Essential Fatty Acids of Grass Carp *Ctenopharyngodon idella*^{*1}Toshio Takeuchi,^{*2} Kyomi Watanabe,^{*2} Wen-Yue Yong,^{*2,3}
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Two feeding experiments were conducted to detect any essential fatty acids (EFA) deficiency and clarify EFA requirement of grass carp *Ctenopharyngodon idella* by feeding diets containing various fatty acids.

The fish fed diet without EFA (fat-free and 12:0 alone diets) showed high percentages of vertebral column curvature (VCC) which exhibited an upward displacement of the column at the region of the 12-15th vertebrae from the caudal end, indicating lordosis. At a 1% level of dietary n-6 or n-3 fatty acids, the diet containing 18:3n-3 performed better than that containing 18:2n-6. However, a higher percentage of the VCC was still observed in the fish fed the 1% 18:3n-3 diet, suggesting that 18:3n-3 in spite of its positive role on growth, could not prevent the occurrence of VCC. On the other hand, the addition of 1% 18:2n-6 to the diet completely prevented the appearance of the VCC. The highest weight gain and feed efficiency were observed in the fish fed the diet containing pollock liver oil plus corn oil or soybean oil mixture. Next in performance came the fish fed both 1% 18:2n-6 and 1% 18:3n-3 or 0.5% n-3HUFA diets. The ratios of 20:3n-9 to 22:6n-3 and 20:3n-9 to 20:4n-6 in polar lipid fractions of hepatopancreas showed less than 0.4 and 0.6 when fish fed the diet containing both 1% n-6 and 0.5 or 1% n-3 fatty acids.

This experiment clearly indicated that VCC is one of the EFA-deficiency symptoms, and also suggested that the requirement of grass carp for dietary n-6 and n-3 fatty acids is found to be 1% and 0.5-1%, respectively.

Till date, many workers have demonstrated the qualitative and quantitative requirements for essential fatty acids (EFA) and the nutritive value of dietary lipids in many cultivable fishes.¹⁻⁶⁾ From the data accrued, a categorization can be made for EFA requirement as follows: marine fish-type (require n-3 highly unsaturated fatty acids: n-3HUFA), rainbow trout-type (require n-3 fatty acids such as linolenic acid and n-3HUFA), carp-type (require both n-3 and n-6 fatty acids) and tilapia-type (require n-6 fatty acids such as linoleic acid).¹⁻⁹⁾ The correlation between dietary habits and fatty acid composition of fish; the inter-species variations in fatty acid profile and the role of environmental water temperature are all well documented.^{1,3,10-13)}

Grass carp *Ctenopharyngodon idella* is a popular herbivorous fish, but no information is available on the EFA requirement of this fish. Therefore, this study was carried out to detect any EFA deficiency, clarify EFA requirement by feeding diets con-

taining various fatty acids like linoleic (18:2n-6), linolenic (18:3n-3), arachidonic (20:4n-6), and n-3HUFA, and investigate the relationship between EFA requirement and food habits. The results, including a new EFA deficiency symptom identified, are presented here.

Materials and Methods

Experimental Conditions

Fingerlings of grass carp were fed an EFA-deficient pre-feeding diet (Table 1) for 6 weeks in Expt. I and 8 weeks in Expt. II, respectively. In the first experiment (Expt. I), the fish were divided into 9 lots of 30 fish each (4.2 g in average). The diets were offered to the fish for a period of 21 weeks except for diet 1 (Table 2). Subsequently, the confirmatory experiment (Expt. II) was carried out with 12 lots of 30 fish each (3.9 g in average), fed the diets shown in Table 2 for a period of 24 weeks. The water temperatures ranged from 23-

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Table 1. Compositions of the pre-feeding and the basal diets in Expts. I and II (%)

Ingredient	Pre-feeding diet		Basal diet	
	Expt. I	Expt. II	Expt. I	Expt. II
Casein	45	40	45	40
α -Starch	20	20	20	20
Dextrin	25	30	20	20
Vitamin mixture	1.5	7	7	7
Choline chloride	0.4			
Vitamin E* ¹	0.1			
Mineral mixture	5	3	3	8
Cellulose	3			
Dietary lipid* ²	0		5	5

*¹ The DL- α -tocopheryl acetate content was 50%.*² See Table 2.**Table 2.** Composition of dietary lipids used in Expts. I and II

