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Enrichment of live food with essential fatty acids and vitamin C: effects on milkfish (*Chanos chanos*) larval performance

R.S.J. Gapasin [a,*], R. Bombeo [a], P. Lavens [b], P. Sorgeloos [b], H. Nelis [c]

[a] *Aquaculture Department Southeast Asian Fisheries Development Center Tigbauan, 5021 Iloilo, Philippines*
[b] *Laboratory of Aquaculture and Artemia Reference Center, Rozier 44, B-9000 Ghent, Belgium*
[c] *Laboratory of Pharmaceutical Microbiology, Harelbekestraat 72, B-9000 Ghent, Belgium*

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Abstract

The effects of essential fatty acids (EFA) and vitamin C-enriched live food on growth, survival, resistance to salinity stress and incidence of deformity in milkfish larvae reared in tanks were investigated. Larvae were either fed rotifers cultured on *Chlorella* sp. and newly hatched *Artemia* nauplii (control), highly unsaturated fatty acid (HUFA)-enriched rotifers and *Artemia* nauplii or HUFA + vitamin C-enriched rotifers and *Artemia* nauplii. Milkfish growth in outdoor nursery ponds was also assessed to compare with growth in indoor tanks. Milkfish fed rotifers/*Artemia* enriched with HUFA (32–48 mg dry weight, DW) or HUFA + vitamin C (33–45 mg DW) exhibited significantly ($P < 0.05$) higher growth than those given unenriched live food (24–27 mg DW) after 40 days of culture. Growth of milkfish in nursery ponds (albeit lower in stocking density) showed similar trends as those reared in tanks. When subjected to salinity stress (Day 25), mortality of the HUFA + vitamin C-treated fish and HUFA-treated fish were significantly lower ($P < 0.05$) than the control fish. Survival of 26-day old milkfish, however, did not differ significantly ($P > 0.05$) among the treatment groups. Forty-day-old milkfish fed HUFA + vitamin C-enriched live food had significantly lower ($P < 0.05$) incidence of opercular deformity (mainly cleft branchiostegal membrane) (8.4–14.7%) compared with those given HUFA-enriched (15.8–23.5%) or unenriched (27.3–33.5%) live food. Results demonstrated the effect of HUFA enrichment in enhancing milkfish larval growth and resistance to salinity stress but not overall survival. Moreover, HUFA and ascorbate supplementation decreased but did

* Corresponding author. Tel.: +63-33-335-1009; fax: +63-33-335-1008; e-mail: seafdec@mozcom.com.

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not totally eliminate incidence of opercular deformity in milkfish larvae.

Keywords: Essential fatty acids; Vitamin C; *Chanos chanos*; Growth; Survival; Deformity; Stress resistance

1. Introduction

Milkfish is an important food fish widely cultured in Southeast Asia notably in Taiwan, the Philippines and Indonesia. About 151,000 metric tons of milkfish were harvested in 1996, valued close to US$380 million (Philippine Fisheries Profile, 1996). Declining supply of milkfish fry from the wild coupled with increased domestic and international demands necessitate a reliable source of hatchery-produced seed to stabilize the milkfish industry. Despite more than a decade of milkfish breeding and seed production studies at SEAFDEC/AQD (Chaudhuri et al., 1978; Liao et al., 1979; Juario et al., 1984; Marte and Lacanilao, 1986; Marte et al., 1988; Gapasin and Marte, 1990; Villegas, 1990; Villegas et al., 1990; Marte and Duray, 1991), problems such as larval mass mortalities, incidences of deformities and variable production are still experienced. Improvements in larval nutrition are necessary to solve some of these problems to come up with a viable and dependable milkfish larviculture technology for commercial scale application.

The polyunsaturated fatty acids, specifically eicosapentaenoic acid (EPA, 20:5$n-3$) and docosahexaenoic acid (DHA, 22:6$n-3$), have been shown to be essential in the diet of marine fish larvae (Watanabe, 1993; Rainuzzo et al., 1995). EFA deficiency signs include poor growth, low feed efficiency, anemia and high mortality (Takeuchi et al., 1979; Roberts and Bullock, 1989; Sargent et al., 1989. Although Bautista and de la Cruz (1988) and Borlongan (1992) demonstrated the nutritional importance of $n-3$ vs. $n-6$ fatty acids in milkfish fingerlings and juveniles, information on the essential fatty acid requirement, particularly EPA and DHA, in milkfish larvae is limited.

Ascorbic acid, or vitamin C, is required in larval fish diets (Sandnes, 1991). Scoliosis, distorted/twisted gill filaments, short operculae and snout are some of the gross signs of ascorbate deficiency (Soliman et al., 1986; Chavez de Martinez, 1990; Dabrowski, 1990). Among hatchery-reared milkfish larvae and postlarvae, opercular deformities have been reported (Brock et al., 1993; Hilomen-Garcia, 1997). May et al. (1979) observed similar abnormalities, as well as scoliosis, in the larvae of the Pacific threadfin, *Polydactylus sexfilis*.

This study was designed to assess the effectiveness of EFA and ascorbate supplementation in improving growth, survival, stress resistance and in eliminating deformities in milkfish larvae.

