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Effect of Dietary Lipid Sources and Their Combinations on Growth and Fatty Acid Composition of Milkfish (*Chanos chanos*) Larvae

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ABSTRACT

Background: The success of larval rearing is greatly influenced by first feeding regimes and the nutritional quality of weaning diets, with dietary lipids being recognized as one of the most important nutritional factors that affect larval growth and survival. Reports are scanty on milkfish larval nutrition and growth and survival unlike other marine species.

Methods: In this investigation during 2020, five larval diets were prepared with 40 g kg^{-1} of entirely fish oil (F4), corn oil (C4) or fish oil and corn oil in 3:1 (F3C1), 2:2 (F2C2) and 1:3 (F1C3) ratios. Each diet was fed to triplicate groups of milkfish larvae (45 mg) in a flow-through rearing system for 42 days.

Result: A growth indices were highest in the F3C1 group, followed by F2C2, F4 and other dietary treatments. The whole-body fatty acid profile was found to change significantly with increasing fish oil replacement with corn oil, *i.e*., the n-3 polyunsaturated and saturated fatty acid proportions decreased linearly, while the n-6 polyunsaturated fatty acids content increased. Overall, this study reveals that growth and survival of milkfish is dependent on dietary lipid source or combinations that meet the essential fatty acid requirements during the early life stages.

Key words: Corn oil, Essential fatty acids, Fish oil, Larval nutrition, Lipid, Milkfish.

INTRODUCTION

Lipids play an important role in the larval nutrition of fish, as they supply the required quantities of energy and essential fatty acids (Borlongan, 1992; Sargent *et al.,* 1999; Izquierdo *et al.,* 2000). Especially in marine fishes, the n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA), namely eicosapentaenoic acid (EPA, 20:5 n-3) and docosah exaenoic acid (DHA, 22:6 n-3), are essential to maintain the structure, fluidity and function of cell membrane permeability and plasticity and also to support prostaglandin production and other essential physiological functions (Tocher, 2003; Faulk and Holt, 2005; Sivaramakrishnan *et al.,* 2017). However, in recent years, plant oil sources are widely used to partially replace fish oil in finfish feeds, as they are abundant, less expensive and free of dioxins and other organic pollutants (Torstensen *et al.,* 2000; Bell *et al.,* 2001; Montero *et al.,* 2003).

Several studies have shown that changes in the dietary fatty acid profile and unsaturated fatty acid concentration (*e.g*., when fish oil is replaced with vegetable oil) influence fatty acid composition and zootechnical indices of animal (Montero *et al.,* 2003; Bransden *et al.,* 2003; Mourente *et al.,* 2005; Lin and Shiau, 2007). For instance, corn oil contains relatively large quantities of n-6 PUFAs such as linoleic acid (18:2 n-6); whereas, n-3 PUFA is rich in fish oil (Bell *et al.,* 2001). The effect of corn oil as a dietary lipid source in juvenile grouper reported that increasing growth and immune responses in partially corn oil replaced diets but decreasing when fish oil was completely substituted (Lin and Shiau, 2007). The choice of corn oil to substitute fish oil in milkfish larval diets in this study was made because of its favourable fatty acid profile and high content of natural antioxidants (vitamin E) and phytosterols (Moreau, 2011).

Milkfish is an important brackishwater food fish cultured in the Indo-Pacific region, with bulk of the production coming from Philippines, Indonesia, Taiwan and other south-east Asian countries (Lim *et al.,* 2002; Bera *et al.,* 2021). The amenability of this fish species for culture in fresh, brackish and marine waters makes it more potential candidate species. Globally, it is one among the top twenty aquaculture

