.7	17.4	16.8	16.3
C16:1	2.59	2.70	2.64	2.72	2.93	2.75
C18:0	4.78	6.49	4.83	6.25	5.72	5.91
C18:1n-9	23.2	19.5	22.4	19.0	18.7	18.5
C18:2n-6	33.3	31.5	28.8	27.2	24.4	22.6
C18:3n-3	4.21	3.88	3.40	3.35	3.25	2.81
C20:3	4.67	5.19	4.65	4.64	5.37	4.66
C20:4n-6	0.0	2.95	5.76	8.51	11.4	14.8
C20:5n-3 (EPA)	4.32	4.77	4.97	5.00	5.25	5.41
C22:6n-3 (DHA)	3.01	3.07	3.28	3.30	3.48	3.56
EPA+DHA	7.33	7.84	8.25	8.3	8.83	8.97

Abbreviations: ARA0.0, low fish meal diet without arachidonic acid; ARA0.3, ARA0.6, ARA0.9, ARA1.2 and ARA1.5, low fish meal diets added graded levels of arachidonic acid as 0.3, 0.6, 0.9, 1.2 and 1.5%.

2.2.2. Fish and feeding trial

The juvenile olive flounder were acclimatized to experimental conditions and facilities for two weeks feeding a control diet. At the end of acclimatization, a total of 432 fish (88.24 ± 0.06g) was randomly selected to stock 24 fish per tank into 118 polyvinyl circular tanks (215L) to be triplicated groups. All the tanks were supplied with a continuously filtered seawater flow (3 L min^{-1}) and an airflow. The average water temperature, salinity, dissolved oxygen, pH and ammonia levels were 15.2±1.2 ˚C, 41±1 ppt, 9.67±0.07 ppm, 8.2±0.3 and 0.063±0.007 ppm during the feeding trial. Fish were hand-fed with one of the diets to apparent satiation daily at 08:30h and 16:30 h for eight weeks. The remaining diets in tanks were collected 30 min after each feeding and weighed for the accurate feed intake calculation.

2.2.3. Sample collection and analysis

At the end of the feeding trial, all individual fish were counted and weighted. The condition factor (CF) was calculated using the individual body weight and length of each fish. Eight randomly selected fish from each tank were anesthetized with 2-phenoxyethanol (200mgl-1) and blood samples were taken from four fish with heparinized syringes. Nitroblue tetrazolium (NBT) activity was measured using whole blood according to Anderson et al. (1992). Plasma was separated by centrifugation at 5000 × g for 10 min and stored at -80 ˚C. Hemoglobin and blood biochemical parameters (plasma levels of glucose, total protein, total cholesterol, triglyceride, alanine aminotransferase/ALT and aspartate aminotransferase/AST levels) were analyzed by an automated blood analyzer. The microhematocrit technique was used to determine the hematocrit percentage (Brown et al., 1980) using the blood drawn using non-heparinized syringes. Serum was separated by allowing to clot at room temperature for 30 min and stored at -80 ˚C to determine non-specific immune parameters. Visceral and hepatic samples were collected for

histological analysis, liver proximate composition and fatty acid profile analysis and samples were weighed to calculate the viscerosomatic index (VSI) and hepatosomatic index (HSI). Lysozyme activity was determined by reaction against *Micrococcus lysodeikticus* bacteria and spectrophotometric analysis with 530 nm absorbance. Plasma immunoglobulin (Ig) levels (Siwicki & Anderson, 1993) and serum MPO activity (Quade & Roth, 1997) were also measured. SOD activity was determined by inhibitory reaction against WST-1 (Water Soluble Tetrazolium dye) following the instructions of the SOD kit (Sigma-Aldrich 19,160). Liver moisture and lipid proximate compositions were measured according to the Association of Official Analytical Chemistry (1995) and Kjeltec Analyzer Unit 2300 (FOSS, Sweden) respectively.

2.2.4. Lipase activity

Three fish were randomly selected after the feeding trial, middle intestine samples (2 cm) were incised and homogenized ten times of chilled distilled water. Homogenate was centrifuged at 15000 rpm for 30 min at 0 ˚C and the supernatant was used as crude enzyme assay. Lipase activity was assayed based on measurements of fatty acid release due to enzymatic hydrolysis of triglycerides in a stabilized standard emulsion of olive oil (Borlongan, 1990). Briefly, crude enzyme mixture (1 ml) was added to 1.5 ml of stabilized olive oil substrate in 1 ml of 0.1 M Tris–HCl buffer at pH 8.0 and incubated for 6 h at 37 ˚C. The reaction was stopped by adding 3 ml of 95% ethyl alcohol and titrated with 0.01 N NaOH using 0.9% (w/v) thymolphthalein in ethanol as the indicator.