2. Materials and methods

2.1. Rotifer enrichment

Rotifers (*Brachionus plicatilis*) were intensively cultured in 200-l cylindro-conical fiberglass tanks and fed a formulated artificial diet (Culture Selco, Inve Aquaculture,

Baasrode, Belgium) following the method of Lavens et al. (1994) with modification. Harvested rotifers were then divided into two lots in 30-l plexiglass tanks. Rotifers in the first group were enriched with HUFA booster diet (Protein Selco, Inve Aquaculture, Baasrode, Belgium), while the second group received Protein Selco supplemented with vitamin C (20% ascorbyl palmitate (AP) inclusion). Ascorbyl palmitate administered through live food organisms has been tested successfully as a dietary vitamin C source for fish (Merchie et al., 1995). In this study, Protein Selco suspension was given in 2 rations (9–10 AM and 7–8 PM) and rotifers were harvested after 24 h enrichment period. Rotifers cultured on the green algae, *Chlorella* sp., served as control.

Rotifers given different diets were sampled regularly and kept at −80°C prior to analysis for fatty acid methyl esters (modified Lepage and Roy, 1984) and ascorbic acid (Nelis et al., 1997). For rotifer dry weight (DW) measurement, a separate 100–150 mg wet samples (FAME analysis, $n = 3$) and 200-mg wet samples (vitamin C analysis, $n = 3$–5) were taken from same batch samples to be analyzed, oven-dried (60°C, 24 h) in preweighed aluminum cups, cooled in a dessicator, weighed and water content calculated.

2.2. *Artemia* enrichment

Artemia cysts (Great Salt Lake, Artemipak brand) were hatched following standard procedures (Sorgeloos et al., 1986). Newly hatched *Artemia* (Instar I) nauplii were divided into two batches in 30-l plexiglass tanks. Enrichment protocol followed the method of Leger et al. (1987). Nauplii in the first tank were given HUFA enrichment (Selco emulsion, Inve Aquaculture, Baasrode, Belgium) at 0.6 g Selco/l of seawater administered in 2 rations. The second tank received Selco plus vitamin C (20% AP inclusion). As in rotifers, enrichment emulsion was given in 2 rations, and nauplii were harvested after 24 h. Newly hatched *Artemia* nauplii served as the control.

Samples of unenriched and enriched *Artemia* were also taken regularly and stored at −80°C. These were later analyzed for fatty acid methyl esters and vitamin C, as well as determination of dry weight (on separate samples) following the same procedures as for rotifers.

2.3. Egg source and incubation

Due to the unpredictability of broodstock spawning, milkfish eggs used in the experiment came from 3 different production cages maintained by SEAFDEC/AQD's Igang Marine Substation (2 egg batches from Cage 60 and 1 egg batch each from Cages 54 and 57). Egg collection and hatching was performed according to standard practice described in Gapasin and Marte (1990).

2.4. Larval culture

Newly hatched (Day 0) milkfish larvae were stocked (30 larvae/l) in 15 circular, flat-bottom fiberglass rearing tanks filled with 350-l filtered seawater. Larval rearing protocol followed the method described by Gapasin and Marte (1990) with modification (Fig. 1).

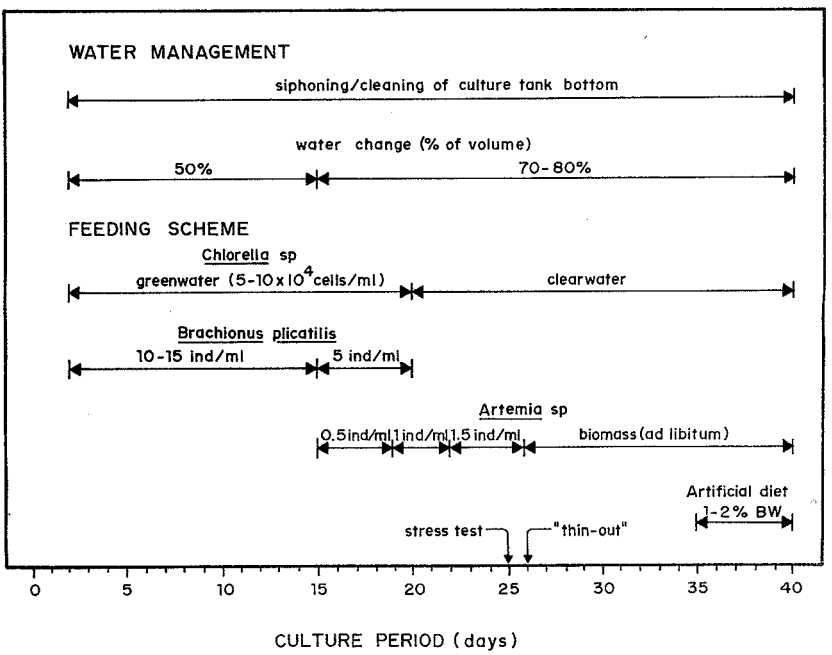

Fig. 1. Water management and feeding scheme for rearing of milkfish (modified from Gapasin and Marte, 1990).

