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Effects of feeding habits and nutrition status of freshwater fishes on muscle with lipid fatty acid composition and tocopherol contents

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Abstract
The objectives of the study was to determine the total lipid content, fatty acid composition and tocopherol content of muscle of eleven fresh water fishes belongs to Cichlidae, Anabantidae, Siluridae, Cyprinidae, Channidae families lived in reservoirs in Sri Lanka and to find high nutritious fresh water fish and the relationship of diet of fish with the feeding habits of fish. Muscle lipid contents were varied between 1.6 - 41.5% of the fishes that having omnivorous, carnivorous and herbivorous feeding habits. Walking catfish (*Clarius brachysoma*) having omnivorous feeding habit showed the highest total lipids (41.5%), polyunsaturated fatty acids (PUFA) (39.9%) and α-tocopherol (29.65mg/Kg) in the muscle. The ratio of total n-3 to n-6 fatty acids in walking catfish was 1 confirmed consumption of catfishes favourable for human health and has a particularly beneficial effect in preventing cardiovascular diseases. Tilapia (*Tilapia mossambica),* and Climbing perch (*Anabas testudineus)* having omnivorous feeding habit showed 15.73 and 12.55% total lipids in the muscle and contained 5.6 and 2.5 mg/Kg α-tocopherol respectively. However, Tilapia (*Tilapia niloticus*) showed very low total lipid (1.69%) and tocopherol (0.08 mg/Kg). Fresh water shark (*Wallago attu*) having carnivorous feeding habit showed 11.29% total lipids, 4.02 mg/Kg α-tocopherol and 36% PUFA in muscle. Similarly, Rohu (*Labeo rohita*) having herbivorous feeding habit contained 10.84% lipids, 6.61mg/Kg α-tocopherol and approximately 90% unsaturated fatty acids. Results of the study revealed that the lipid content, fatty acid composition and tocopherol content in the muscle of the freshwater fishes in Sri Lanka was not significantly influenced by the feeding habits of the fishes and influenced the composition of diet of fish and no relationship with the families.

Keywords: Lipids, fatty acids, tocopherol, feeding habits, fresh water fishes

1. Introduction
Freshwater fishes contain saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and long-chain polyunsaturated fatty acids (PUFAs) that have significant role in human health (Gladyshev *et al*. 2012) [1]. Polyunsaturated fatty acids (PUFAs) are particularly important due to their ability to prevent cardiovascular disease, psychiatric disorders, and some other illnesses such as atherosclerosis, thrombogenesis, high blood pressure, cancer, and skin diseases (Gladyshev *et al*. 2012) [1]. Fish meat and oils are good sources of unsaturated omega-3fatty acids, eicosapentacenoic acid (EPA, 20:5n-3) and docasahexaenoic acid (Calder,2004) [2], which are known to be cardio-protective, anti-atherosclerotic, antithrombotic, and anti-arrythmitic and also play a role in reducing the cholesterol level (Potter and Kiss, 1995) [3] regulate prostaglandin synthesis and hence induce wound healing (Bowman and Rand, 1980) [4] and stabilizing the electrical activity of the heart cells (Dallongeville, 1981) [5]. The fish contains n-3-PUFAs which gives good heart health and concentrations of n-3 PUFAs and essential FAs are varied among fresh fishes (Gladyshev *et al*. 2012) [1]. These PUFAs are not synthesized in the human body and therefore inclusion of fish in human diets is a necessity (Jabeen and Chaudhry, 2011) [6]. However, different species have variation in their FA composition and levels (Özparlak *et al*. 2013) [7]. Fish meat containing high-3/n-6 ratios of PUFAs are the most beneficial in terms of human health (Dhanapal, 2011) [8]. PUFAs are one of the most important components of fish muscle providing energy reserves and components of cell bio-membranes. Fish lipids are dominated by saturated fatty acids and myristic (C14:0) acids followed by stearic acid and palmitic (C16:0).

whereas the monounsaturated fatty acids (MUFA) are oleic and palmitoleic acids (Kolakowska *et al*. 2002) [9] and in Polyunsaturated fatty acids (PUFA) are EPA and DHA, which may reduce the risk of coronary heart diseases (Dhanapal, 2011) [8]. Lipid composition in muscle is primarily dependent on the feeding habits of fish. Fatty acid composition of different fish species from different regions are available in literature (Haliloglu *et al*. 2004 [10]; Jahkar *et al*. 2012) [11].