2.2.5. Intestinal histology

The intestinal histology analysis was performed as previously discussed by Lee et al. (2017). Tissue sections of the anterior intestine (one cm after the pyloric caeca) were removed and fixed in 10% formalin. Tissue sections were dehydrated in graded ethanol solutions and fixed in

paraffin blocks. Sliced sections (5 μm thick) of blocks were taken with a rotary microtome (CUT 4055, MicroTec, Germany) and stained with hematoxylin and eosin (H&E). A light microscope (AX70 Olympus, Tokyo, Japan) was used with an image analyzer software (Image J 1.32j; National Institute of Health, USA) and a digital camera (DIXI Optics, Daejeon, Korea) to observe the samples. Comparisons were made based on the measurement of average villi lengths per intestinal section and data were presented as mean values in μm.

2.2.6. Statistical analysis

All the experimental diet groups were subjected to a complete randomized design. Data were analyzed by one-way ANOVA using the SPSS (version 18.0) program. When ANOVA identified differences among groups, the differences in mean values were compared using Duncan's multiple range test ($P \leq 0.05$). Data were provided as mean ± standard deviation (mean ± SD). Percentage data were arcsine transformed before all the statistical analyses.

Figure 2.1. Diet preparation, feeding trial, blood sampling and blood analysis by an automatic blood analyzer.

2.3. Results

The fish fed low fish meal diet containing 0.6% ARA showed significantly highest body weight compared to other treatments. Weight gain percentage and specific growth rate were significantly higher in the ARA0.6 group compared to ARA0.0, ARA1.2 and ARA1.5 groups. The feed conversion ratio and feed efficiency ratio were significantly improved in fish fed ARA0.6 diet. The hepatosomatic index was significantly higher in ARA0.0 diet fed fish compared to all the other diets.

The liver fatty acid composition of C18:2n-6 was significantly reduced according to increasing levels of ARA, C20:4n-6, and liver fatty acid level of ARA significantly increasing with the increasing dietary levels of it.

The higher blood glucose level observed in ARA0.0 level treatment. The liver AST level was significantly higher in ARA0.0 level diet compared to ARA0.6 and ARA1.2 treatment. A higher ALT level was observed in control diet compared to ARA0.3 and ARA0.9.

The nitroblue tetrazolium activity was significantly higher in the ARA0.6 compared to ARA0.0 and ARA1.2 and ARA 0.6 and ARA0.9 treatments showed significantly higher NBT levels compared to ARA0.0 level. The highest SOD level was observed in ARA0.9 treatment than ARA0.0 and ARA0.3 treatments. A significantly higher MPO level was observed in ARA0.9 diet fed fish than ARA0.0 and ARA1.2 diet fed fish. The immunoglobulin level was significantly increased in the fish fed the ARA0.3 diet fish fed compared to control treatment. The lysozyme activity was significantly improved in ARA0.3 diet fed compared to ARA0.0 and ARA1.5 levels. The antiprotease activity was increased with ARA0.6 and ARA0.9 diets compared to ARA0.0 diet.

The moisture content of liver fish fed ARA0.3 diet compared to ARA0.0 diet and ARA0.3 diet fed fish showed lower liver lipid composition compared to control diet fed fish.

Table 2.3. Growth performance and biometric indexes of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

	ARA0.0	ARA0.3	ARA0.6	ARA0.9	ARA1.2	ARA1.5
IBW[1]	88.19±0.10	88.21±0.07	88.21±0.03	88.33±0.07	88.22±0.08	88.25±0.03
FBW[2]	147.4±2.26^{c}	153.1±2.24^{bc}	160.1±3.70^{a}	154.5±6.40^{bc}	144.7±5.16^{c}	146.1±3.46^{c}
WG[3]	67±3.0^{b}	74±3.0^{ab}	81±4.0^{a}	75±7.0^{ab}	64±6.0^{b}	66±4.0^{b}
SGR[4]	0.82±0.02^{b}	0.90±0.02^{ab}	0.90±0.04^{a}	0.90±0.07^{ab}	0.80±0.06^{b}	0.80±0.04^{b}
FI[5]	81.7±7.7	83.6±6.96	81.88±5.58	87.56±5.44	79.19±6.10	86.38±5.59
FCR[6]	1.38±0.11^{ab}	1.28±0.07^{ab}	1.14±0.08^{b}	1.33±0.17^{ab}	1.14±0.13^{ab}	1.50±0.10^{a}
PER[7]	1.50±0.12^{ab}	1.58±0.09^{ab}	1.78±0.12^{a}	1.54±0.20^{ab}	1.45±0.11^{ab}	1.37±0.09^{b}
Survival	91.7±8.33	100±0	98.6±2.41	97.2±4.81	100±0	90.3±8.67
CF[8]	1.036±0.07	1.001±0.086	1.057±0.068	1.022±0.022	1.057±0.055	1.014±0.023
HSI[9]	2.37±0.09^{a}	2.09±0.04^{b}	2.11±0.06^{b}	2.05±0.08^{b}	1.99±0.09^{b}	2.02±0.07^{b}
VSI[10]	5.74±0.36	6.36±0.57	6.00±0.63	5.93±0.53	5.85±0.56	5.76±0.52

Abbreviations: ARA0.0, low fish meal diet without arachidonic acid; ARA0.3, ARA0.6, ARA0.9, ARA1.2 and ARA1.5 are low fish meal diets added graded levels of arachidonic acid as 0.3, 0.6, 0.9, 1.2 and 1.5%.