The three treatments (in a completely randomized design with 5 replicates per treatment) were: (I) larvae fed rotifers cultured on *Chlorella* sp. and newly hatched *Artemia* nauplii; (II) larvae fed HUFA-enriched rotifers and *Artemia* nauplii; and (III) larvae fed HUFA + vitamin C-enriched rotifers and *Artemia* nauplii. Treatment I served as control, as this is the milkfish larval rearing procedure practised at SEAFDEC/AQD (Gapasin and Marte, 1990) and elsewhere (Eda et al., 1990; Tamaru et al., 1993). Larval culture for all treatments was conducted using 'greenwater' (*Chlorella* sp. density: $5–10 \times 10^4$ cells/ml). A total of 4 larviculture trials was conducted. Physico-chemical variables were monitored every morning prior to water change and feeding. Water quality was within optimum range: temperature (28.4–30.9°C), salinity (29.3–31.5 g/l), dissolved oxygen (5.6–6.2 mg/l), pH (7.2–7.8), nitrite (0.2–0.3 mg/l) and total ammonia (0.2–0.5 mg/l).

2.5. Morphometrics and growth performance

Ten larvae were randomly sampled from each replicate tank every 10 days (until Day 40). Samples were oven-dried at 60°C for 24 h and constant weights determined.

Twenty five day-old milkfish larvae were subjected to salinity stress test following the method described by Dhert et al. (1992). Briefly, the test involved immersing the fish (10 larvae/replicate tank) in a pre-aerated 65 g/l (saline) medium and mortality was recorded every 5-min interval. The test was terminated once 100% mortality was observed in any of the replicate samples.

Fish larvae were harvested and individually counted at Day 26. Larval samples from each treatment group (pooled from 4 trials) were analyzed for fatty acids and vitamin C following the methods of Lepage and Roy (1984) and Nelis et al. (1997), respectively. Determination of fish dry weight followed the same method used for rotifers. Survival was expressed as a percentage of the total number harvested over the initial stock. Larvae from the surviving stock were then randomly sampled and restocked (or 'thinned-out') in the same tank. Stocking was based on the lowest survival count. Fish were reared until Day 40 (age at which deformities are readily perceived by the naked eye) following the rearing protocol in Fig. 1. Random samples ($n = 100$) from each of the replicate tanks were collected with a glass beaker and individually examined for deformities, i.e., live fish were viewed ventrally from the beaker bottom and categorized as deformed when the branchiostegal membrane is cleft with the gills exposed, and normal when the branchiostegal membrane is intact and completely covers the gills. Deformity was expressed as percentage of abnormal fish relative to total fish sampled.

To assess growth performance under natural conditions, excess 26-day-old milkfish from the 'thinned-out' population were pooled according to treatment and stocked separately in three 10 m × 100 m earthen nursery ponds (Dumangas, Iloilo, Philippines) at a density of 3–4 larvae/m^2. The ponds were fertilized and an algal mat ('lab-lab') was allowed to grow abundantly prior to stocking the fish. After 6 weeks of extensive culture (fish subsist on natural food only and were not given any supplementary feed), 20 fish (Day 68) from each pond were randomly sampled, anaesthesized with 2-phenoxy-ethanol, and total length (TL) and wet weight (WW) taken. As there was only one pond per treatment, two replicate trials were conducted.

2.6. Statistical analysis

Length, weight, survival and deformity data were log- or arcsine-transformed where appropriate before subjecting to one-way analysis-of-variance (ANOVA) followed by Duncan's multiple range test (DMRT) to determine significant differences among treatment means at $\alpha = 0.05$.

Linear regression curves of fish mortality (after salinity stress test) per treatment were calculated and subjected to regression analysis at $\alpha = 0.05$. All analyses were conducted using the SAS program (SAS Institute, 1988).

3. Results

3.1. Rotifer enrichment

Fatty acid profiles of rotifers given different diets is presented in Table 1. Absolute amounts of 14:0, 16:0, 16:1$n-7$ and 20:4$n-6$ were generally higher in *Chlorella*-cultured rotifers than in HUFA- or HUFA + vitamin C-enriched rotifers. However, the levels of 18:1$n-9$, 18:2$n-6$, 18:3$n-3$ and 20:1$n-9$ were higher in the fish fed enriched diet than the control. Although rotifers cultured on *Chlorella* sp. contained higher amounts of $n-3$ and $n-6$ polyunsaturates than rotifers enriched with HUFA or

Table 1
Certain fatty acids (mg FA/g DW) of the rotifers *Brachionus plicatilis* cultured on *Chlorella* sp. (A), rotifers enriched with HUFA (B), and rotifers enriched with HUFA + vitamin C (C)