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species with an annual production of nearly one million metric tonnes in 2018 (Bera *et al.,* 2019). At present, in India, milkfish larvae production is non-intensive, relatively less wide-spread and traditional due to the lack of quality seed and feed. Recently, ICAR-CIBA has made a major breakthrough in the captive maturation, breeding and hatchery rearing of milkfish and is further fine-tuning the seed production protocols for extended breeding periods Bera *et al.,* 2021). Moving forward, it is very important to understand the larval nutritional requirements and diet preferences of milkfish to facilitate mass-scale production of healthy milkfish fry and fingerling. Particularly, optimizing the dietary lipid composition and supply of essential a fatty acids based on critical early life requirements is prerequisite for the development of an efficient larval feed. Previous studies have revealed the importance of dietary phospholipid content for milkfish larval development (Balito-Liboon *et al.,* 2018; Sivaramakrishnan *et al.,* 2021). Also, DHA and arachidonic acid were found to be conserved during the larval stages of milkfish, possibly due to its essentiality in the development process (Borlongan, 1992; Sivaram akrishnan *et al.,* 2021). Besides this, there is no information on the effect of dietary lipid source and essential fatty acid requirements of milkfish during their early life stages. In this context, the present study was undertaken to examine the effect of different dietary lipid sources (fish oil and corn oil) and their combinations on growth, survival, feed utilisation and fatty acid composition of milkfish larvae.

MATERIALS AND METHODS

Experimental diets

For this study, 5 micro extruded and marumerized (MeM) experimental diets were prepared and tested. The experimental diets were isonitrogenous (~580 g kg^{-1} crude protein) and iso-energetic (20.5 MJ kg^{-1} gross energy and 120 g kg^{-1} crude lipid). These experimental diets were prepared with incorporation of fish oil (F4), corn oil (C4), blend of fish and corn oil at the ratio of 3:1 (F3C1), 1:1 (F2C2) and 1:3 (F1C3) at the level of 40 g kg^{-1}. Other than lipid sources and its inclusion level, the ingredient composition of the experimental diets were kept same (Table 1). The mixture was then thoroughly blended to form a dough with the addition of required quantity of water. Subsequently, the dough was steam-cooked and cooled, prior to the addition and blending of vitamin and mineral mixtures. Finally, the feed mixture was passed through a micro extruder (MG-55 Multi-xtruder™ Fuji paudel, Japan) and marumerised (QJ-230T Laboratory

Table 1: Ingredient and chemical composition of the experimental diets.

Ingredient composition (g per kg feed)	F4	F3C1	F2C2	F1C3	C4
Fish meal	550	550	550	550	550
Acetes, dried	150	150	150	150	150
Squid meal	50	50	50	50	50
Wheat gluten	90	90	90	90	90
Dry yeast	25	25	25	25	25
Fish oil	40	30	20	10	0
Corn oil	0	10	20	30	40
Soy lecithin	30	30	30	30	30
Spirulina	10	10	10	10	10
Fish hydrolysate	10	10	10	10	10
Vitamin premix[a]	15	15	15	15	15
Mineral premix[b]	15	15	15	15	15
Binder	10	10	10	10	10
Astaxanthin	1	1	1	1	1
Choline chloride	2	2	2	2	2
Vitamin C	2	2	2	2	2
Chemical composition of feed (g per kg dry matter basis)					
Crude protein	584.1	580	580	588.4	582.8
Ether extract	124.1	123.2	122.7	121.5	121.1
Gross energy, MJ/g DM	20.5	20.6	20.6	20.5	20.4
Protein to energy ratio, g CP/MJ	28.5	28.2	28.2	28.7	28.6

[a]Composition of vitamin premix (quantity/kg); Vitamin A, 500,000 IU; Vitamin D3, 250,000 IU; Vitamin E, 2000 mg; Vitamin K, 1000 mg; Thiamin, 100 mg; Riboflavin, 2000 mg; Pyridoxine, 500 mg; Cyanacobalamin, 400 mg; Calcium Pantothenate, 2500 mg; Niacin, 4000 mg; Biotin, 4000 mg; Folic acid 200 mg.

