Effect of dietary linolenic acid (18:3*n*–3)/linoleic acid (18:2*n*–6) ratio on growth performance, tissue fatty acid profile and histological alterations in the liver of juvenile *Tor tambroides*

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Received: February 2013 Accepted: May 2013

Abstract

This study was conducted to determine optimal ratios of dietary linolenic acid (18:3*n*-3, LnA) to linoleic acid (18:2*n*-6, LA) for *Tor tambroides*. Juveniles were fed three trial diets with different ratios of LnA/LA (0.0, 0.5 and 1.0) for 10 weeks. Another diet contained 100% palm oil, which was similar tothe diet with 0.0 LnA/LA ratio but different in total amounts of C18polyunsaturated fatty acid, was also used as a control. At the end of the experiment, no significant difference in growth performance was observed among treatments. The overall fatty acid composition in muscle of *T. tambroides* fed experimental diets was similar in terms of saturated fatty acid and monounsaturated fatty acid. The muscle of fish fed diet with LnA/LA ratio of 0.0 contained significantly lower ($p<.05$) amount of overall *n*-3 PUFA than those fed the other diets. Intense accumulation of lipid in the liver parenchyma of all fish except for those fed control diet led to severe degeneration of hepatocytes indicating fatty liver. However, most of the hepatocytes of fish fed control diet were also swollen with nuclei migrated. *T. tambroides* fed diet with LnA/LA ratio of 0.0 showed degenerated enterocytes with an epithelium with disrupted edges. In conclusion, using vegetable oils contained high level of either LA or LnA in *T. tambroides* diet seemed to have no advantage over using palm oil contained high level of saturated fatty acid.

Keywords: Omega-3, Nutrition, Fatty acid, Malaysian mahseer, *Tor tambroides*

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[DOR: 20.1001.1.15622916.2014.13.1.16.6]

Introduction

Fish cannot synthesize *de novo* polyunsaturated fatty acids (PUFAs) due to lack of Δ12 and Δ15 desaturase enzymes, but requires obtaining these fatty acids through the diet (Tanet al., 2009). In fish nutrition, provision of adequate amounts of correct PUFAs is quite important in order to meet the requirements for normal growth and development as well as maintenance of cellular structure and function. Since almost all freshwater species studied so far could bio-convert linolenic acid (LnA, 18:3 *n*-3) to eicosapentaenoic acid (20:5 *n*-3) and docosahexaenoic acid (22:6 *n*-3), and could also bio-convert linoleic acid (LA, 18:2 *n*-6) to arachidonic acid (20:4 *n*-6), the PUFA requirements of freshwater teleosts may be generally met by LA and LnA (Sargentet al., 2002). These fatty acids, therefore, can be considered as true essential fatty acid in freshwater fish (Tanet al., 2009). Better understanding of essential fatty acid nutritional roles and interactions may lead to produce a more efficient fish oil deprived diet (Senadheera et al., 2010).

Although LnA and LA have been considered as the essential fatty acid for freshwater teleost, not both of these fatty acids are needed for all the freshwater fish. The species such as salmonids and whitefish require mainly *n*-3 PUFA while tilapia is one of the few fish require only LA (Sargent et al., 2002). Earlier studies have been shown that carp needs both LnA and LA fatty acids and its optimal growth is obtained with a diet containing 1% LnA and 1% LA (Henderson and Tocher, 1987). There is a permanent competition between the *n*-3 and *n*-6 PUFAs for using the elongase and desaturase enzymes (Jankowskaet al., 2010). Therefore, precise requirements of LnA and LA are determined not only by the absolute amounts of each fatty acid, but also by the optimal balance between them. The optimal LnA/LA ratio has recently been studied for some freshwater teleosts such as silver perch, *Bidyanus bidyanus* (Smithet al., 2004), yellow catfish, *Pelteobagrus fulvidraco* (Tanet al., 2009) and Murray cod, *Maccullochella peelii peelii* (Senadheeraet al., 2010). A diet rich in *n*-6 PUFA affects liver lipid deposition in some fish. It is shown that the increase of liver lipid deposition can be considered as a main indication of imbalance dietary *n*-3/*n*-6 ratio (Robainaet al., 1998). Fish oil substitution with vegetable oil usually lowers the percentage of *n*-3 PUFA in fish tissues. However, the rate of this reduction is highly influenced by the LnA/LA ratio in the diet (Senadheeraet al., 2010).

The Malaysian mahseer, *Tor tambroides* (Bleeker) is one of the most valued and sought after species of mahseer, *Tor* spp. (Ramezani-Fard et al., 2011a). The culture of this species has recently been initiated to meet the high demand for *T. tambroides* in Southeast Asia. Accordingly, efforts have been made to formulate a suitable diet, which can provide good health and optimal growth for fish as well as high quality fillet composition for human consumers. The aim of the present study was to evaluate the effect of different LnA to LA

[DOR: 20.1001.1.15622916.2014.13.1.16.6]

ratios on growth performance, body liver and muscle fatty acid profile of juvenile Malaysian mahseer.