Diet no.	Dietary lipid* ¹
Expt. I	
1	Fat-free* ²
2	5.0% 12:0
3	4.5% 12:0+0.5% 18:2n-6
4	4.5% 12:0+0.5% 18:3n-3
5	4.0% 12:0+0.5% 18:2n-6+0.5% 18:3n-3
6	3.0% 12:0+1.0% 18:2n-6+1.0% 18:3n-3
7	4.5% 12:0+0.5% 20:4n-6
8	4.5% 12:0+0.5% n-3HUFA* ³
9	3.0% Soybean oil+2.0% Pollock liver oil
Expt. II	
10, 11	5.0% 12:0
12* ⁴	5.0% 12:0
13, 14	4.0% 12:0+1.0% 18:2n-6
15, 16	4.0% 12:0+1.0% 18:3n-3
17, 18	3.0% 12:0+1.0% 18:2n-6+1.0% 18:3n-3
19, 20	3.5% 12:0+1.0% 18:2n-6+0.5% n-3HUFA* ³
21	3.0% Corn oil+2.0% Pollock liver oil

*¹ Fatty acids used were all methyl esters except for diets 9 and 21.*² Composition is the same as the pre-feeding diet.*³ n-3HUFA: highly unsaturated fatty acids, mainly 20:5n-3 and 22:6n-3.*⁴ Recovery test.

25°C in Expt. I and 18–25°C in Expt. II. Other details of fish care and feeding were described previously.¹⁴⁾

Experimental Diets

The compositions of the basal diet and the dietary lipids for both experiments are shown in Tables 1 and 2. The ingredients were commercially available products and the vitamin and mineral mixtures were prepared as described earlier.^{15–17)}

The purity of methyl laurate (12:0) obtained from Tokyo Chemical Industry Co. Ltd., and methyl esters of 18:2n-6, 18:3n-3 and 20:4n-6 from Sigma Chemical Co., were checked by gas liquid chromatography (GLC), and were found

to be more than 99, 99, 95 and 90%, respectively. A mixture of methyl esters of n-3HUFA prepared from sardine oil by vacuum distillation contained 30% 20:5n-3, 49% 22:6n-3 and 3% other n-3HUFA (20:3n-3, 22:5n-3). Soybean oil, corn oil and pollock liver oil were purchased from commercial sources.

In Expt. I., a fat-free diet (diet no. 1) was the basal diet. In both Expts. I and II, diet 2 and diets 10 to 12 containing 5% 12:0 as the sole lipid source were also arranged as the EFA-deficient diet. Diet 12 was substituted by the diets containing 3.5% 12:0 plus 1% 18:2n-6 and 0.5% n-3HUFA (diets 19 and 20) after 15 weeks, as a recovery test. In order to examine the effects of

n-6 and n-3 fatty acids on grass carp, 12:0 in diet was replaced by an equal amount of 0.5 and/or 1% of 18:2n-6, 18:3n-3, 20:4n-6 and n-3HUFA in diets 3-8 and diets 13-20. Diets 9 and 21 containing 2% pollock liver oil and 3% soybean oil or 3% corn oil were arranged as the control groups. These experimental diets were provided to fish in a dry pellet form.

Analytical Methods

The hepatopancreas were taken from fish in each treatment at the end of the experiment, pooled and stored at -70°C for lipid analysis. Lipids were extracted by the method of Folch *et al.*¹⁸⁾ Polar and nonpolar lipid fractions were separated by employing silicic acid column chromatography (Sep-pak) as noted by Juaneda and Recquelin.¹⁹⁾ The fatty acid profiles of polar fractions were determined by GLC with a capillary column, SUPELCOWAX-10 (30 m $\times$ 0.32 mm I.D., with isothermal set at 205°C and helium as carrier gas).⁹⁾ Photograph of vertebral columns in fish was taken by soft X ray (Sofron NST-M630 Model, Sofron Co. Ltd.).

Results and Discussion

Feed Performance

The effects of dietary lipids on mean weight

gain, feed efficiency, mortality and vertebral column curvature (VCC) in Expts. I and II are summarized in Table 3 and Fig. 1.