[b]Composition of mineral premix (quantity/kg); Manganese oxide, 500 mg; Potassium iodide, 500 mg; Ferrous sulphate, 10 g; Zinc oxide, 1000 mg; Copper sulphate, 250 mg; Cobalt carbonate, 2 mg; Sodium selenite, 10 mg; Chromium chloride, 100 mg; Calcium lactate, 250 g; Calcium phosphate (monobasic), 350 g; Magnesium sulphate, 50 g.

marumerizer) into MEM particles of 300, 500 and 800 microns size. The finished diets were dried at 40°C to a moisture content of less than 10% and stored in airtight containers under refrigerated conditions until use. During the feeding trial, the different feed sizes (300, 500 and 800 µ) were used for feeding 18-33, 34-47 and 48-63 day post-hatch (dph) milkfish larvae, respectively.

Experimental fish and feeding trial

Milkfish larvae were sourced from fish hatchery and feeding experiment was conducted during April to May 2020 at nutrition wet laboratory facility of Muttukadu Experimental Station (MES) of ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India. One thousand and five hundred larvae of 18-dph (mean weight 45.00±0.08 mg) were randomly distributed into 15 fibre reinforced plastic (FRP) rectangular tanks of 100 L capacity (n=100) in a flow through rearing system. Tanks were arranged in triplicate following a complete randomized design (CRD) to compare effects of five experimental dietary groups on milkfish larvae. All the major water quality parameters were regularly monitored and maintained in optimal levels without any variability among the dietary.

Determination of zootechincal indices and survival

The larvae were individually weighed in an electronic balance with an accuracy of 0.001 g (Shimadzu, BL220H) at the beginning and end of the experiment. To ascertain the growth and survival the larvae were counted individually and twenty larvae from each experimental tank were weighed fortnightly to ascertain the growth and feed requirement. Survival rate (%) was determined at the end of the experiment from each tank by counting the individual larvae. The total feed given during the experiment was recorded for each tank and used for estimating feed utilisation. Indices of growth, feed utilisation, body indices and larval survival were calculated based on standard formulae (SivaramaKrishnan *et al.,* 2017).

Proximate composition analysis of diets

The proximate composition of experimental diets was analysed according to the standard methods of AOAC (1995).

Analysis of fatty acid composition

Extraction of total lipids from feed and whole larvae was carried out as per the standard method (Folch *et al.,* 1957), with slight modifications. Following that, fatty acid methyl esters (FAME) was prepared by acid catalysed transme thylation of total lipids as described in the standard AOAC method (AOAC, 1995). For fatty acid composition analysis, separation of fatty acid methyl esters was performed using a gas chromatograph (GC-2014, Shimadzu, Japan), on an RT wax capillary column (100 m length × 0.25 mm internal diameter × 0.2 µm film thickness). Nitrogen was used as carrier gas at a linear velocity of 20.9 cm s^{-1} with 3 ml min^{-1} of purge flow. Individual fatty acids were identified by comparing the retention times with a 37 component standard FAME mixture (Supelco-Sigma, USA) as described by Sivaramakrishnan *et al.* (2021).

Statistical analysis

Data in the tables, figures and text is presented as mean ± standard deviation. All the variables were normalized and made homogeneousn of variance using the Kolmogorov-Smirnoff and Leven's tests respectively. The arcsine transformation was done for the data that did not comply with the normal distribution (rate of survival and deformity) before proceeding further statistical analysis. One-way analysis of variance (ANOVA), followed by Duncans' post-hoc multiple range test were used to estimate the statistical differences between the dietary treatments. All the statistical analyses were performed using the software SPSS V21.0.

RESULTS AND DISCUSSION

Feed intake, nutrient utilization, growth and survival

Milk-fish larvae studied in this experiment showed good palatability for all the diets as evidenced from excellent acceptability and there were no issues among encountered related to the intake of the micro-extruded and marumerised larval diets. The feed intake during the course of the experiment showed a decreas trend as the dietary corn oil inclusion level was enhanced, the differences being non-significant and highest feed intake observed in F3C1 dietary group was significantly different from other four experimental dietary groups.