In marine animals, α-tocopherol has been found to be the principal tocopherol (Syväoja *et al*. 1985 [12]; Gotoh *et al*., 2009) [13]. α-tocopherol, the fat –soluble vitamin, is an important antioxidant to comfat free radical–induced diseases, particularly protection of membrane and lipoprotein particles (Kalogeropoulos *et al*. 2007) [14]. Studies on the unsaponifiable lipid components (α-tocopherol) in fish and sea food have been conducted in Italy (Orban *et al*. 2011) [15]. The tocopherol content of fish foods is influenced by a large number of factors e.g. seasonal differences, and significant losses may occur during processing and storage of foods (Deshpande *et al*. (1996) [16]. Fish are unable to synthesize the vitamin, resulting in characteristic tocopherol levels in the tissue of different fish species related to diet. For example, López *et al*. (1995) [17] found the highest α-tocopherol concentrations in juvenile rainbow trout, but no significant difference was found between sexes in adult samples. Considerable differences in α-tocopherol concentration have been reported between light and dark fish muscle. Pettillo *et al*. (1998) [18] observed a loss in α-tocopherol content from both light and dark muscle of mackerel following an 11-day storage period on ice, whereas a 10% loss of α-tocopherol was measured in sardine fat over six days and, in channel catfish, no decrease was reported after 7days of storage (Erickson,1992) [19]. Parazo *et al*., (1998) [20] stated that both α-tocopherol and γ-tocopherol were effective stabilisers of salmon muscle lipids during frozen storage. European catfish muscle (Ng *et al.,* 2004) [21], and microalgae (Huo *et al.,* 1997) [22].

The main objective of this study was to determine the lipid content, fatty acids composition (percentages) and α-tocopherol of 13 species of fresh water fishes of Sri Lanka where people from low land and rural people consume the fresh water fishes as their nutritious diets. Therefore, detailed information about their lipid content, fatty acids composition and α-tocopherol was important from nutritional point of view and are needed because it influenced quality in frozen storage of some fresh water fish species. Thus, this study was carried out to evaluate the lipid content and fatty acid profile and α-tocopherol of commercially important fresh water fishes of both Sri Lanka and Japan and find the possibilities to select as farm species in terms of essential fatty acids, α.tocopherol and feeding habits.

2. Materials and Methods

2.1 Collection

Thirteen species of freshwater fish from five families were purchased from the Eravur, Chenkalady, and Valaichennai Markets of Batticaloa, Sri Lanka. Muscle (100 g) was removed from the mid abdomen of the fish and flushed with cold water. The samples were packed with dry ice and kept at-80°C until air shipment to Tokyo University of Marine Science and Technology, Japan. Two fresh water fish a belonging to 02 families were collected from Tokyo markets and packed with ice in polythene bags before immediate transfer to the laboratory. All fish were kept in the deep freezer at -80°C prior to tocopherol analysis. For lipid extraction, fish were washed with cool water and 10 g muscle was removed.

2.2 Lipid extraction

The total lipid (TL) content was extracted from fish muscle using the method of Bligh and Dyer (1959) [23]. Muscle tissue (10g) was homogenized in 20 volumes of methanol (2:1,v/v), followed by 10 volumes of chloroform, in a homogenizer (Nissei Ace Homogenizer, AM Japan) for 4 min at 10000 to 15000rpm. The homogenate was filtered (using a suction funnel with No.1 filter paper) to recover the liquid phase and the filter residue was re-homogenised with a second volume of chloroform: methanol (1:2,v/v). The filtrate of both was extractions was combined and treated with 2 volumes of 0.88% KCl, and centrifuged (Kubota, Japan, 2010) for 10 min at 3000 to 4000rpm.The lower phase of chloroform containing the lipids was collected and evaporated under vacuum in a rotator evaporator (EYELA, DPE-2120) to a volume of 1ml. Further evaporation of chloroform was done under nitrogen gas stream exposure, and the residue was weighed to quantify the amount of lipid extracted. The lipid residue was dissolved again in 25ml of chloroform and stored in a 25-ml bottle protected from light with exposure to argon gas at -20°C until needed.

2.3 Preparation of fatty acid methyl esters (FAME)

Esterification of the extracted lipids was performed according to Association of Analytical Communities method (AOAC, 2000) [24]. Fatty acid methyl esters (FAMEs) were obtained from the isolated lipids by heating the lipid extract with 1 mL of methanolic-NaOH (0.5N NaOH–MeOH) at 100°C for 15 min, followed by cooling at room temperature and treatment with 2 mL of boron trifluoride (BF_3) methanol solution. One milliliter of *n*-hexane was added to recover the methyl esters in the organic phase. The mixture was washed with a saturated solution of NaCl. Two phases were formed after washing; the upper *n*-hexane phase was collected in 10-mL glass chromatography vials for GC analysis.