Values are means form triplicate groups of fish where the values in each row with the different superscripts are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]FInitial body weight (g); [2]Final body weight (g); [3]Weight gain (%); [4]Specific growth rate (%); [5]Feed intake (g/fish); [6]Feed conversion ratio; [7]Protein efficiency ratio; [8]Condition factor; [9]Hepatosomatic index (%); [10]Vicserosomatic index (%).

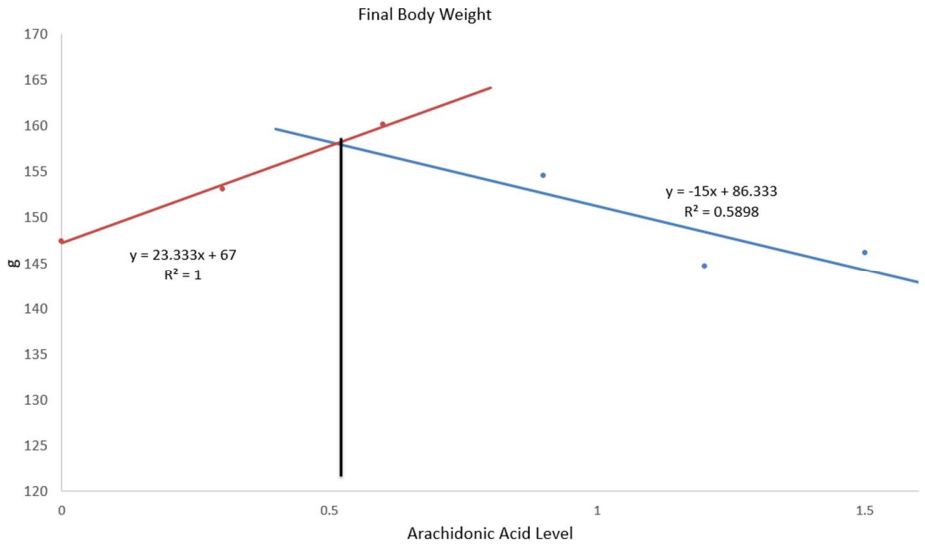


Figure 2.2. The optimum dietary arachidonic acid level for optimum body weight of olive flounder (*Paralichthys olivaceous*).

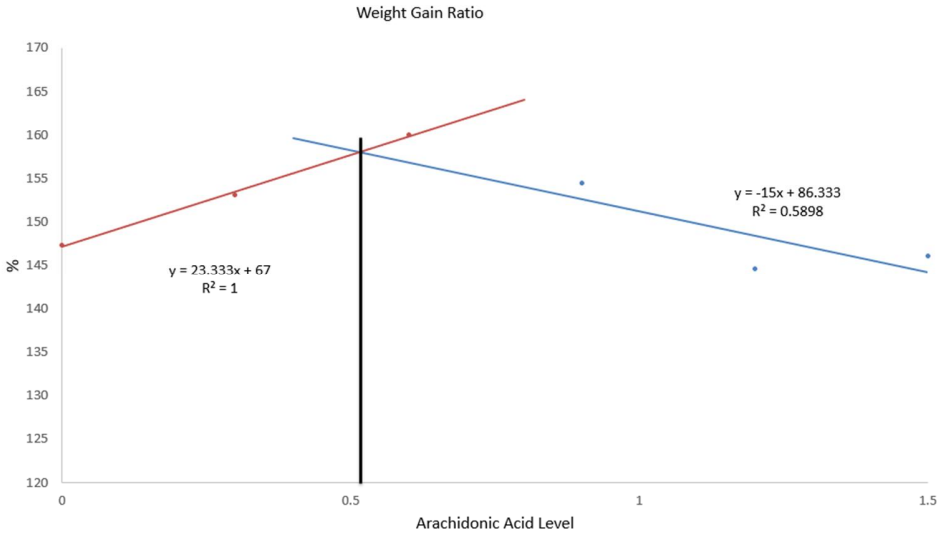


Figure 2.3. The optimum dietary arachidonic acid level for optimum weight gain ratio of olive flounder (*Paralichthys olivaceous*).