Fatty acid	A	B	C
14:0	2.67 ± 0.42	1.35 ± 0.35	1.35 ± 0.35
16:0	15.00 ± 3.00	7.60 ± 0.85	10.35 ± 1.41
16:1*n* − 7	8.60 ± 1.21	4.70 ± 1.76	3.60 ± 1.27
18:0	2.80 ± 0.44	3.20 ± 0.42	2.90 ± 0.57
18:1*n* − 7	2.80 ± 0.53	2.30 ± 0.56	1.75 ± 0.49
18:1*n* − 9	2.47 ± 0.57	10.95 ± 2.76	8.30 ± 2.26
18:2*n* − 6	2.90 ± 0.36	5.25 ± 0.78	4.60 ± 0.85
18:3*n* − 3	0.10 ± 0.00	0.80 ± 0.14	0.65 ± 0.21
18:4*n* − 3	0.10 ± 0.00	0.15 ± 0.07	0.15 ± 0.07
20:1*n* − 9	0.80 ± 0.10	1.55 ± 0.35	1.25 ± 0.49
20:4*n* − 3	0.13 ± 0.06	0.70 ± 0.42	0.75 ± 0.07
20:4*n* − 6	3.57 ± 0.46	1.00 ± 0.14	0.80 ± 0.14
20:5*n* − 3	9.60 ± 2.09	4.10 ± 0.14	3.05 ± 0.35
22:5*n* − 3	4.37 ± 1.04	2.20 ± 0.00	1.70 ± 0.14
22:6*n* − 3	0.40 ± 0.00	2.90 ± 0.28	2.25 ± 0.35
mg FA / g DW			
Σ*n* − 3	14.70 ± 3.10	11.10 ± 1.13	8.75 ± 1.20
Σ*n* − 6	8.21 ± 0.29	6.80 ± 0.71	5.95 ± 0.78
ΣFA	61.01 ± 8.50	52.60 ± 8.77	46.95 ± 9.12
DHA/EPA[1]	0.04 ± 0.01[b]	0.71 ± 0.04[a]	0.74 ± 0.03[a]
Σ*n* − 3/Σ*n* − 6[1]	1.79 ± 0.26	1.63 ± 0.00	1.47 ± 0.01

Data are mean ± S.D. of 2–3 assays.
[1] Values within rows with different letter superscripts are significantly different ($P < 0.05$).

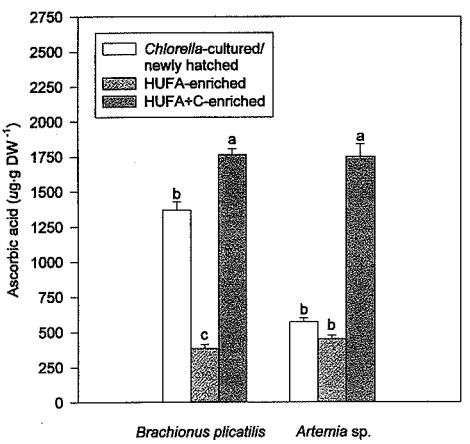

Fig. 2. Ascorbic acid levels (mean ± S.E.M.) in two live food organisms: *Brachionus plicatilis*—was either *Chlorella*-cultured ($n = 8$ assays), HUFA-enriched ($n = 10$ assays) or HUFA + vitamin C-enriched ($n = 7$ assays). *Artemia* sp.—was either newly hatched nauplii ($n = 5$ assays), HUFA-enriched ($n = 5$ assays) or HUFA + vitamin C-enriched ($n = 5$ assays). For each organism, different letter symbols denote treatment means that are significantly different ($P < 0.05$).

HUFA + vitamin C, the $n-3{:}n-6$ ratios did not differ significantly among the treatment groups. While rotifers reared on *Chlorella* sp. had higher levels of EPA than those enriched with HUFA or HUFA + vitamin C, its DHA level was the lowest among the treatment groups. Moreover, the DHA:EPA ratio in the *Chlorella*-cultured rotifers (0.04) was significantly lower compared with the HUFA-enriched (0.71) or HUFA + vitamin C-enriched (0.74) rotifers. Between the latter two, the DHA:EPA ratios were not significantly different.

Ascorbate levels in rotifers given different diets is shown in Fig. 2. HUFA + vitamin C-enriched rotifers had significantly higher amounts of ascorbic acid compared with the rotifers cultured on *Chlorella* sp. or enriched with HUFA only. As expected, the HUFA-enriched rotifers had the lowest ascorbate levels, since there was no ascorbyl palmitate (AP) supplementation.

3.2. *Artemia* enrichment

Fatty acid content of newly hatched and enriched *Artemia* is shown in Table 2. Unlike in rotifers, the individual fatty acid levels were consistently higher (except for $18{:}3n-3$) in the HUFA- and HUFA + vitamin C-enriched nauplii than in the newly

Table 2
Certain fatty acids (mg FA/g DW) of newly hatched *Artemia* nauplii (A), *Artemia* enriched with HUFA (B), and *Artemia* enriched with HUFA + vitamin C (C)