Materials and methods

Three isonitrogenous and isolipidic diets containing 40% crude protein and 5-6% crude lipid (Ramezani-Fard et al., 2012a) were formulated to contain different LnA/LA ratios. Linseed oil as the primary source of LnA and sunflower oil as the primary source of LA were mixed with palm oil in different proximate composition, tissue histology, and ratio in order to produce the oil mixtures with different ratio of LnA/LA (0.0, 0.5 and 1.0). A diet contained 100% palm oil was also used as a control (CD). Diets CD and D0 were similar in LnA/LA ratio but different in total amounts of C_{18} PUFA. There were at least 1.1% *n*-3 long chain polyunsaturated fatty acid (*n*-3 LC-PUFA) residues derived from fishmeal in all the diets. Proximate and fatty acid compositions of the experimental diets are presented in Table 1.

Table 1: Feed ingredients and chemical and fatty acid compositions of the experimental diets

Ingredient (% diet)	Diet*			
	CD	**D0**	**D0.5**	**D1.0**
Fish meal (Malaysian)[a]	23.0	23.0	23.0	23.0
Soy meal	50.0	50.0	50.0	50.0
Corn meal	21	21	21	21
Palm oil	3.0	1.5	1.5	1.5
Sunflower oil	0.0	1.5	0.7	0.0
Linseed oil	0.0	0.0	0.8	1.5
Vitamin premix[b]	2.0	2.0	2.0	2.0
Mineral premix[c]	1.0	1.0	1.0	1.0
Proximate analysis (% as fed basis)				
Crude protein	38.6	38.8	39.2	38.1
Crude lipid	6.7	6.9	6.5	6.8
Ash	9.7	9.7	9.7	9.8
Carbohydrates[d]	33.5	33.4	33.6	33.6
Moisture	11.4	11.3	10.9	11.6
Gross energy (kJ g^{-1})	18.0	18.0	18.2	18.1
Fatty acid composition (% of total fatty acid)				
14:0	1.0	0.9	0.7	0.8
16:0	31.0	22.6	23.1	21.4

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Continue Table 1:

16:1*n*-7	0.7	0.7	0.8	0.6
18:0	4.9	5.0	4.8	4.3
18:1*n*-9	34.4	30.8	29.2	26.2
18:2*n*-6	24.6	36.0	26.5	23.6
18:3*n*-3	1.4	1.7	12.7	21.2
20:0	0.8	0.7	0.7	0.5
20:1*n*-9	0.4	0.4	0.3	0.3
20:5 *n*-3	0.4	0.4	0.6	0.3
22:1*n*-11	0.2	0.1	0.0	0.1
22:6*n*-3	0.7	0.8	0.7	0.8
∑ SFA	37.7	29.2	29.2	27.1
∑ MUFA	35.3	31.9	30.3	27.1
∑ *n*-3 PUFA	2.5	2.9	14.0	22.2
∑ *n*-6 PUFA	24.6	36.0	26.5	23.6
Total C_{18} PUFA	26	37.7	39.1	44.8
n-3/ *n*-6	0.1	0.1	0.5	0.9
LnA/LA[e]	0.06	0.04	0.47	0.90

[*] CD, control diet; D0, diet with LnA /LA ratio of 0; D0.5, diet with LnA/LA ratio of 0.5; D1.0, diet with LnA / LA ratio of 1.0;

[a] Malaysian fish meal (58% crude protein)

[b] Vitamin premix (g kg^{-1} premix): ascorbic acid, 45; myo-inositol, 5; choline chloride, 75; niacin, 4.5; riboflavin, 1; pyridoxine, 1; thiamin mononitrate, 0.92; Ca-pantothenate, 3; retinyl acetate, 0.6; cholecalciferol, 0.083; vitamin K menadione, 1.67; α-tocopheryl acetate (500 IU/g), 8; biotin, 0.02; folic acid, 0.09; vitamin B_{12}, 0.001; cellulose, 845.11

[c] Mineral premix (g kg^{-1} premix): KCL, 90; KI, 0.04; CaHPO4.2H_2O, 500; NaCl, 40; $CuSO_4$.5H_2O, 3; $ZnSO_4$.7H_2O, 4; $CoSO_4$, 0.02; $FeSO_4$ 7H_2O, 20; $MnSO_4$.H_2O, 3; $CaCo_3$, 215; MgOH, 124; Na_2SeO_3,0.03; NaF, 1

[d] Carbohydrates = Dry matter – [protein + lipid + ash]