After 42 days, there were significant differences in the survival rates and growth performances among the experimental groups (Table 2). Larvae fed with F3C1 diet showed the best final body weight (FBW), which was significantly different from those fed the other four experimental diet. Furthermore, growth parameters such as weight gain (WG), average daily gain (ADG), specific growth rate (SGR) and weight gain percentage (WG%) of larvae showed (P<0.05) significant differen up to 2% corn oil replaced diets, followed by fish fed with F1C3 and the lowest growth observed in C4 diet (Table 2). Similarly, the higher FER compared to larvae fed the other four experimental diets but there was no statistically significant difference among dietary treatment groups. Fish fed the C4 diet had a lower survival rate (52%) than fish on other dietary treatments. Based on comparative performance analysis among the dietary groups, the present study revealed that up to half the quantity of fish oil in milkfish larval diets can be replaced by corn oil (at the tested inclusion levels, *i.e.*, 2% fish oil and 2% corn oil F2C2 group), without affecting their growth parameters and wellbeing. Our earlier study showed that the optimal dietary soy lecithin inclusion level for larval diet of milkfish was 5.75 g kg^{-1} whereas 3.5 g kg^{-1} of dietary soy lecithin inclusion meets the minimal requirement (Sivaramakrishnan *et al*., 2021).

Whole body larvae fatty acid composition

The fatty acid composition of milk fish early fry at the end of the feeding experiment is presented in Table 3. In terms of fatty acid profile, the n-3 polyunsaturated fatty acids concentration in F2C2 diet accounted for 13.95% of total fatty acids (Table 3), which is equivalent to 1.71-2.0% of the

diet. This suggests that a dietary content of 1.71-2.0% of n-3 PUFA could meet the essential fatty acid requirement of milkfish and effectively support growth and survival during their early life stages. Similarly, other marine fishes such as red sea bream (Fujii *et al.,* 1976), turbot (Gatesoupe *et al*., 1977) and gilthead sea bream (Kalogeropoulos *et al*., 1992) and grouper (Lin and Shiau, 2007) showed maximal growth at 0.5, 0.8 and 1% of n-3 PUFA, respectively.

The presence of eicosatrienoic acid (20:1n-9) in tissues is an indicator of deficiency of essential fatty acid in striped bass and palmetto bass (Webster *et al*., 1994). Similarly, oleic acid (18:1 n-9) occurrence was also reported to be the indictor for the deficiency of essential fatty acids in red sea bream (Fujii *et al*., 1976) and gilthead sea bream (Rodriguez *et al*., 1994). In this study, 20:1n-9 was linearly increasing with increasing the dietary corn level and highest concentration

Table 2: Growth performance, feed utilisation and survival of *C. chanos* larvae fed experimental diets with graded replacement of fish oil with corn oil.

Parameters	F4	F3C1	F2C2	F1C3	C4
IBW (g)	0.045±0.08	0.045±0.08	0.045±0.08	0.045±0.08	0.045±0.08
FBW (g)	0.94^{a}±0.08	1.14^{b}±0.14	1.05^{ab}±0.12	0.93^{a}±0.08	0.89^{a}±0.03
WG (g)	0.90^{a}±0.07	1.10^{b}±0.14	1.01^{ab}±0.13	0.89^{a}+0.08	0.85^{a}±0.03
SGR	3.38^{a}±0.09	3.6^{b}±0.14	3.5^{ab}±0.13	3.36^{a}±0.10	3.32^{a}±0.04
WG (%)	2001^{a}±182.00	2450^{b}±316.55	2252^{ab}±196.95	1974^{a}±287.09	1882^{a}±87.50
ADG (mg/d)	15.01^{a}±2.13	18.38^{b}±1.48	16.66^{ab}±1.54	14.80^{a}±2.03	14.11^{a}±1.18
FER*^{NS}	0.99±0.06	1.08±0.05	0.99±0.37	0.92±0.10	0.95±0.06
PER*^{NS}	1.71±0.10	1.87±0.10	1.76±0.14	1.64±0.18	1.58±0.11
Survival (%)	86^{c}±4.0	87^{c}±4.0	87^{c}±3.0	74^{b}±2.0	52^{a}±6.0