Fatty acid	A	B	C
14:0	1.40 ± 0.00	3.10 ± 0.57	2.25 ± 0.21
16:0	17.25 ± 0.64	21.25 ± 1.77	20.45 ± 1.34
$16{:}1n-7$	7.05 ± 0.21	11.85 ± 0.49	7.60 ± 0.28
18:0	6.05 ± 0.35	7.35 ± 0.78	7.35 ± 0.78
$18{:}1n-7$	12.60 ± 0.71	14.55 ± 0.78	13.00 ± 0.14
$18{:}1n-9$	28.95 ± 1.63	36.30 ± 2.55	29.85 ± 0.49
$18{:}2n-6$	8.75 ± 0.49	13.75 ± 1.06	9.40 ± 0.57
$18{:}3n-3$	32.20 ± 1.13	26.85 ± 1.63	24.15 ± 0.49
$18{:}4n-3$	3.95 ± 0.07	3.75 ± 0.35	2.75 ± 0.35
$20{:}1n-9$	0.60 ± 0.10	1.45 ± 0.21	1.15 ± 0.21
$20{:}4n-3$	0.55 ± 0.07	1.65 ± 0.21	0.80 ± 0.14
$20{:}4n-6$	1.70 ± 0.14	3.45 ± 0.21	2.70 ± 0.28
$20{:}5n-3$	8.00 ± 0.42	32.90 ± 3.11	19.80 ± 0.57
$22{:}5n-3$	–	2.85 ± 0.49	1.70 ± 0.14
$22{:}6n-3$	0.10 ± 0.00	13.65 ± 1.20	6.50 ± 0.57
mg FA / g DW:			
$\Sigma n-3$	45.45 ± 1.77	82.23 ± 3.89	56.55 ± 1.91
$\Sigma n-6$	11.05 ± 0.64	18.35 ± 1.06	12.80 ± 0.85
ΣFA	135.00 ± 6.22	201.33 ± 10.68	154.80 ± 1.41
DHA/EPA[1]	0.01 ± 0.00[c]	0.41 ± 0.00[a]	0.33 ± 0.02[b]
$\Sigma n-3/\Sigma n-6$[1]	4.11 ± 0.08	4.48 ± 0.47	4.42 ± 0.14

Data are mean ± S.D. of 2 assays.
[1] Values within rows with different letter superscripts are significantly different ($P < 0.05$).

hatched nauplii. The PUFA $n-3$:$n-6$ ratio of the newly hatched *Artemia* nauplii did not differ significantly compared with the HUFA-enriched or HUFA + vitamin C-enriched *Artemia*. However, the EPA and DHA levels were highest in the HUFA-enriched *Artemia* followed by the HUFA + vitamin C-enriched *Artemia* with the newly hatched *Artemia* having the lowest amount of EPA and DHA. The DHA:EPA ratio of the HUFA-enriched *Artemia* (0.42) was significantly higher compared with HUFA + vitamin C-enriched *Artemia* (0.33) or the newly hatched *Artemia* nauplii (0.01). The HUFA-enriched *Artemia*, on the other hand, had a significantly higher DHA:EPA ratio than the newly hatched *Artemia*.

Vitamin C concentration in *Artemia* nauplii fed different diets is shown in Fig. 2. *Artemia* enriched with HUFA + vitamin C had significantly higher ascorbate concentration than the newly hatched *Artemia* or the HUFA-enriched *Artemia*. Ascorbic acid levels of the latter two were not significant.

3.3. Fatty acid and ascorbate levels in milkfish larval tissues

Table 3 shows the whole-body fatty acid profile of milkfish larvae given different diets. Tissue fatty acid levels in fish fed enriched diets were generally higher than those fed the unenriched diets. Noteworthy were the levels of palmitic (16:0), oleic (18:1$n-9$),

Table 3
Whole-body fatty acid composition (mg FA/g DW) of 26-day old milkfish larvae fed *Chlorella*-cultured rotifers/newly hatched *Artemia* nauplii (A), larvae fed HUFA-enriched rotifers/*Artemia* nauplii (B), and larvae fed HUFA + vitamin C-enriched rotifers/*Artemia* nauplii (C)

Fatty acid	A	B	C
14:0	0.60	1.00	1.40
16:0	18.30	20.50	22.70
16:1$n-7$	4.60	7.60	6.60
18:0	8.10	10.20	10.00
18:1$n-7$	9.80	13.10	11.80
18:1$n-9$	20.10	27.30	24.30
18:2$n-6$	5.00	7.90	7.00
18:3$n-3$	12.40	12.40	12.90
18:4$n-3$	1.80	1.60	1.50
20:1$n-9$	0.50	1.00	0.70
20:4$n-3$	1.80	1.60	1.20
20:4$n-6$	3.40	3.80	3.80
20:5$n-3$	7.80	10.00	10.20
22:5$n-3$	4.00	5.40	4.40
22:6$n-3$	2.30	12.10	10.40
mg FA / g DW			
$\Sigma n-3$	30.80	44.10	41.20
$\Sigma n-6$	9.90	12.90	11.90
ΣFA	105.70	141.30	139.90
DHA/EPA	0.29	1.21	1.02
$\Sigma n-3/\Sigma n-6$	3.11	3.42	3.46

Fatty acids were determined from a single pooled sample.

eicosapentaenoic (20:5$n-3$) and docosahexaenoic (22:6$n-3$) acids. The DHA:EPA ratio was quite low (0.29) in the fish fed *Chlorella*-cultured rotifers/newly hatched *Artemia* nauplii (control) compared with those given HUFA-enriched rotifers and *Artemia* (1.21) or HUFA + vitamin C-enriched rotifers/*Artemia* (1.02). The docosahexaenoic acid (DHA) levels in the group treated with HUFA (12.10) and those treated with HUFA + vitamin C (10.40) were much higher than the control group (2.30). The $n-3$:$n-6$ ratios in the 3 treatment groups, however, were comparable.

No tissue ascorbate data on milkfish larvae fed different diets were available, as fish samples deteriorated during transport to Belgium.