2.4 Fatty acid analysis using gas chromatography

FAMEs were analyzed on a GC -14A model gas chromatograph (Shimadzu, Japan) equipped with a flame ionization detector(FID) and fitted with a capillary column (Omegawax 320, 30m length, 0.32mm internal diameter, 0.25 µm film thickness, Sigma-Aldrich Co. LLC, St. Louis, MO). Injector and detector temperatures were 250°C and 260°C, respectively. The oven was initially held at a temperature of 100°C for 5 min. The temperature was then increased at a rate of 1°C/ min and held at175°C and 240°C for 10 min each. The total run time was 90 min. Helium (2.5 mL/min) was used as a carrier gas. Samples were run in a split mode (100:1). Three replicate analyses were performed per sample. Fatty acids were identified by comparing the retention times of the FAME peaks in the sample to those obtained for the standards (Supelco 37 Component FAME Mix, Sigma-Aldrich Co. LLC). The fluorescence intensity signals were integrated with Chromatopak CR6A chromatographic integrators (Shimadzu Corporation, Kyoto, Japan). Results are expressed as relative percentages of peak area obtained from GC-FID chromatogram.

2.5 Determination of Tocopherols using High Pressure Liquid Chromatography (HPLC) Analysis

The basic extraction procedure and fast HPLC method were used as described by Nirungsan and Thongnopnua (2006) [25] to determine the level of tocopherol in freshwater and marine fish. HPLC analyses were conducted using a Shimadzu Prominence HPLC apparatus (Shimadzu, Kyoto, Japan) equipped with a RF-10AXL fluorescence detector and pump (Shimadzu, Jasco, PU-1580). The column was Inertsil HPLC, ODS-P 6mm, (4.6 × 250mm) purchased from GL. Sciences Inc. Japan, and was packed with reversed-phase Develosil 5µm RP AQUEOUS (150 × 4.6mm).

Tocopherol standard, α-tocopherol (Food Science & Technology, TUMAST), was used for the determination of tocopherol content from the lipid extract of each fish. The mobile phase consisted of acetonitrile and methanol. Chromatographic separation was carried out using continuous isocratic elution with HPLC-grade acetonitrile (eluent A) and methanol (eluent B). The HPLC isocratic profile was 60% acetonitrile and 40% methanol, and the flow rate was 1.0 mL/min throughout the whole separation. The total separation time was 12 min and the isocratic elution was run for 15 min to ensure full separation. The injection volume was 10 µl and detection was monitored with a UV fluorescence detector (RF-10AXL, Shimadzu Fluorescence detector, Kyoto, Japan) at wavelengths of 298 and 330 nm for excitation and emission, respectively. The signals of fluorescence intensity were integrated with a C-R5A Chromatopak (Shimadzu, Kyoto, Japan). The detected signals were quantified using a standard calibration curve prepared using authentic standards (Food Science and Technology Laboratory, TUMSAT, Japan). The regression coefficient of the four replicate standard injections was 0.998 for each tocopherol at 0, 2, 4,6,8,10 and 12 µg/mL

2.6 Feeding habits of fish

The collection of fish was made from local fish landing centres and observation of food composition were made to show the food choice, and feeding habit of the fish. Just after collection 10% formalin solution was injected into the gut of all the fishes in order to stop digestion of food items. Stomach analysis was carried out in the laboratory of the Dept. of Zoology, Eastern University, Sri Lanka. Some fresh and live specimens were also examined. The stomach of the fishes were dissected with the help of a simple scissors and the stomach contents were taken into a petridish and the food items were identified by (Magnification 5X, 10X, 40X). The frequency occurrence of food items in the stomach were studied and confirmed the possible food and feeding habits of fish.