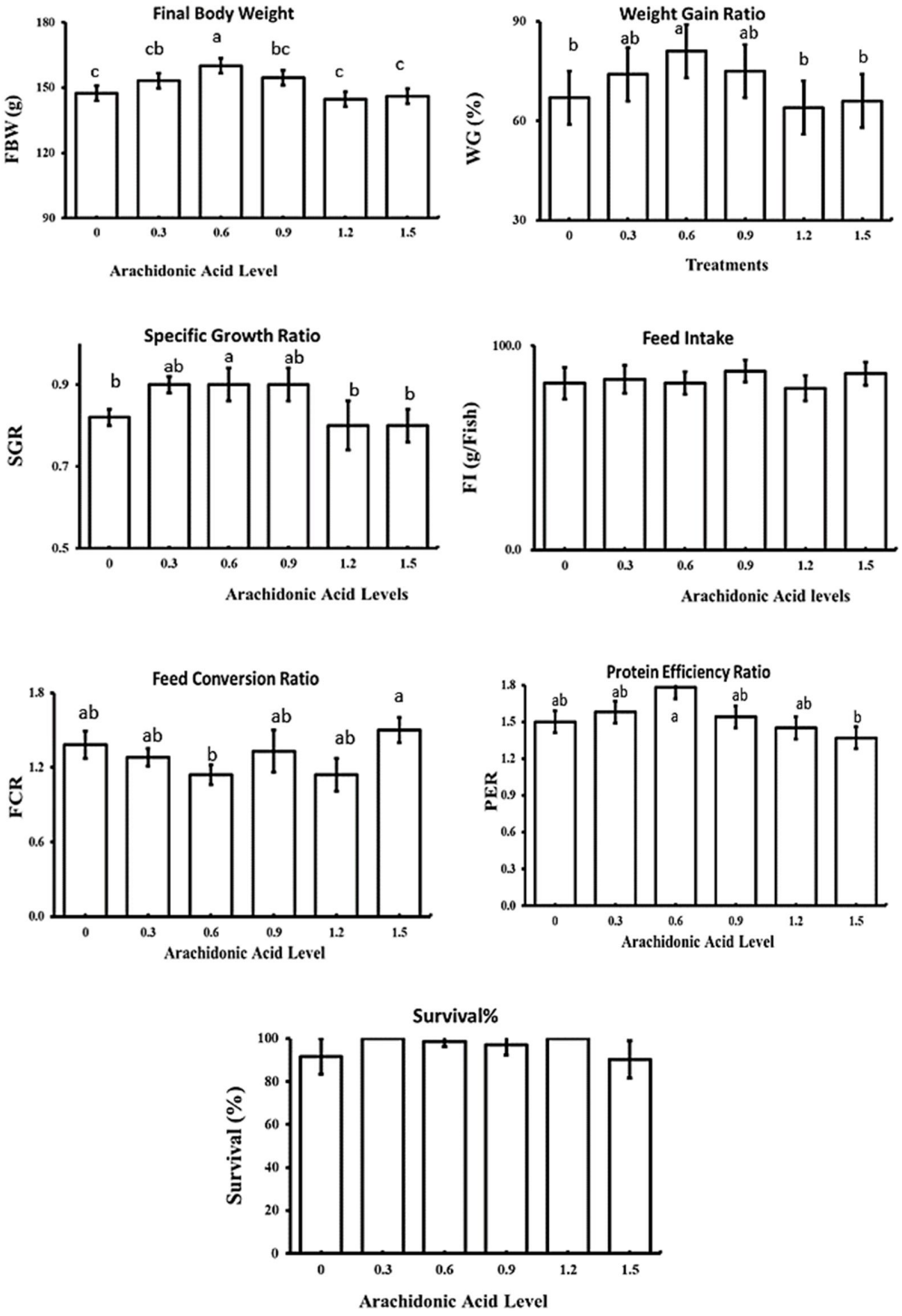


Figure 2.4. Growth performance of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

Table 2.4. Fatty acids composition of liver in olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

	ARA0.0	ARA0.3	ARA0.6	ARA0.9	ARA1.2	ARA1.5
Fatty acids (% of fatty acid)						
C14:0	2.64±0.23	2.69±0.36	2.45±0.18	2.29±0.16	2.55±0.43	2.23±0.37
C16:0	15.5±1.24	16.3±1.94	15.7±0.59	15.3±0.64	15.8±0.78	14.9±0.76
C16:1	3.67±0.36	3.47±0.33	3.29±0.35	3.24±0.18	3.32±0.52	3.08±0.27
C18:0	3.09±1.11	4.18±1.77	3.37±0.91	3.69±1.04	4.23±0.56	4.5±1.14
C18:1n-9	30.2±1.50	28.1±4.67	27.7±2.72	24.4±3.64	24.8±3.50	24.9±5.38
C18:2n-6	31.7±3.67^{a}	28. ±6.44^{ab}	28.7±1.12^{abc}	27.2±2.40^{abc}	23.4±2.41^{bc}	20.9±0.76^{c}
C18:3n-3	3.18±1.23	2.59±0.43	2.51±0.26	2.46±0.25	2.17±0.28	2.79±0.98
C20:2	1.83±0.21	1.88±0.25	1.48±0.51	1.41±0.36	1.12±0.17	1.55±0.38
C20:3	2.12±0.14	2.24±0.39	2.33±0.27	2.35±0.46	2.23±0.31	2.24±0.52
C20:4n-6	0.8±1.38^{e}	4.10±1.40^{de}	6.48±0.84^{cd}	11.2±1.55^{bc}	13.7±1.70^{ab}	16.2±3.15^{a}
Fatty acids (% of fatty acid)						
C20:5n-3 (EPA)	2.63±0.06	2.91±0.75	2.98±0.18	3.34±0.14	3.19±0.54	3.09±0.68
C22:6n-3 (DHA)	2.66±0.96	3.06±0.21	2.90±1.11	3.16±1.04	3.43±0.79	3.64±1.56
EPA+DHA	5.29±1.01	5.97±0.96	5.88±1.25	6.49±1.13	6.62±1.05	6.74±2.22