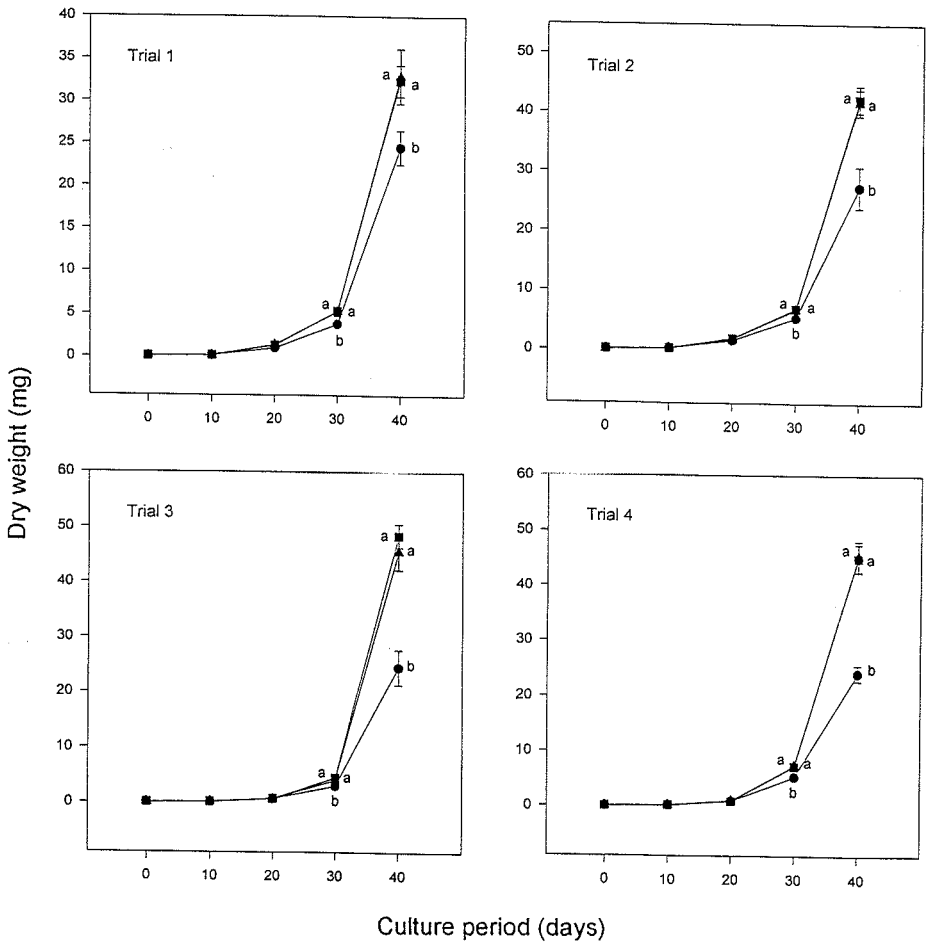

Fig. 3. Growth rates of 40-day-old milkfish fed *Chlorella*-cultured rotifers/newly hatched *Artemia* nauplii (●), fish fed rotifers/*Artemia* enriched with HUFA (■) and fish fed rotifers/*Artemia* enriched with HUFA + vitamin C (▲). Each point represents mean dry weight (± S.E.M.) of 30–50 fish samples. For each time point, different letter symbols denote means that are significantly different ($P < 0.05$). Four trials were conducted.

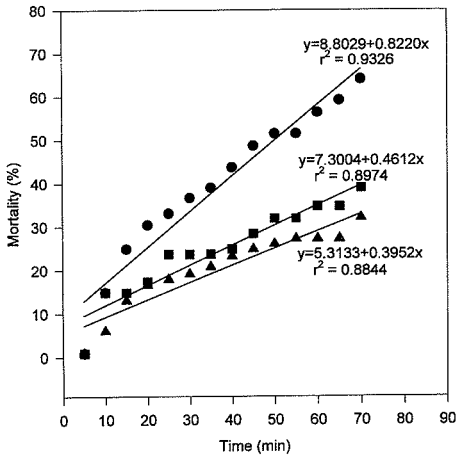

Fig. 4. Mortality rates of 25-day-old milkfish subjected to salinity stress. Fish were fed *Chlorella*-cultured rotifers/newly hatched *Artemia* nauplii (●), fish fed rotifers/*Artemia* enriched with HUFA (■) and fish fed rotifers/*Artemia* enriched with HUFA + vitamin C (▲). Each data point represents mean of 5 replicates.

3.4. Larval performance

Milkfish fed rotifers and *Artemia* enriched with HUFA (32–48 mg DW) or HUFA + vitamin C (33–45 mg DW) exhibited significantly higher ($P < 0.05$) growth rates than those given the unenriched diet (24–27 mg DW) after 40 days of culture (Fig. 3).

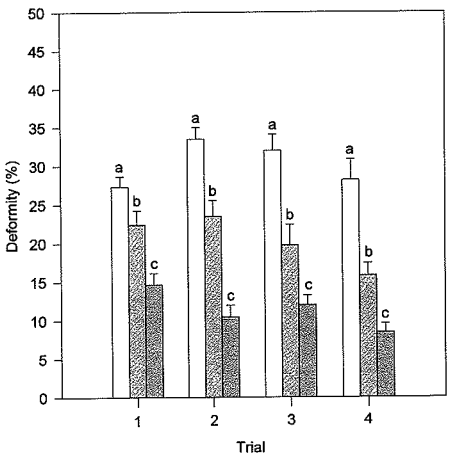

Fig. 5. Percentage incidence of opercular deformity (mainly cleft branchiostegal membrane) among 40-day-old milkfish fed *Chlorella*-cultured rotifers/newly hatched *Artemia* nauplii (blank square with shadow), fish fed rotifers/*Artemia* enriched with HUFA (right diagonal-shaded square with shadow) and fish fed rotifers/*Artemia* enriched with HUFA + vitamin C (left diagonal-shaded square with shadow). For each trial, different letter symbols denote treatment means (±S.E.M.) that are significantly different ($P < 0.05$).