[e] Linolenic acid/linoleic acid ratio

Domesticated wild caught juveniles *T. tambroides* were obtained from a local supplier and acclimatized to laboratory condition in aquaculture experimental station for two weeks. Fish were fed a practical diet contained 40% crude protein and 5% crude fat during acclimatization. A total of 120 juveniles (initial weight of 3.5 ± 0.4 g; mean ± S.D) were then randomly distributed into 12 rectangular shaped glass aquaria, filled with 65L dechlorinated public utility water and equipped with a biofilteration system (flow rate of approximately 3 L / min). Water quality was checked two times per week. Oxygen level

[DOR: 20.1001.1.15622916.2014.13.1.16.6]

was maintained above 7 mg/L. Temperature ranged from 27.8 to 29.3 °C and pH ranged 8.1 to 8.7. The ammonia ($NH3^{+}$) level was always below 0.01 mg/L and photoperiod was left at natural condition. Three replicate groups of fish were fed to visual satiety twice a day (0900 and 1600 h). The experiment was conducted for 10 weeks. Fish in each aquarium were batch-weighed at the start and end of the experiment as well as every two weeks.

At the end of the experiment, weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) were estimated using the following formulae. Six fish per treatment (two per replicate) were sacrificed, weighed, and stored at -80 °C for subsequent whole-body proximate analysis. Six more fish per treatment (two per replicate) were sacrificed, individually weighed, and dissected in order to determine the hepato-somatic index (HSI) and viscera-somatic index (VSI). The dissected fish were subsequently dressed and muscle from the area between the lateral and dorsal line was removed. The liver and muscle samples were immediately stored at -80 °C for further fatty acid analyses.

WG = Final Body Weight – Initial Body Weight

SGR = [(ln final mean weight – ln initial mean weight) / experimental days]×100

FCR = dry feed intake (g) / wet weight gain (g)

HSI = 100 × liver weight (g) / body weight (g)

VSI = 100 × visceral weight (g) / body weight (g)

Prior to biochemical analysis, the whole-body and fillet of fish were lyophilized in triplicate groups per sample for 48 h and the lost moisture was calculated. The crude protein of experimental diets and whole-body of fish samples were determined by the Kjeldahl method and the crude lipid were determined by ether extraction with the Soxtec system (Association of Official Analytical Chemists, AOAC, 1997). The ash content was determined by incinerating the dry samples at 600 °C for 4 h and the gross energy of diet determined by direct combustion in an adiabatic bomb calorimeter.

For fatty acid analysis, lipid were extracted from feed, liver, and lyophilized fillet with a chloroform:methanol (2:1 v:v) mixture (Folchet al., 1957). After a series of liquid / liquid phase separation, centrifugation, and evaporation under nitrogen the lipid fraction was methylated. Fatty acid methyl esters (FAMEs) were then injected into a gas chromatograph (Agilent 7890N) fitted with a fused silica capillary column (Supelco SP-2330: 30 m in length, 0.25 mm in diameter, and 0.20 µm in film thickness) and a flame ionization detector. Fatty acid profiles were identified by comparing the relative retention times with 37 components FA mix standards (Supelco, USA) and menhaden oil, and expressed as the area percentage of total fatty acids.

Three fish per treatment were also sacrificed and dissected and their visceral organs including liver were extracted. The extracted tissues were fixed in Bouin's solution at room temperature for 24 h, then washed and stored in 70% ethanol until the

[DOR: 20.1001.1.15622916.2014.13.1.16.6]

wax embedding (Ramezani-Fard et al., 2011b). Serial 5µm sections were prepared, stained with haematoxylin-eosin and slides were examined under a light microscopy (Zeiss Primo Star) fitted with a digital camera (Canon A640).

All the experimental data were reported in mean ± SE and subjected to a one-way ANOVA. The mean differences were evaluated using Duncan's Multiple Range test. Percentage data were arcsin transformed when necessary before being statistically analyzed. Statistical analysis was carried out using SPSS 15 for Windows (SPSS Inc., Chicago, IL) and the difference was considered significant at $p<.05$.

Results

No significant differences ($p>.05$) were observed in weight gain, SGR, FCR and HSI of fish fed different experimental diets at the end of the experiment (Table 2). The VSI values, however, were significantly decreased ($p<.05$) with the increase of dietary LnA/LA ratio.

Table 2: Growth performance and body indices of *T. tambroides* juvenile fed the experimental diet for 10 weeks

Parameters	Diet*			
	CD	D0	D0.5	D1.0
IW[1]	3.4 ± 0.2	3.4 ± 0.1	3.8 ± 0.2	3.6 ± 0.3
FW[2]	7.1 ± 0.3	6.8 ± 0.3	6.9 ± 0.1	7.0 ± 0.2
WG[3] (g)	5.2 ± 0.2	5.3 ± 0.5	5.5 ± 0.1	5.3 ± 0.5
WG (%)	109.8 ± 8.9	102.7 ± 5.8	81.4 ± 6.0	96.9 ± 9.9
SGR[4] (% day^{-1})	1.1 ± 0.1	1.0 ± 0.0	0.8 ± 0.1	1.0 ± 0.1
FCR[5]	1.8 ± 0.0	1.6 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
HSI[6]	2.0 ± 0.1	2.2 ± 0.0	2.2 ± 0.0	2.0 ± 0.1
VSI[7]	9.9 ± 0.3^{a}	8.8 ± 0.4^{ab}	8.1 ± 0.5^{b}	7.9 ± 0.5^{b}