IBW= Initial body weight; FBW= Final body weight; WG= Weight gain; SGR= Specific growth rate; ADG= Average daily growth rate; FER= Feed efficiency ratio; PER= Protein efficiency ratio. Data are presented as Means±SD (n=3) Values in the same row with different superscript differ significantly (P<0.05). *NS: Not significant.

Table 3: Whole body fatty acid composition of milkfish fed experimental diets with graded replacement of fish oil with corn oil (expressed as % of total fatty acids).

Fatty acid	F4	F3C1	F2C2	F1C3	C4	S.D
C14:0	4.61^{c}	4.34^{c}	2.90^{b}	2.38^{ab}	1.68^{a}	1.06
C15:0	0.60^{ab}	0.67^{c}	0.57^{ab}	0.64^{bc}	0.45^{a}	0.09
C16:0*^{NS}	23.66	24.24	21.11	20.09	19.34	2.17
C17:0	1.24^{b}	1.19^{b}	1.11^{ab}	0.92^{ab}	0.80^{a}	0.19
C18:0*^{NS}	6.91	6.47	7.01	6.06	6.17	0.43
C20:0	0.41^{a}	0.33^{ab}	0.35^{b}	0.33^{ab}	0.27^{a}	0.16
C21:0	0.77^{a}	0.87^{b}	1.13^{c}	1.36^{d}	1.56^{e}	0.33
C22:0	0.83^{c}	0.62^{b}	0.66^{b}	0.55^{ab}	0.47^{a}	0.14
SFA	39.03^{e}	38.73^{d}	34.84^{c}	32.33^{b}	30.47^{a}	3.80
C16:1	6.24^{c}	5.97^{c}	4.51^{b}	3.78^{b}	2.95^{a}	1.41
C18:1	15.10^{a}	16.89^{b}	18.57^{c}	21.06^{d}	22.42^{e}	2.98
C20:1 n9	0.47^{b}	0.43^{a}	1.33^{c}	1.91^{d}	2.45^{e}	0.89
MUFA	22.5^{a}	23.95^{b}	25.14^{c}	26.87^{d}	28.81^{e}	2.47
C18:2 n6	13.63^{a}	16.9^{b}	19.31^{c}	23.09^{d}	24.75^{e}	4.52
C18:3 n3	1.15^{a}	1.60^{b}	1.57^{b}	1.77^{c}	1.79^{c}	0.26
C20:3 n6	1.16^{a}	1.31^{b}	1.67^{c}	1.81^{c}	2.13^{d}	0.39
C20:4 n6	3.89^{c}	3.69^{c}	3.25^{b}	2.97^{a}	2.85^{a}	1.22
C20:5 n3	4.76^{c}	3.35^{c}	2.91^{bc}	2.20^{ab}	1.55^{a}	1.46
C22:6 n3	11.87^{b}	10.99^{b}	9.42^{a}	8.78^{a}	8.48^{a}	0.38
Total PUFA	36.46^{a}	37.89^{a}	38.13^{c}	40.62^{d}	41.55^{e}	0.08
n3 PUFA	17.78	15.94	13.9	12.75	11.82	0.22
n6 PUFA	18.68	21.9	24.23	27.87	29.73	1.26
n3/n6	0.95	0.72	0.57	0.45	0.39	0.09
ARA/EPA	0.81	1.10	1.11	1.35	1.83	3.10
EPA/DHA	0.40	0.30	0.30	0.25	0.18	2.42