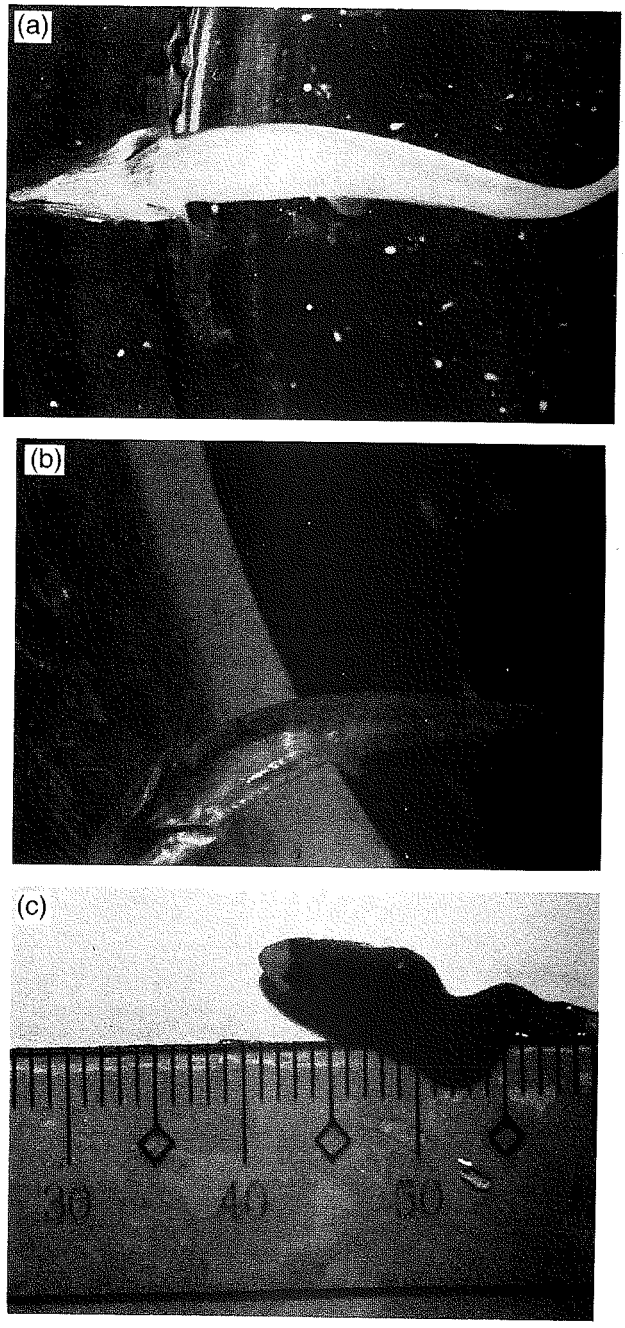

Fig. 6. Fish that is normal with branchiostegal membrane intact (a), deformed with cleft branchiostegal membrane (b) and scoliotic (c).

Although growth differences could already be seen after 30 days of culture, significant results were apparent at Day 40. Growth trends were consistent in all the 4 trials and observed to be relatively better in the HUFA-treated or HUFA + vitamin C-treated fish compared with the control. In terms of $n-3{:}n-6$ ratios, the values were comparable among the treatment groups (Table 3).

When 25-day-old milkfish were subjected to salinity stress, mortality rates (i.e., slopes) of the HUFA + vitamin C-treated and HUFA-treated fish were significantly lower ($P < 0.05$) than the untreated fish (Fig. 4).

Fish treated with HUFA + vitamin C (8.4–14.7%) had significantly lower incidence of opercular deformity (mainly branchiostegal membrane) compared with those treated with HUFA only (15.8–23.5%) or the control (27.3–33.5%) fish (Fig. 5). Fish having intact branchiostegal membrane were considered normal (Fig. 6a), while those with cleft branchiostegal membrane exposing the gills were regarded as deformed (Fig. 6b) (Hilomen-Garcia, 1997). Ascorbate supplementation decreased, but did not totally eliminate incidence of opercular deformity in milkfish larvae. Scoliotic larvae (Fig. 6c) were encountered but constituted a mere 0.1% of the HUFA-treated or control fish populations in trial 3 only.

After 26 days of culture, percentage survival did not differ significantly among the treatment groups in each trial (Fig. 7). However, survival was not consistent in the 4 trials, and this may be attributed to the eggs coming from different broodfish and different spawned-egg batches. Eggs used in trial 1 came from Cage 57 broodstock, trial 3 egg batch came from Cage 54 spawners, and trials 2 (July-spawned egg batch) and 4 (October-spawned egg batch) came from Cage 60 broodfish.