Abbreviations: ARA0.0, low fish meal diet without arachidonic acid; ARA0.3, ARA0.6, ARA0.9, ARA1.2 and ARA1.5, low fish meal diets added graded levels of arachidonic acid as 0.3, 0.6, 0.9, 1.2 and 1.5%.

Values are means form triplicate groups of fish where the values in each row with the different superscripts are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

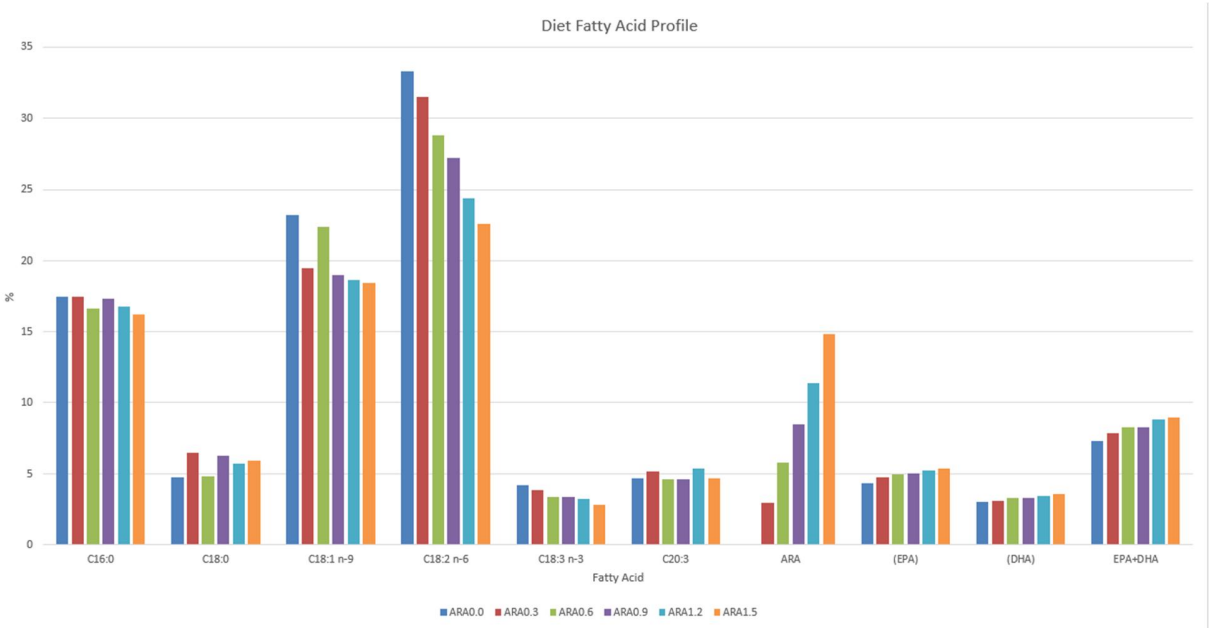


Figure 2.5. Fatty acids composition of the experimental diets for olive flounder (*Paralichthys olivaceus*).