Mean ± SE (n=3); Values within the same row with different superscript are significantly different at $p<.05$

* See Table 1 for diet abbreviations

[1] Initial body weight (g)

[2] Final body weight (g)

[3] Weight gain

[4] Specific growth rate

[5] Feed conversion ratio

[6] Hepato-somatic index

[7] Viscera-somatic index

[DOR: 20.1001.1.15622916.2014.13.1.16.6]

Table 3: Whole body proximate composition and muscle lipid content (% wet weight) of juveniles *T. tambroides* fed the test diets for 10 weeks.

	Diet*			
	CD	**D0**	**D0.5**	**D1.0**
Moisture	68.4 ± 0.3	68.3 ± 0.3	68.2 ± 0.5	68.4 ± 0.7
Protein	15.5 ± 0.1[a]	15.8 ± 0.[2ab]	14.5 ± 0.2[c]	15.9 ± 0.1[b]
Fat	12.5 ± 0.1[a]	11.5 ± 0.2[b]	13.4 ± 0.2[c]	11.7 ± 0.2[b]
Ash	3.6 ± 0.2	3.3 ± 0.1	3.6 ± 0.2	3.6 ± 0.1
Muscle fat	3.7 ± 0.2[a]	3.3 ± 0.2[b]	3.7 ± 0.1[a]	3.2 ± 0.1[b]

Mean ± SE; n=3; Values within the same row with different superscript are significantly different at p<.05.

* See Table 1 for diet abbreviations

Table 4: Fatty acid composition (% of total fatty acid) of muscle tissue of juveniles *T. tambroides* fed the test diets for 10 weeks.

Fatty acid	Diet*			
	CD	**D0**	**D0.5**	**D1.0**
14:0	2.9 ± 0.1	3.1 ± 0.1	2.9 ± 0.3	3.1 ± 0.1
16:0	29.3 ± 0.3	28.8 ± 0.5	28.1 ± 0.4	29.2 ± 0.2
16:1*n*-7	3.7 ± 0.2	3.6 ± 0.2	4.2 ± 0.3	4.3 ± 0.5
18:0	9.0 ± 0.3	8.3 ± 0.3	8.5 ± 0.5	8.5 ± 0.4
18:1*n*-9	31.6 ± 0.8	32.3 ± 1.1	30.5 ± 0.6	32.2 ± 0.3
18:2*n*-6	10.8 ± 0.4[a]	13.7 ± 0.4[b]	13.0 ± 0.3[b]	11.1 ± 0.4[a]
18:3*n*-3	1.4 ± 0.2[a]	1.1 ± 0.1[a]	2.5 ± 0.0[b]	3.5 ± 0.1[c]
20:1*n*-9	1.3 ± 0.0	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
22:0	1.4 ± 0.0[a]	1.4 ± 0.2[a]	1.2 ± 0.1[ab]	0.8 ± 0.1[b]
22:1*n*-11	2.3 ± 0.3	2.1 ± 0.2	2.3 ± 0.2	1.4 ± 0.1
20:5*n*-3	1.0 ± 0.0[a]	0.6 ± 0.1[b]	1.0 ± 0.0[a]	0.9 ± 0.0[a]

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Continue Table 4:

22:5*n*-3	1.1 ± 0.1[a]	0.5 ± 0.1[b]	0.6 ± 0.0[b]	0.6 ± 0.0[b]
22:6*n*-3	4.2 ± 0.3	3.2 ± 0.3	3.7 ± 0.0	2.9 ± 0.4
∑ SFA	42.6 ± 0.2	41.6 ± 0.4	40.7 ± 0.4	41.6 ± 0.4
∑ MUFA	39.0 ± 0.3	39.3 ± 0.6	38.4 ± 0.5	39.3 ± 0.7
∑ *n*-3 PUFA	7.7 ± 0.3[a]	5.3 ± 0.6[b]	7.9 ± 0.1[a]	8.0 ± 0.5[a]
∑ *n*-3 LC-PUFA	6.3 ± 0.3[a]	4.2 ± 0.5[b]	5.4 ± 0.0[ab]	4.4 ± 0.5[b]
∑ *n*-6 PUFA	10.8 ± 0.4[a]	13.7 ± 0.4[b]	13.0 ± 0.3[b]	11.1 ± 0.4[a]
n-3/*n*-6	0.7 ± 0.0[a]	0.4 ± 0.0[c]	0.6 ± 0.0[b]	0.7 ± 0.0[a]
LnA/LA[a]	0.12 ± 0.02[a]	0.08 ± 0.01[b]	0.19 ± 0.01[c]	0.32 ± 0.01[d]