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; S.D: Standard deviation; *NS: Not significant.

was observed in C4 group. As milkfish is a brackishwater fish with generally scanty D5-desaturase and it is likely that 20:1 n-9 can be produced. These findings were in agreement with our previous study in milkfish larvae where linoleic acid (C18:2 n-6) and oleic acid (C18:1 n-9) content increased with higher inclusion of soy lecithin in the experimental diets. In contrary presence of higher EPA and DHA level was associated with lower growth; whereas, the increasing proportion of phospholipid addition to neutral lipid was found to be superior for growth, feed utilisation and survival in the milkfish larvae (Sivaramakrishnan *et al*., 2021). A similar result also reported in European sea bass larvae (Cahu *et al*., 2003). The fatty acid bioconversion ability has been documented in the previous studies in milkfish as reported by Benitez and Gorricita (1985) the presence of significant amounts of PUFA in the liver in spite of their deprivation in the natural food. If the fish were on nutrient plane that was essentially fatty acid-deficient, it is more likely that 20:2 n-9 and/or 18:2 n-9 would be produced (Lin and Shiau, 2007).

According to, Wu *et al.* (2002) and Lin and Shiau (2007) a high tissue 20:1 n-9 concentration in tissues of grouper to be a indicator deficiency of essential fatty acid and in the present study, elevated 20:1 n-9 concentrations in milkfish larvae fed the F1C3 and C4 diets (Table 3) also indicate a sign of essential fatty acid deficiency. This may explain the poor growth of the two groups. The omega-3 fatty acids such as alpha linolenic acid, EPA and DHA were critical fatty acids which is more important than omega-6 fatty acid like. linoleic acid for milkfish larvae (Borlongan, 1992; Borlongan and Benitez, 1992; Sivaramakrishnan *et al.,* 2021). The whole-body nutrient composition of milkfish larvae fed with diet containing various lipid sources in the diets must have influenced by the composition of the dietary fatty acids (Borlongan and Benitez, 1992; Kumar *et al.,* 2014; Balito-Liboon *et al.,* 2018; Sivaramakrishan *et al.,* 2021).

In the current work, DHA (docosahexaenoic acid, 22:6 n-3) and EPA (eicosapentaenoic acid, 20:5 n-3) concentrations in the F4 diet were 11.87 and 4.76% of the total lipid concentration respectively (Table 3), which was equivalent to 1.47 and 0.59% of diet. The n3/n6 ratio in this diet was calculated to be about 0.95. This ratio seemed to be an equal against earlier recommendation of n3/n6 1 to for enhanced growth of marine fishes (Lin and Shiau 2007; Wu *et al.,* 2002). It is interesting to observe growth of milk fish larvae was actually suppressed when fed diet having n3/n6 ratio is 0.57. Thus, the F4 diet (n3/n6: 0.95) of the current study meets the requirement and is to be used as the requirement.

CONCLUSION

The present study is revealing that milkfish larvae require 1.7-2.0% of n-3 PUFA and 2.7-3.0 % of n-6 PUFA (n-3 to n-6 ratio of 0.6-0.7) to support maximum growth and survival. DHA (docosahexaenoic acid, 22:6 n-3) and EPA (eicosapentaenoic acid, 20:5 n-3) concentrations in the F4 diets were 11.87 and 4.76% of the total lipid concentration, which is equivalent to 1.47 and 0.59% of diet, respectively. The higher abundance of n-9 monounsaturated fatty acids (C18:1 and C20:1) in F1C3 and C4 dietary groups possibly indicates essential fatty acid deficiency, which might underlie the lower fish survival observed in these groups. Overall, this study reveals that growth and survival of milkfish is dependent on dietary lipid source or combinations that meet the essential fatty acid requirements during the early life stages. Further investigations are needed to investigate the fatty acid biosynthesis pathway as well as potential in milkfish larvae.

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