Experiment I: The highest weight gain was observed in the fish fed diet 9 containing mixture of 3% soybean oil and 2% pollock liver oil. This finding is the same as that in carp¹⁶⁾ and eel.²⁰⁾ The fish fed the fat-free diet (diet 1), showed low weight gain and feed efficiency together with appearance of VCC which have been observed from the 11th week onwards. There after a larger percentage of the animals were affected with further deterioration of the vertebral column. The feeding was terminated after 19 weeks for the fat-free diet (diet 1) because the cumulative mortality by the 18-th week was 65%. The dead fish were characterized by pale gills and swollen pale livers. The fish fed diet 2 containing 12:0 as a sole lipid source showed the highest percentage of VCC. However, no mortality was observed in this group. Almost the same weight gain and feed efficiency were obtained in the fish fed the diet containing only 0.5% of 18:2n-6, 18:3n-3 or 20:4n-6. Percentage of VCC was still higher in the fish fed the diet containing n-3 fatty acids alone (diets 4 and 8). On the other hand, improved feed performance was observed in the fish fed 1% each of both 18:2n-6 and 18:3n-3 (diet 6). These results suggested that amount of

Table 3. Results of the 21-week and 24-week feeding trials in Expts. I and II

Diet no.	Av. body wt. (g)		Percent gain	Feed efficiency (g gain/g feed)	Mortality (%)	Vertebral column curvature (%) (VCC)
	Initial	Final				
Expt. I						
1* ¹	4.2	45.4	981	0.89	65	30
2	4.2	48.3	1050	0.88	0	85
3	4.2	47.4	1029	0.83	0	0
4	4.2	47.4	1029	0.89	0	0
5	4.2	48.9	1064	0.89	0	0
6	4.2	62.3	1383	0.94	0	0
7	4.2	49.4	1076	0.86	0	0
8	4.2	43.2	929	0.85	0	5
9	4.2	81.1	1831	0.95	0	0
Expt. II						
10, 11* ²	3.9	21.2	444	0.87	3	14
12* ³	2.7	19.0	600	0.88	7	7
13, 14* ²	3.8	41.1	982	1.07	0	0
15, 16* ²	3.9	48.2	1136	1.14	0	14
17, 18* ²	3.9	47.1	1108	1.12	0	0
19, 20* ²	3.7	49.8	1246	1.09	0	0
21	2.9	54.5	1798	1.19	0	0

*1 19-week feeding.

*2 Mean of duplicate experiments.

*3 Recovery test: fish fed the diet containing 3.5% 12:0 + 1.0% 18:2n-6 + 0.5% n-3HUFA after the 15th week.

Fig. 1. Effect of dietary lipids on the growth of grass carp in Expt. II. Each mark was resulted from the mean of duplicate experiment, except for recovery test and control diet (natural oil mixture).

Fig. 2. Effect of dietary lipids on vertebral columns of grass carp. The fish fed the EFA-deficient diet (5% 12:0) showed the vertebral column curvature (VCC) in photograph A. The fish fed the mixture of 3% corn oil and 2% pollock liver oil showed normal vertebral column in photograph B.

0.5% of n-6 or n-3 fatty acids in diet does not satisfy EFA.

Experiment II: The highest weight gain and feed efficiency were observed in the fish fed diet 21 containing mixture of 3% corn oil and 2% pollock liver oil. The next in performance came the fish fed both 1% 18:2n-6 and 0.5% n-3HUFA diet. On the other hand, fish fed the diet containing 12:0 alone showed the lowest weight gain, feed efficiency and high incidence of the VCC. Growth was hardly improved in the fish fed the recovery test diet (1% 18:2n-6 and 0.5% n-3HUFA) (Fig. 1). At a 1% level of dietary n-6 or n-3 fatty acids, the diet containing 18:3n-3 performed better than 18:2n-6. However, a higher percentage of VCC was still observed in the fish fed 1% 18:3n-3 diet (diets 15 and 16). This tendency was the same as that in Expt. I, suggesting that 18:3n-3 in spite of its positive role on growth, could not prevent the occurrence of VCC. On the other hand, the addition of 1% 18:2n-6 to the diet prevented completely the appearance of VCC.