Mean ± SE; *n*=3; Values within the same row with different superscript are significantly different at $p<.05$; * See Table 1 for diet abbreviations

[a] Linolenic acid/Linoleic acid ratio

The dietary LnA / LA ratios significantly affected ($p<.05$) *T. tambroides* whole body protein and fat contents (Table 3). The whole body fat content significantly decreased ($p<.05$) with the increase of dietary LnA / LA ratio except for fish fed diet D0.5 those contained the highest whole body fat percentage. The lowest level of whole body protein content was also observed among fish fed diet D0.5. The dietary levels of LnA / LA ratio also significantly affected *T. tambroides* muscle fat content. The muscle fat content, similar to whole-body fat content, significantly decreased ($p<.05$) with the increase of dietary LnA/LA ratio except for fish fed diet D0.5.

At the end of the experiment, the overall fatty acid compositions of muscle in *T. tambroides* fed all the test diets were similar in terms of saturated fatty acid and MUFA. The muscle of fish fed a diet with LnA / LA ratio of zero (Table 4) contained significantly lower ($p<.05$) amount of overall *n*-3 PUFA (5.3%) compared to those fed diets CD, D0.5 and D1.0 (7.7, 7.9 and 8.0%, respectively). Within the *n*-3 PUFA class, the content of LnA clearly reflected that of diets and significantly increased ($p<.05$) with the increase of dietary LnA / LA ratio. However, the range of these changes in the muscle lipid was noticeably narrower than that found in the dietary lipids. The LnA concentration varied between 1.4 and 21.2% in the diet while it varied between 1.1 and 3.5% in the muscle of fish. The highest ($p<.05$) overall *n*-3 LC-PUFA content was observed in the muscle of fish fed control diet. However, there was no significant difference ($p>.05$) between the muscle *n*-3 LC-PUFA content of fish fed the other diets. The muscle 20:5 *n*-3 content was also significantly lower ($p<.05$) in fish on diet D0 than those fed the other diets. The muscle *n*-3/*n*-6 ratio significantly increased ($p<.05$) with the increase of dietary

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LnA/LA. However, there were no significant differences ($p>.05$) between the muscle *n*-3/*n*-6 ratios of fish fed control diet and those on diet D1.0. The muscle LnA/LA ratios simply reflected this ratio in the diets.

Similar to the muscle, the overall saturated fatty acid and MUFA contents of *T. tambroides* liver were not significantly different ($p>.05$) at the end of the experiment (Table 5). However, within the MUFA class, liver 20:1 *n*-9 and 22:1 *n*-11 levels significantly decreased ($p<.05$) with the increase of dietary LnA/LA ratios except for the 22:1 *n*-11 of fish on diet D1.0. The liver *n*-3 PUFA concentrations varied from 1.7% in fish on diet D0 to 6.0% in those fed diet D1.0. The concentrations were greatly enhanced by increasing dietary LnA/LA ratio. However, there was no significant difference ($p>.05$) between fish on control diet and those on a D0.5. Within the *n*-3 PUFA class, the content of LnA noticeably reflected the LnA content of diets. The liver LnA / LA ratio also increased significantly ($p<.05$) with the increase of dietary LnA/LA ratio. However, the ranges of LnA concentration were clearly narrower in the livers (0 – 2.1) than the diets (1.4 – 21.2). The highest liver 20:5 *n*-3 and 22:6 *n*-3 concentrations observed in fish fed diet D1.0, followed by those fed control diet. The lowest liver contents of these fatty acids were observed among fish on diets D0 and D0.5.

Liver parenchyma of *T. tambroides* fed control diet is shown in Fig. 1. Most of the hepatic cells were swollen, with a roundish polygonal cell body containing a clear spherical nucleus. A number of hepatocytes with nuclei migrated due to lipid accumulation were also observed. Lipid accumulation led to the degeneration in few of the hepatocytes.

Table 5: Fatty acid composition (% of total fatty acid) of liver tissue of juveniles *T. tambroides* fed the test diets for 10 weeks.

Fatty acid	Diet*			
	CD	D0	D0.5	D1.0
14:0	3.4 ± 0.2[a]	4.2 ± 0.1[b]	3.4 ± 0.0[a]	3.6 ± 0.1[a]
16:0	27.9 ± 0.9	28.9 ± 0.3	28.6 ± 0.6	27.7 ± 0.7
16:1*n*-7	4.7 ± 0.3	4.2 ± 0.1	4.8 ± 0.0	3.9 ± 0.3
18:0	11.2 ± 0.6[a]	8.9 ± 0.5[b]	9.5 ± 0.2[b]	9.4 ± 0.5[b]
18:1*n*-9	34.5 ± 1.3	37.7 ± 0.9	39.2 ± 1.1	38.7 ± 1.4
18:2*n*-6	7.3 ± 0.1[ab]	8.0 ± 0.4[a]	8.5 ± 0.6[a]	6.5 ± 0.3[b]
18:3*n*-3	0	0	1.4 ± 0.1[a]	2.1 ± 0.0[b]
20:1*n*-9	1.8 ± 0.1[a]	1.6 ± 0.2[ab]	1.5 ± 0.1[b]	1.2 ± 0.1[c]