2.7 Statistical Analysis

The results are presented as mean ± standard deviation for triplicate samples. For tocopherol determination, triplicates samples were used to calculate means. Statistical differences were calculated using standard t-tests, one sample t-tests, and one way ANOVA (SPSS 10.0) with

$p < 0.05$ considered significant. Data were subjected to Tukey and T-tests where differences were detected for homogenous subsets. All statistical analyses were performed using SPSS-10 for Windows and EXCEL, 2010

3. Results and Discussion

3.1 Lipid Content

The Sri Lankan fresh water species *Tilapia niloticus* (nile tilapia), *Etroplus suratensis* (green chromide), *Channa striata* (Snakehead Murrel), *Anabas testudineus* (Climbing Perch), *Wallago attu* (Fresh water shark),*Cyprinus carbio* (Common carp), *Labeo rohita* (Rohu), *Channa orientalis* (Snake head),*Etroplus maculate* (Banded chromide), *Clarius brachysoma* (Walking catfish),*Tilapia mossambica* and Japanese fresh water species, *Salvelinus leucomaenis* (Iwana) and *Plecoglossus altivelis* (Ayu) were selected for the study because of their economical importance and consumer demand in the aquaculture. As shown in Table 1, the total lipid content of muscle was significantly high in *C. brachysoma* (41.50%) and this fish showed high demand among pregnant and feeding women in market and it was stated that walking catfish is cooked with its oil itself. Results showed the difference of lipid content among species *T. niloticus* (1.69%) and *T. mossambica* (15.73%) whereas the *E. suratensis* (2.98%) and *E. maculata* (2.62%) showed no significant difference in lipid content. So, this difference could be related with the maturity and breeding cycle of *T. mossambica*. Total lipid content in body parts varied in different fresh water fishes studied by Swapna *et al.* [26]. 2010 and was observed comparatively high content of lipid in muscle of all fresh water fishes studied.

Table 1: Lipid content (%), Feeding habits of thirteen fresh water fishes

Scientific Name	Family	Common Name	Habitat	Feeding Habit	Total Lipid (%)
Tilapia niloticus	Cichlidae	Tilapia	Fresh (Sri Lanka)	Omnivorous	1.69
Etroplus suratensis	Cichlidae	Orange chromide	Fresh (Sri Lanka)	Omnivorous	2.98
Channa striata	Channidae	Snakehead	Fresh(Sri Lanka)	Carnivorous	3.64
Wallago attu	Siluridae	Fresh water shark	Fresh (Sri Lanka)	Carnivorous	11.29
Anabas testudineus	Anabantidae	Climbing perch	Fresh(Sri Lanka)	Omnivorous	12.55
Labeo rohita	Cyprinidae	Rohu	Fresh(Sri Lanka)	Herbivorous	10.84
Cyprinus carpio	Cyprinidae	Common carp	Fresh(Sri Lanka)	Omnivorous	1.68
Channa orientalis	Channidae	Snakehead	Fresh(Sri Lanka)	Carnivorous	8.31
Etroplus maculatus	Cichlidae	Green chromide	Fresh(Sri Lanka)	Omnivorous	2.62
Clarias brachysoma	Siluridae	Walking cat fish	Fresh(Sri Lanka)	Omnivorous	**41.50**
Tilapia mossambica	Cichlidae	Tilapia	Fresh(Sri Lanka)	Omnivorous	**15.73**
Salvelinus leucomaenis	Oithonidae	Iwana	Fresh (Japan)	Algal feeder	4.40
Plecoglossus altivelis	Plecoglossidae	Ayu	Fresh (Japan)	Larval feeder	1.89

3.2 Food composition in stomach of fish

Food composition of fresh water fishes were studied with frequency occurrence method. Among fresh water fishes studied plants and animal foods were found in *T. mossambica, T. niloticus*, *C. brachysoma E. maculatus C.carpio, E. suratensis,* and *Anabas testudineus* whereas animal particles were found in *C. striata*, *C orientalis* and *W. attu* and they are carnivorous species. In *S. leucomaenis* (Iwana), all food

particles were green algae and planktons and *P. altivelis* (Ayu)had insect food particles, Among fishes with omnivorous feeding habits, lipid content had significantly varied and *T. mossambica* had significantly high content of lipid as it can explained that maturity and spawning change the lipid content in muscle (Table1).