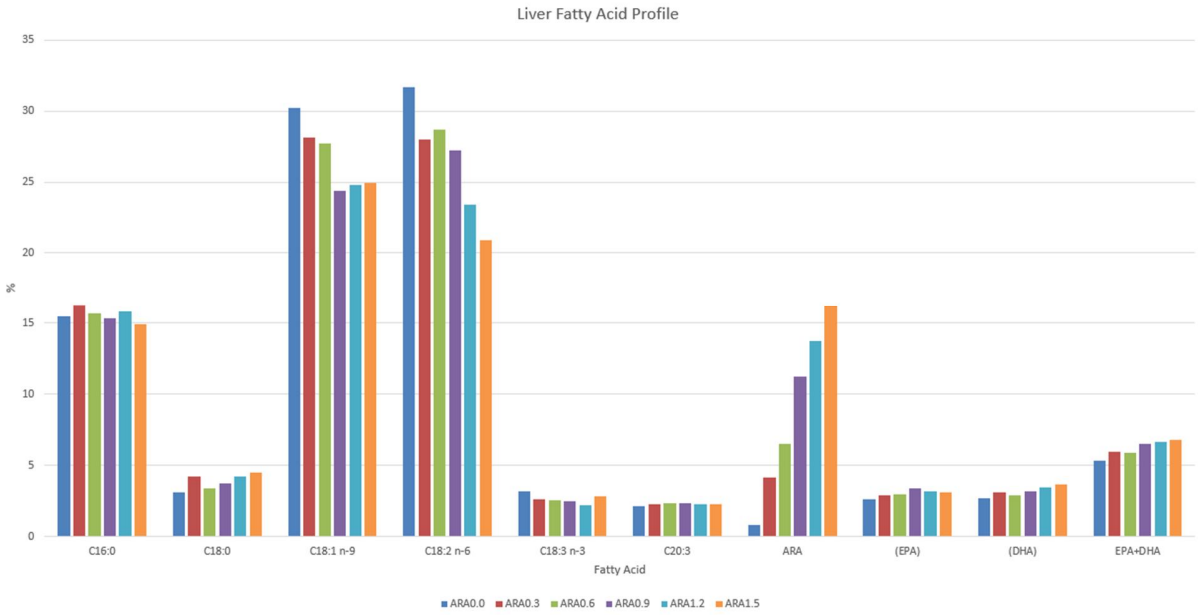


Figure 2.6. Fatty acids composition of liver in olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

Table 2.5. Lipase activity of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

	ARA0.0	ARA0.3	ARA0.6	ARA0.9	ARA1.2	ARA1.5
Lipase activity (units/mg protein)	22.75±1.89	22.58±1.88	24.50±2.29	23.42±0.63	22.00±1.30	24.00±2.38

Abbreviations: ARA0.0, low fish meal diet without arachidonic acid; ARA0.3, ARA0.6, ARA0.9, ARA1.2 and ARA1.5, low fish meal diets added graded levels of arachidonic acid as 0.3, 0.6, 0.9, 1.2 and 1.5%.

Values are means form triplicate groups of fish.

Table 2.6. Hematological parameter of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

Treatment	ARA0.0	ARA0.3	ARA0.6	ARA0.9	ARA1.2	ARA1.5
Hb[1]	5.08±0.6	4.97±0.5	5.91±0.7	6.06±1.4	5.23±1.8	5.19±1.7
Ht[2]	27.6±3.6	29.0±2.6	27.4±4.0	26.9±1.3	26.4±3.7	25.9±1.8
Glucose	37.1±2.4^{a}	28.3±1.7^{b}	27.5±3.9^{b}	29.8±3.7^{ab}	26.1±2.1^{b}	25.9±1.7^{b}
TP[3]	14.8±0.5	15.41±0.9	14.61±1.5	15.15±1.1	15.29±1.0	15.00±1.6
TG[4]	622±37.9	638±1.5	636±13.3	632±40.3	621±36.0	628±21.5
CHOL[5]	242±2.1	243.6±3.5	243.1±8.6	244.2±13.3	249.0±6.0	236.2±14.0
AST[6]	61.7±2.2^{a}	55.7±3.4^{ab}	50.3±3.0^{b}	56.1±2.0^{ab}	48.5±4.9^{b}	55.8±1.4^{ab}
ALT[7]	31.7±2.9^{a}	26.4±1.6^{b}	28.9±0.5^{ab}	27.3±1.5^{b}	29.4±0.6^{ab}	29.5±1.3^{ab}

Abbreviations: ARA0.0, low fish meal diet without arachidonic acid; ARA0.3, ARA0.6, ARA0.9, ARA1.2 and ARA1.5, low fish meal diets added graded levels of arachidonic acid as 0.3, 0.6, 0.9, 1.2 and 1.5%.

Values are means form triplicate groups of fish where the values in each row the different superscripts are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Hemoglobin (g/dL); [2]Hematocrit (%); [3]Total protein (g/dL); [4]Triglyceride (mg/dL); [5]Cholesterol (mg/dL). [6]Aspartate aminotransferase (U/L); [7]Alanine aminotransferase (U/L);

Table 2.7. Non-specific immune parameters of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

Treatment	ARA0.0	ARA0.3	ARA0.6	ARA0.9	ARA1.2	ARA1.5
NBT[1]	0.739±0^{c}	0.813±0^{abc}	0.885±0^{a}	0.842±0^{ab}	0.786±0^{bc}	0.814±0^{abc}
SOD[2]	59.7±3.3^{b}	63.8±2.0^{b}	66.7±4.7^{ab}	71.9±1.8^{a}	64.1±3.1^{ab}	66.4±0.3^{ab}
MPO[3]	2.71±0.0^{b}	2.78±0.1^{ab}	3.04±0.2^{ab}	3.23±0.2^{a}	2.72±0.1^{b}	2.84±0.3^{ab}
Ig[4]	40.0±3.2^{b}	50.0±4.2^{a}	44.0±2.7^{ab}	42.1±2.2^{ab}	44.3±3.4^{ab}	44.2±2.9^{ab}
Lysozyme[5]	22.0±1.2^{c}	29.1±1.1^{a}	28.8±1.7^{ab}	27.8±1.0^{ab}	28.2±2.8^{ab}	24.2±2.3^{bc}
Antiprotease[6]	8.6±1.0^{b}	10.3±0.8^{ab}	12.2±1.1^{a}	11.0±0.8^{a}	9.9±0.8^{ab}	10.0±0.7^{ab}

Abbreviations: ARA0.0, low fish meal diet without arachidonic acid; ARA0.3, ARA0.6, ARA0.9, ARA1.2 and ARA1.5, low fish meal diets added graded levels of arachidonic acid as 0.3, 0.6, 0.9, 1.2 and 1.5%.