Gross Symptoms of Fish in Essential Fatty Acid Deficiency

Photographs of soft X-ray of normal and abnormal vertebral column are presented in Fig. 2. Abnormal fish exhibited an upward displacement of the column at the region of the 12–15th vertebrae from caudal end, indicating lordosis. However, the inter-bone distance was normal. No scoliosis was observed. So far symptoms of EFA deficiency such as impaired pigmentation, "shock syndrome", swollen pale liver, high hepatosomatic index, etc. have been reported for rainbow trout, chum salmon, carp and eel.^{14, 21, 22)} This is perhaps the first documentation linking vertebral deformity to EFA deficiency. It is well known that vertebral abnormality such as scoliosis, lordosis, dwarfism etc. appear when fish are fed diets without all the trace elements,²³⁾ phosphorus, magnesium, manganese, zinc,²⁴⁾ vitamin E,²⁵⁾ vitamin C,^{26, 27)} and/or tryptophan.²⁸⁾ In this experiment, no variations in the levels of above nutrients were observed between normal and abnormal bone and hepatopancreas (data not presented here). The fish with the VCC also showed swollen pale livers, and these symptoms were prevented in the fish fed n-6 or n-6 and n-3 fatty acids. These results suggest that the VCC of grass carp might be one of the symptoms of EFA deficiency.

Table 4. Effect of dietary lipids on lipid compositions of hepatopancreas in Expts. I and II

Diet no.	% in Hepatopancreas		
	Total	Polar	Nonpolar
Expt. I			
1	11.8	1.5	10.3
2	8.1	1.5	6.6
3	7.8	1.3	6.5
4	6.3	1.3	5.0
5	5.3	1.3	4.0
6	10.7	1.4	9.3
7	7.5	1.5	6.0
8	7.6	1.5	6.1
9	12.9	1.5	11.4
Expt. II			
11	12.4	0.9	11.5
12* ¹	10.8	1.2	9.6
13, 14* ²	9.8	1.0	8.8
15, 16* ²	7.5	0.9	6.6
17, 18* ²	6.6	1.0	5.6
19, 20* ²	8.4	1.1	7.3
21	10.0	1.0	9.0

*1, 2 See the footnote of Table 3.

Lipid Composition of Hepatopancreas

The effect of dietary fatty acids on lipid composition of hepatopancreas from grass carp is shown in Table 4. In both Expts. I and II, the fish fed the fat-free (diet 1) or 12:0 alone diet (diets 2 and 11) showed a high content of total lipid due to increase of neutral lipid, but the lipid content was reduced by supplementation with n-6 and/or n-3 fatty acids in all cases. However, high contents of total lipid were also observed in the fish fed natural oils (diets 9 and 21). This similar pattern in lipid content between EFA deficient groups (diets 2 and 11) and natural oils fed groups might be due to the difficulty in distinguishing tissue and deposit fat in the latter group. These findings are almost the same as that in carp.¹⁶⁾ There was no marked difference in the polar lipid content between the treatments in Expts. I and II.

Fatty Acid Composition of Polar Lipid Fractions in Hepatopancreas

The fatty acid composition of polar lipid fractions from hepatopancreas in Expts. I and II are shown in Tables 5 and 6, respectively. The percentage of 20:3n-9 was higher in the fish fed the fat-free (diet 1) and the EFA-free diet (12:0 alone; diets 2 and 11), and was reduced by supplementation of n-6 and/or n-3 fatty acids. Dietary 20:4n-6 or n-3HUFA was found to be more ef-

Table 5. Fatty acid composition of polar lipids from hepatopancreas in Expt. I (area %)

Fatty acid	Diet no.								
	1	2	3	4	5	6	7	8	9
16:0	22.5	23.8	28.7	29.3	28.8	26.1	28.7	26.5	34.1
16:1n-7	5.8	5.2	4.2	4.2	2.5	2.4	3.5	3.1	2.3
18:0	5.8	6.1	9.2	9.5	9.8	12.1	9.3	9.0	14.0
18:1n-(9+7)	27.3	25.1	18.7	28.8	14.4	14.1	17.0	18.1	14.0
18:2n-6	0.2	0.1	0.9	0.1	1.4	2.2	0.1	0.1	2.9
18:3n-3	tr	tr	tr	0.3	0.3	0.7	tr	tr	0.1
20:2n-6	0.6	0.6	0.3	0.4	0.3	0.2	0.3	0.5	0.1
20:3n-9	24.8	26.7	12.5	14.5	6.3	2.8	8.4	11.9	1.4
20:4n-6	1.0	0.9	9.6	0.5	9.3	9.8	11.7	1.3	7.9
20:5n-3	0.1	0.1	tr	1.3	1.3	2.0	0.1	1.1	1.0
22:5n-6	0.4	0.3	8.0	0.1	2.1	3.2	11.6	0.6	1.8
22:6n-3	1.8	1.3	1.1	11.5	12.9	16.4	2.5	20.6	11.8
20:3n-9/22:6n-3	13.8	20.5	11.4	1.2	0.5	0.2	3.4	0.6	0.1
20:3n-9/20:4n-6	24.8	29.7	1.3	28.6	0.7	0.3	0.7	9.2	0.2