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Continue Table 5:

20:4*n*-6	2.0 ± 0.2[a]	1.3 ± 0.1[b]	1.0 ± 0.1[b]	0.9 ± 0.2[b]
22:0	1.5 ± 0.1[a]	1.3 ± 0.1[a]	0.6 ± 0.0[b]	1.0 ± 0.1[c]
22:1*n*-11	2.4 ± 0.2[a]	2.1 ± 0.1[a]	0.7 ± 0.0[b]	1.2 ± 0.1[c]
20:5*n*-3	0.9 ± 0.1[a]	0.5 ± 0.0[b]	0.6 ± 0.0[b]	1.2 ± 0.0[c]
22:6*n*-3	2.3 ± 0.1[b]	1.3 ± 0.2[a]	1.3 ± 0.1[a]	2.8 ± 0.1[c]
∑ SFA	44.1 ± 1.5	43.3 ± 0.3	41.6 ± 0.8	41.7 ± 1.2
∑ MUFA	43.3 ± 1.5	45.7 ± 0.7	45.7 ± 1.2	45.0 ± 1.7
∑ *n*-3 PUFA	3.3 ± 0.1[a]	1.7 ± 0.2[b]	3.3 ± 0.1[a]	6.0 ± 0.1[c]
∑ *n*-3 LC-PUFA	3.2 ± 0.1[a]	1.7 ± 0.2[b]	1.9 ± 0.1[b]	3.9 ± 0.1[c]
∑ *n*-6 PUFA	9.4 ± 0.1[a]	9.3 ± 0.6[a]	9.4 ± 0.6[a]	7.4 ± 0.4[b]
n-3/ *n*-6	0.4 ± 0.0[a]	0.2 ± 0.0[b]	0.4 ± 0.0[a]	0.8 ± 0.0[c]
LnA/LA[a]	0	0	0.16 ± 0.00[a]	0.32 ± 0.01[b]

Mean ± SE; *n*=3; Values within the same row with different superscript are significantly different at $p<.05$; *

See Table 1 for diet abbreviations

[a] Linolenic acid / Linoleic acid ratio

Intense accumulation of lipid in the liver parenchyma of fish fed a diet with LnA/LA ratio of zero led to severe degeneration of hepatocytes indicating fatty liver (steatosis). Degenerated hepatocytes showed intense cell swelling as well as cytoplasmic clearing (Fig. 2). Similar characteristics were also observed for fish fed diets D0.5 and D1.0. A section of normal intestine of *T. tambroides* on control diet is shown in Fig. 3. The intestinal wall consisted of mucosa, lamina propria, muscularis and serosa. The folds were deep with smooth edges. Similar characteristics were observed among fish fed diets D0.5 and D1.0. However, *T. tambroides* on diet D0 showed accumulation of free lipid droplets in the enterocytes cytoplasm, which led to degenerated enterocytes and the epithelium with disrupted edges (Fig. 4).

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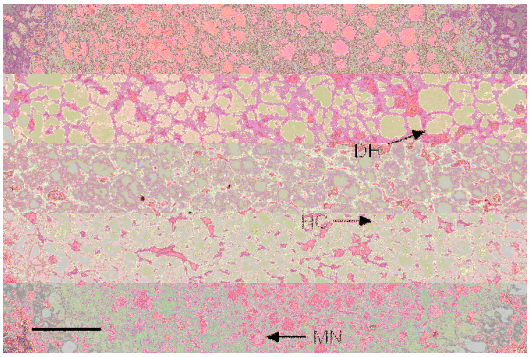

Figure1: Histological appearance of the liver of fish fed control diet showed mild lipid accumulation after 10 weeks. scale bar = 50µm; HC, swollen hepatocytes; MN, hepatocytes with migration of nuclei; DH, degenerated hepatocytes

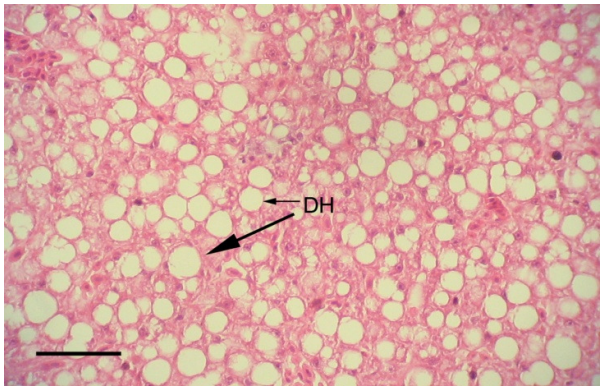

Figure 2: Histological appearance of fatty liver of fish fed diet with LnA/LA ratio of 0 showed an intense degeneration of hepatocytes (DH). scale bar = 50µm