Values are means form triplicate groups of fish where the values in each row the different superscripts are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

[1]Nitroblue tetrazolium activity (absorbance at 540); [2]Superoxide dismutase (units /mg protein); [3]Myeloperoxidase (absorbance at 450nm); [4]Immunoglobulin (mg/mL); [5]Lysozyme (μg/mL); [6]Antiprotease activity (% inhibition).

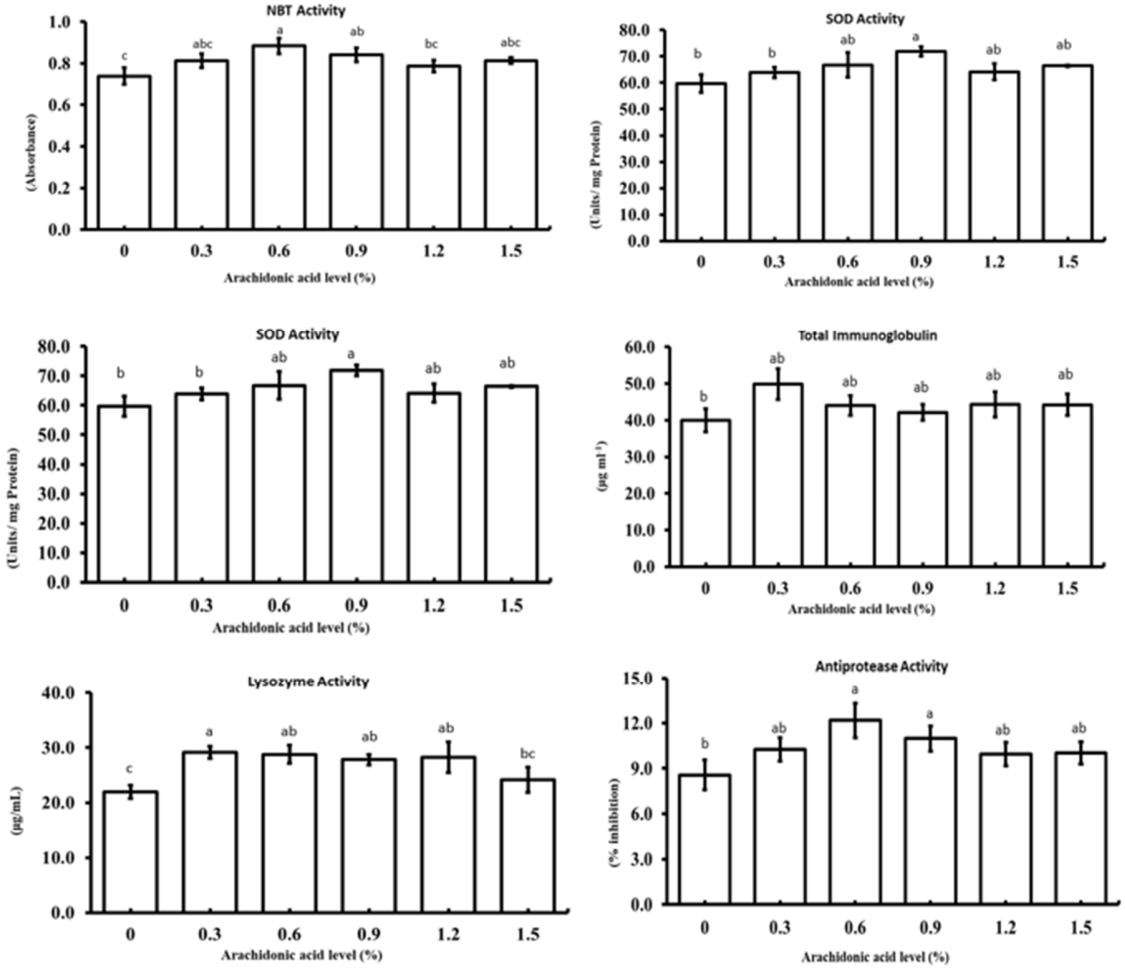


Figure 2.7. Non-specific immune parameters of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

Table 2.8. Liver composition (%) of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

Treatment	ARA0.0	ARA0.3	ARA0.6	ARA0.9	ARA1.2	ARA1.5
Moisture	61.2±0.6[b]	65.45±2.1[a]	62.43±1.7[ab]	62.20±3.6[ab]	63.27±1.5[ab]	64.45±2.1[ab]
Crude lipid	22.08±4.2[a]	17.03±1.4[b]	19.72±1.3[ab]	19.52±0.4[ab]	19.97±4.1[ab]	16.66±1.3[ab]

Abbreviations: ARA0.0, low fish meal diet without arachidonic acid; ARA0.3, ARA0.6, ARA0.9, ARA1.2 and ARA1.5, low fish meal diets added graded levels of arachidonic acid as 0.3, 0.6, 0.9, 1.2 and 1.5%.