3.3 α-Tocopherol content

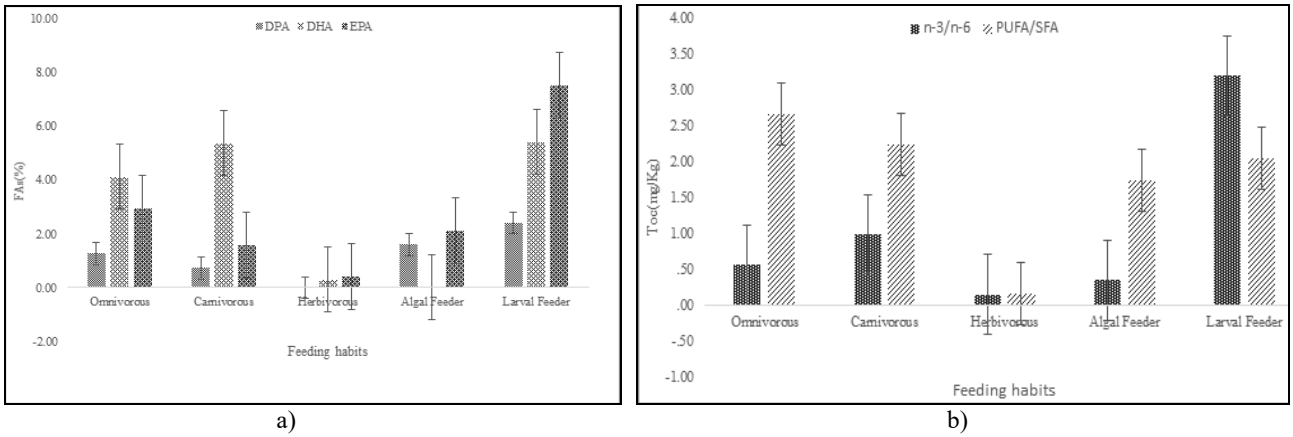
Among the fresh waters studied, α- tocopherol was significantly high in *C. brachysoma* (29.65mg/Kg) ($p<0.01$) and other fishes, such as *W. attu* (7.43mg/Kg), *Labeo rohita* (6.61mg/Kg), *T. mossambica* (5.64mg/Kg) were found relatively low level of α- tocopherol. Azrina *et al*., (2015) [27] studied the α- tocopherol of marine fishes which showed very higher amount than fresh water fishes. Because of the low content of α- tocopherol (antioxidant) in muscle, fresh water fishes were rapidly spoiled than marine fishes and because of that these fishes are processed by smoking rather than drying in rural area. Ozyurt *et al*. (2009) [28] evaluated vitamin E contents of fresh water species and they reported 0.94, 0.46 and 0.80 mg/100g of vitamin E for pike, common carp and European catfish, respectively. Tocopherol in marine fishes reported by Gotoh *et al*. (2009) [13] and Matsushita *et al*. (2010) [29].

3.2 Essential Fatty acids

Fatty acids in fishes are derived from two main sources, namely biosynthesis and diet. The chain length varies from C_{14}-C_{24} of varying degree of unsaturation, from saturated to polyunsaturated. The dominant fatty acids in the lipid extract from muscle of different fresh water fishes were arachidonic acid (maximum 21.2%), gamma –linoleic acid (19%) erucic acid (maximum 29.60%), stearic acid (maximum 22.20%) and palmitic acid (maximum 7.50%). But, Ackman *et al*. (1994) [30] observed that palmitic, oleic and linoleic acids were dominated in their study. However, oleic acid was significantly very low in muscle of fresh water fishes recorded in all study. Among PUFA,, arachidonic acid was significantly high in *L. rohita* (21.20%) where as γ- Linolenic acid was low in muscle of fresh water fishes in this study. Erucic acid was significantly high in *T. mossambica* (29.60%) and Nervonic acid was high in *C. brachysoma* (13.1%) among all thirteen fresh water fishes. However, Swapna *et al*., 2010 mentioned that tilapia had vey low oleic acid and γ- Linolenic acid (12.2%) in their result. Palmitoleic acid was commonly found low amount in all fresh water species. Among SFA, stearic acid and arachidic acid were found high in *W. attu* (22.2%) and *C. striata* (15.30%) respectively(Table 3).DPA is significantly very lower in almost all fresh water fishes whereas the DHA is relatively higher than DPA in fresh water fishes, particularly, *Anabas testudineus* (13.70%) consisted of significantly ($p<0.05$) higher DHA in its muscle. EPA content in freshwater fishes such as *E. maculates* (8.20%) and *Plecoglossus altivelis* (7.50%) was seen relatively lower compared to other freshwater fishes studied. These results are agreed with the result study of commercial important fresh water fishes in Brazil (Gutierrez and de Silva, 1993) [31]. The n-3/n-6 ratio was higher in *Anabas testudineus* (2.21) and *Plecoglossus altivelis* (3.19). PUFA/SFA ratio was significantly high in *T. niloticus* (7.15) and *Channa striata* (2.55). Fatty acid composition among the fishes were different (Table 2).