Table 6. Fatty acid composition of polar lipids from hepatopancreas in Expt. II (area %)

Fatty acid	Diet no.						
	11	12	13, 14*	15, 16*	17, 18*	19, 20*	21
16:0	25.0	25.1	29.1	30.3	29.0	29.4	29.9
16:1n-7	6.2	4.1	5.2	3.5	3.0	3.0	3.0
18:0	7.1	10.2	9.2	11.7	11.5	11.7	13.4
18:1n-(9+7)	23.7	19.3	16.8	18.8	13.4	12.5	13.6
18:2n-6	0.1	1.6	1.6	0.1	2.6	2.3	2.5
18:3n-3	tr	tr	0.1	0.8	0.6	tr	tr
20:2n-6	18.8	6.7	7.0	9.8	0.5	0.5	0.4
20:3n-9					3.9	4.9	3.8
20:4n-6	0.9	9.2	9.7	0.4	8.4	9.0	8.3
20:5n-3	0.1	0.3	tr	2.4	1.3	0.3	0.5
22:5n-6	0.2	4.0	10.3	tr	3.2	4.9	4.6
22:6n-3	1.7	9.8	0.6	15.3	12.6	12.9	11.1
20:3n-9/22:6n-3	11.1	0.7	11.7	0.6	0.3	0.4	0.3
20:3n-9/20:4n-6	20.9	0.7	0.7	24.5	0.5	0.5	0.5

* See the footnote of Table 3.

fective for reduction of 20:3n-9 than dietary 18:2n-6 or 18:3n-3 at the same level of supplementation (Expt. I). The dietary 18:2n-6 or 18:3n-3 was converted to 20:4n-6 and 22:5n-6 or 22:6n-3 respectively, as in other freshwater fish.^{1,8,5-7,18)} The lower level of 22:5n-6 observed in the fish fed both 18:2n-6 and 18:3n-3 may indicate an apparent inhibition of conversion of 18:2n-6 to 22:5n-6 due to the presence of n-3 fatty acids. The fatty acid profile was almost same in fish fed 1% 18:2n-6 and 1% 18:3n-3 or 0.5% n-3HUFA (diets 17, 18 and 19, 20), and the natural oil mixture (diet 21). In the recovery test, the percentage of 20:3n-9 was effectively reduced by feeding the diet with 1% 18:2n-6 and 0.5% n-3HUFA diet after 15 weeks.

The ratios of 20:3n-9 to 20:4n-6 and 20:3n-9 to 22:6n-3 were high in the fish fed the fat-free and 12:0 alone diets (diets 1, 2 and 11), respectively, and was reduced by supplementations of both n-6 and n-3 fatty acids. These two ratios in liver polar lipid are already proposed as an EFA index for carp.¹⁷⁾ Since carp require fatty acids of both the 18:2n-6 and 18:3n-3 families for growth, it is reasonable to consider the ratios of 20:3n-9/20:4n-6 as well as 20:3n-9/22:6n-3 for the EFA index. When both ratios are applied to carp as indices of EFA-deficiency, the former ratio becomes less than 0.4 and the latter less than 0.6, if the carp are receiving a sufficient amount of both n-6 and n-3 fatty acids. In this experiment, grass carp also requires both n-6 and n-3 fatty acids for improve-

ment of growth, feed efficiency and normal bone formation. If both ratio can be used as an EFA index for grass carp, the fish fed both 1% 18:2n-6 and 1% 18:3n-3 or 0.5% n-3HUFA might be satisfying its EFA requirement.

This experiment clearly indicated that the VCC is one of the EFA-deficiency symptoms. It is also suggested that the requirement of grass carp for dietary n-6 and n-3 fatty acids is found to be 1% and 0.5–1%, respectively. This quantitative requirement of grass carp is almost the same as that of carp,¹⁷⁾ but it was higher than that of eel.²⁰⁾ The present study has led to many new lines of thinking. It is necessary to investigate as to why grass carp shows the VCC, hitherto an unknown EFA deficiency symptom; what the causative mechanisms are; and whether the VCC is the same symptom as that of the vitamin E-deficiency of carp²¹⁾ or not. These questions may be partly answered in an on-going experiment to compare feed performance, appearance of the VCC and physiological relationship between EFA deficiency and vitamin E for grass carp.

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