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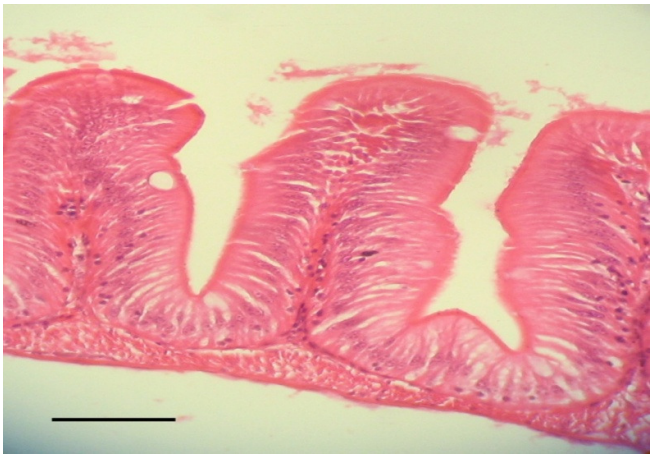

Figure 3: Normal intestine wall in fish on control diet. scale bar = 50µm

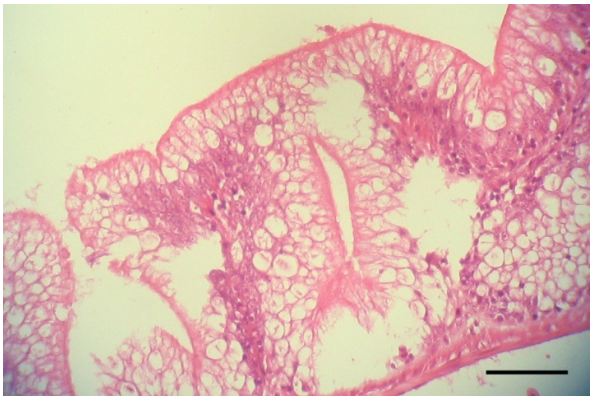

Figure 4: Lipid vacuoles in the enterocytes of T. tambroides on diet with LnA / LA ratio of 0. scale bar = 50µm.

Discussion

Our earlier studies have shown that a diet containing either 50% or 100% linseed oil with an *n*-3/*n*-6 ratio of above 1.0, compared to a diet containing 100% palm oil as the oil source, do not improve the growth performance of *T. tambroides* (Kamarudin et al., 2012). However, the effects of *n*-3/*n*-6 ratios lower than 1.0 is rather confusing. This experiment was designed to investigate the effects of the dietary *n*-3/*n*-6 ratio lower than 1.0, specifically the underlying effect of LnA and LA, on the growth performance, tissue fatty acid profile and histology of Malaysian mahseer. The results revealed that increasing dietary LnA/LA ratio up to 1.0 did not improve the growth performance of *T. tambroides*. However, the best growth performance of juvenile yellow catfish, *Pelteobragus fulvidraco*, is achieved at dietary LnA/LA ratio of 1.17, while decreasing the ratio to 0.35 severely decreases the weight gain and SGR (Tanet al., 2009). In contrast, Senadheera et al.

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(2010) observed no significant difference between growth performances of Murray cod fed different LnA / LA ratios from 0.3 to 2.9 after a 74-days experimental period. Good growth performances of fish fed different dietary LnA/LA levels in this study suggested that either *n*-3 PUFA is not an essential fatty acid for growth performance of *T. tambroides* or the essential *n*-3 PUFA can be met by the residual LC-PUFA from fishmeal. Both control diet and D0 were similar in LnA/LA and *n*-3/*n*-6 ratios and different in the amounts of LA. The lack of significant difference between fish fed these two diets confirmed that excess amount of LA from sunflower oil cannot improve the growth performance of *T. tambroides*.

The higher VSI value in fish fed low dietary LnA / LA ratio suggesting larger size of the epididymal fat in these fish than the other experimental fish. Reduction of whole body fat content with the increase of LnA / LA ratio may support this notion. However, the increased whole body fat content in fish fed diet D0.5 was also associated with the increase of fat content of muscle in this group of fish. Fluctuation in muscle fat content indicated that lipid deposition in muscle of *T. tambroides* was influenced by dietary LnA / LA ratio. Similar to mahseer, lipid deposition in muscle of yellow catfishis also influenced by dietary *n*-3/*n*-6 ratio (Tanet al., 2009). However, there is no direct relationship between muscle fat content and dietary *n*-3/*n*-6 ratio in tench, *Tinca tinca* (Turchiniet al., 2007).