Table 2: α- Tocopherol (mg/Kg), Essential fatty acids (%), n3: n6 and PUFA/SFA ratio of the selected fresh water fishes

Fish Name	α-Toc(mg/Kg)	DPA(%)	DHA(%)	EPA(%)	n3/n6	PUFA/SFA
Tilapia niloticus	0.08	1.00	3.50	1.00	0.60	7.15
Etroplus suratensis	1.21	2.10	5.50	3.90	0.78	2.13
Channa striata	1.37	0.00	1.80	0.50	0.22	2.55
Anabas testudineus	2.38	1.30	13.70	0.20	2.21	1.90
Wallago attu	7.43	0.30	2.80	1.70	0.22	1.86
Cyprinus carbio	3.33	0.00	0.30	0.40	0.14	0.15
Labeo rohita	6.61	0.30	4.20	0.60	0.14	0.00
Channa orientalis	0.03	0.90	0.60	4.00	0.52	2.27
Etroplus maculata	2.3	1.70	3.80	8.20	0.63	4.37
Clarius brachysoma	29.65	2.10	5.50	3.90	1.00	2.60
Tilapia mossambica	5.64	1.40	3.50	1.30	0.50	0.51
Salvelinus leucomaenis	1.39	1.60	0.00	2.10	0.34	1.73
Plecoglossus altivelis	3.97	2.40	5.40	7.50	3.19	2.05



a) b)

Fig 1a: Variation of essential fatty acid content (%) with the feeding habits of the fresh water fishes. b) n3/n6 ratio and PUFA/SFA ratio with feeding habits of fishes. Data represented as mean±SE.

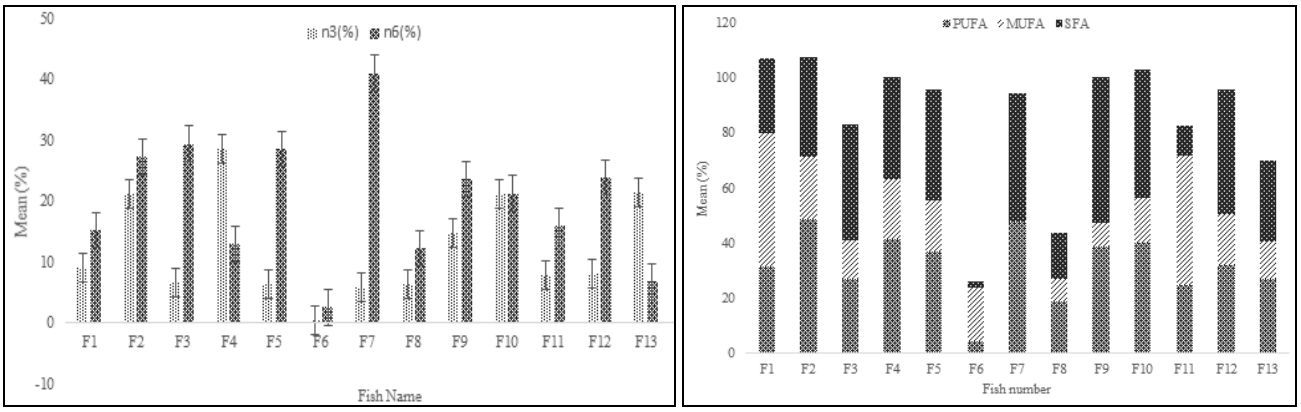


Fig 2c: Variation of content of n-3 and n-6 fatty acids among fresh water fishes (n=13), d) Variation of total PUFA, MUFA and SFA in fishes. F1- *Tilapia niloticus,* F2- *Etroplus suratensis*, F3- *Channa striata*,F4-*Anabas testudineus*, F5- *Wallago attu*, F6- *Cyprinus carbio*, F7- *Labeo rohita*,F8- *Channa orientalis*, F9- *Etroplus maculata*, F10- *Clarius brachysoma*, F11- *Tilapia mossambica*, F12- *Salvelinus leucomaenis*, F13- *Plecoglossus altivelis*

4. Conclusion

Fresh water fishes with omnivorous habits had considerably high level of essential fattyacids and α- tocopherol than other feeding habits. The essential fatty acids EPA, DHA, DPA were very less amount in muscle except climbing perch. The dominant fatty acids were arachidonic acid, gama linolenic acid, erucic acid and stearic acid were found high amount in muscle of fresh water fishes. α- Tocopherol content was poor in muscle except, walking cat fish. Because of the reason, fresh water fishes were not able to do freeze processing and generally muscle of fresh water fishes are processed either smoked and deep dry salted and cannot be kept longer period in the processed stage.

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