Values are means form triplicate groups of fish where the values in each row the different superscripts are significantly different ($P<0.05$). The lack of superscript letter indicates no significant differences among treatments.

2.4. Discussion

The fish fed ARA 0.3, ARA0.6 and ARA0.9 diets showed comparatively higher performance of numerous parameters after the feeding trial. The replacement of dietary fishmeal with a high level of plant protein could inhibit growth performance and induce histopathological changes including detrimental inflammatory responses in the gut of turbot (Li et al., 2019) and Atlantic salmon (Egertonet al., 2020). The ARA-derived eicosanoids are known to protect tissues against inflammatory and autoimmune damages (Harizi et al., 2008). Further, ARA is important to develop cell membranes in tissue development at severe tissue damages. The growth of olive flounder was improved by dietary supplementation with 0.3-0.9% ARA in this study, indicating that extra dietary ARA is beneficial for maintaining normal growth performance in olive flounder. Our result also agrees with previous studies in juvenile yellow catfish, *Pelteobagrus fulvidraco* (Ma et al., 2018), gilthead seabream (Koven et al., 2001) and turbot, *Scophthalmus maximus* (Wei et al., 2020).

The replacement of soybean oil with ARA-rich oil has significantly reduced the C18:2 n-6 fatty acid composition both in diet and liver. Further, liver ARA content has significantly increased with the increasing levels of ARA. However, the same trend could identify in the fatty acid composition of the diet. Chee et al. (2020) observed a similar trend in Malabar red snapper (*Lutjanus malabaricus*) fed with diets that included graded levels of ARA.

The lipase activity of olive flounder was not significantly changed with the increasing levels of ARA in this study. Lipase is secreted from the pancreas and pancreatic juice and bile releasing is stimulated by the cholecystokinin hormone. Cholecystokinin secretion is stimulated when the lipid molecules contact the chemical receptors located in the intestinal epithelium. Blood cholesterol level is also an indicator of released bile level because bile contains cholesterol

and those cholesterol and bile acids are reabsorbed into the blood in the intestine. In this experiment, blood cholesterol or lipase activity was not significantly changed indicating that there was no significant impact on lipase secretion with the increasing levels of ARA.

The plasma levels of glucose, AST and ALT enzymes were significantly increased in the control fish group. This could be due to stress conditions induced by dietary inclusion of plant protein sources. ARA might have reduced the stress in olive flounder. ARA-derived endocannabinoids reduce stress and increase the brain reward signaling in the motivational process of animals (Tallima and Ridi, 2018). Moderate levels of ARA are known to reduce the triglyceride absorption in the intestine and reduced lipid deposition in the liver (Xu et al., 2018). Accordingly, this may reduce the liver damage and it might have reduced the AST and ALT enzyme production in this study.

It is reported that the antinutritional factors in plant protein sources can trigger oxidative stress in fish (Li et al., 2019). The NBT, SOD and MPO are vital parameters used to evaluate the antioxidant status in organisms (Li et al., 2019; Xu et al., 2010). In the present study, oxidative stress induced by the high level of dietary plant protein was observed in the CON group with reduced NBT, SOD and MPO activity. Dietary supplementation of 0.3-0.9% ARA significantly increased the antioxidant capacity in olive flounder in this study. The improvement of the antioxidant capacity and reduction of oxidative damage caused by dietary ARA has also been reported in Japanese seabass (Xu et al., 2010), juvenile oriental river prawn, *Macrobrachium nipponense* (Ding et al., 2018) and turbot (Wei et al., 2020).

Antiprotease, lysozyme and immunoglobulin play important roles in the immune responses of marine animals (Li et al., 2019). The lysozyme protects against microbial infections by degrading the β-1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine

in the peptidoglycan of bacterial cell walls (Zheng et al., 2017). Antiproteases are important enzymes that inhibit bacterial invasions and act against proteases secreted by bacteria that destroy host first line defense mechanisms (Bowden et al., 1997). Further, immunoglobulin plays a critical part in the immune response by specifically recognizing and binding to particular antigens, such as bacteria or viruses, aiding them to destroy. Increased levels of lysozyme, antiprotease and immunoglobulin indicated that 0.3-0.9% ARA supplementation could enhance the immune function in olive flounder.

In this study, we observed the positive effects of ARA on fish growth and immune system. Nevertheless, the role of ARA in the innate immune system makes it a crucial functional fatty acid in aquafeed. In fact, studies in mammals have found multiple physiological roles and potential health benefits of ARA (Pompeia et al., 2000; Tallima & Ridi, 2018).

In conclusion, the supplementation of 0.3-0.9% ARA improves growth, innate immunity and hematological parameters of olive flounder. The increasing levels of ARA content in diet significantly increase the liver ARA content. ARA supplementation could reduce the lipid deposition in the liver.

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