The LnA and LA contents of fish tissues directly reflected those amounts in the diets. Increased levels of tissue LnA concentration were associated with the increase of LnA / LA ratio in the diet. However, the C_{18} PUFA levels were clearly lower in all fish tissues than the diets, indicating this PUFA class tended to be more catabolised or bio-converted by *T. tambroides*. Selective depletion of LnA in the tissue has been observed in other freshwater species such as yellow catfish (Tanet al., 2009) and Murray cod (Senadheera et al., 2010). However, the LA can be deposited in the tissues of fish fed a high LA diet (Trushenskiet al., 2008; Senadheeraet al., 2010). The depletion or deposition of a specific C_{18} PUFA appeared to be species and/or diet dependant characteristics. Franciset al. (2009) found that Δ-6 desaturase activity of fish fed high amount of C_{18} PUFA have a greater tendency to act on LnA rather than LA, while a reduction of dietary C_{18} PUFA shifted the substrate preference of Δ-6 desaturase from LnA to LA. Accordingly, *T. tambroides* fed the diet with highest LnA / LA ratio (1.0) and highest amounts of C_{18} PUFA had a significant trend to the increase of liver *n*-3 LC-PUFA percentage, which may show active elongation and desaturation of LnA to longer chain *n*-3 LC-PUFA in these fish. Lack of similar *n*-3 LC-PUFA elevation in the muscle was probably due to low concentrations of very low-density lipoproteins (VLDL), a major vehicle complex for transporting lipids from the liver to the other tissues (Sheridan, 1988; Nantonet al., 2001; Martinset al., 2007). Low

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concentration of VLDL could consequently caused accumulation and deposition of the *n*-3 LC-PUFA in the liver of fish fed a diet with LnA / LA ratio of 1.0.

Total *n*-3 PUFA content of muscle increased with the increase of LnA / LA ratio in the diet. However, such elevation was a direct reflection of increased LnA content in the diet. Interestingly, muscle total *n*-3 LC-PUFA content was significantly higher in fish on the control diet with a LnA / LA ratio of 0.06 than the other diets. Control diet had a total saturated fatty acids and MUFA amounts of 73% (of total fatty acid) while in the other diets, there were a total of 54.2 - 61.1% of saturated fatty acids and MUFAs classes. Adequate amounts of dietary saturated fatty acid and MUFA have been recommended to spare and maintain the level of *n*-3 PUFAs in the tissues of freshwater fish (Mishra and Samantaray, 2004). This recommendation has been confirmed in *T. tambroides* by our earlier study (Ramezani-Fard et al., 2012b). Therefore, a balanced saturated fatty acid to *n*-3 LC-PUFA ratio in the diet of *T. tambroides* seems to be more important for optimizing the tissue fatty acid profiles of fish than the *n*-3/*n*-6 ratio.

Within the hepatocytes, free fatty acids may be either oxidized for energy production or converted to phospholipids and cholesterol esters. Remaining fatty acids are then esterified to triglycerides, forming very low density or low-density lipoproteins and are released into the plasma to be transferred to the other tissues. The presence of excessive lipid within the liver is termed as fatty liver or steatosis and it occurs when the rate of triglyceride accumulation inside the hepatocytes is more than the rate of fatty acid catabolism or fatty acid releasing into plasma (McGavinet al., 2000). In this experiment, fish fed control diet showed a moderate fatty liver while fish on the other diets had intense steatosis. Similarly, gilthead seabream, *Sparus aurata* showsslight to intense levels of lipid accumulation and steatosis when 70% of fish oil in its diet is substituted with palm oil, rapeseed oil or soy oil (Fountoulakiet al., 2009). An insufficient amount of dietary essential fatty acid increases the rate of *de novo* fatty acid synthesis as well as reduces the rate of fatty acid removal from the liver into the circulation (Farkas et al., 1978). The higher rate of lipid synthesis in the liver together with the lower amount of fatty acid removal to blood stream increases the rate of lipid accumulation in the liver. However, intense lipid accumulation in the liver of fish fed diet with LnA/LA ratio of 1.0 (with high amounts of *n*-3 PUFA) may be associated with the low concentrations of VLDL as described above.

The pathogenic effects of steatosis are not well understood yet. Many researchers believe that fatty liver condition is a physiological state due to an imbalanced diet and it is reversible through a balanced diet (Segner and Witt, 1990; Caballeroet al., 2004). However, some researchers emphasized the pathological characteristics of steatosis and cautioned that, this hepatocyte disturbance would lead to liver necrosis in the long-term if the diet is not corrected (Storch et al., 1984; Mosconi-Bac, 1990). Further long-term studies are needed to confirm whether this notion is also applicable for *T. tambroides*. As a

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conclusion, this study showed that use of vegetable oils with high levels of either LA or LnA in *T. tambroides* diet have no advantage over using palm oil as the dietary oil source. However, the presence of dietary fishmeal with residual fish oil should be noted.

Acknowledgement

This study was supported through a Malaysian government E-Science grant no. 05-01-04-SF0209.

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