When reared extensively in earthen nursery ponds, milkfish that were fed (during the hatchery phase) live food enriched with HUFA (117.9 ± 3.2 mm TL/14.5 ± 0.8 g WW)

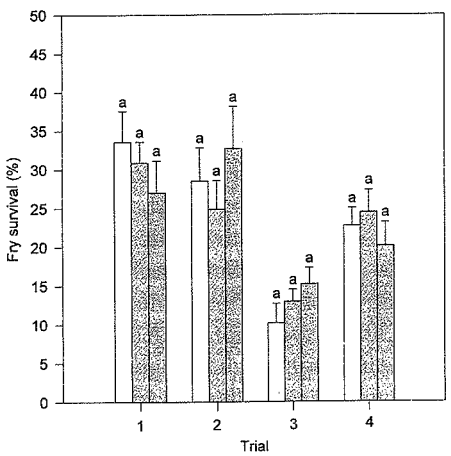

Fig. 7. Percentage survival of 26-day-old milkfish fed *Chlorella*-cultured rotifers/newly hatched *Artemia* nauplii (blank square), fish fed rotifers/*Artemia* enriched with HUFA (left diagonal-shaded square with shadow) and fish fed rotifers/*Artemia* enriched with HUFA + vitamin C (right diagonal-shaded square with shadow). Different letter symbols denote treatment means (± S.E.M.) that are significantly different ($P < 0.05$).

or fed HUFA + vitamin C (119.0 ± 2.5 mm TL/14.6 ± 0.7 g WW) exhibited significantly better growth ($P < 0.05$) than those given the unenriched diet (108.1 ± 3.0 mm TL/11.4 ± 0.6 g WW). Milkfish growth in nursery ponds followed a similar trend as those in tanks.

4. Discussion

Several studies have demonstrated the positive effect of enriched live food on the growth performance of various aquaculture species. Striped bass and palmetto bass larvae given HUFA-enriched *Artemia* nauplii exhibited better growth and survival (Tuncer and Harrell, 1992; Ozkizilcik and Chu, 1994). Gilthead sea bream larvae also grow better if fed rotifers enriched with high $n-3$ HUFA (Mourente et al., 1993). In contrast to the findings of Tamaru et al. (1993), the present study did not find any significant differences in the growth of 10-day-old milkfish larvae fed different diets; however, HUFA- and HUFA-treated fish exhibited significant growth than the control after 30 days of culture (Fig. 3.). On the other hand, survival of 26-day-old milkfish fed various diets were not significantly different corroborating the results of Tamaru et al. (1993). Corollary to the preceding observations, Tuncer and Harrell (1992) pointed out that growth is more sensitive than survival in determining essential fatty acid deficiency in fish.

Red sea bream larvae given rotifers enriched with high DHA showed better growth and higher survival after a vitality test (Watanabe et al., 1989). Mourente et al. (1993) reported best growth rate in gilthead sea bream larvae given enriched rotifers high in DHA:EPA ratio. When 15-day-old mullet larvae were exposed to air in a stress test, Ako et al. (1994) observed no or few mortalities among fish fed *Artemia* enriched with menhaden oil (high DHA:EPA ratio) compared to high mortalities among fish fed unenriched *Artemia*. Red sea bream and marble sole larvae given diets containing DHA and lecithin tolerated temperature and salinity changes, low oxygen and air exposure better than the larvae given DHA and lecithin-free diets (Kanazawa, 1995). Furuita et al. (1996a,b) reported that yellowtail larvae and red sea bream juveniles fed *Artemia* enriched with DHA exhibited higher survival in a vitality test than those fed *Artemia* enriched with EPA. In the present study, milkfish larvae fed rotifers and *Artemia* enriched with HUFA or HUFA + vitamin C (high DHA:EPA ratios) showed better growth and increased resistance to salinity stress than those given unenriched diet (low DHA:EPA ratio) corroborating previous findings. The high body tissue DHA:EPA ratios of 1.21 (in fish fed HUFA-enriched live food) and 1.02 (in fish fed HUFA + vitamin C-enriched live food) compared to a low 0.29 (in fish fed unenriched diet) seemed to suggest higher biological value of DHA over EPA in fish, notably marine species, as proposed by Watanabe et al. (1989), Koven et al. (1993) and Zheng et al. (1995). In related studies by Tocher and Harvie (1988) and Bell and Dick (1991), the DHA:EPA ratios in fish brain and neural tissues were found to be higher compared to all other tissues. Bell and Dick (1993) found that the appearance of retinal rods correlates closely with the appearance of di-22:6$n-3$ phospholipids in the larva's eyes. Tocher et al. (1992) and Masuda et al. (1995) showed that $^{14}C-DHA$ was incorporated in the brain

of juvenile flounder and retina, brain, and vertebrae of yellowtail larva, respectively. Low predation efficiency were observed among herring larvae fed *Artemia* nauplii deficient in 22:6$n-3$ (Bell et al., 1995). These reports underscore the importance of DHA in the neural and visual functions of the larval fish. Fish larvae are generally visual feeders.

Ascorbic acid is an important micronutrient in fish. It is needed in the synthesis of collagen necessary in the formation of connective tissues and bone matrix (Sandel and Daniel, 1988). Using ^{14}C-radiolabelled ascorbic acid, Halver (1972) was able to identify the skin, caudal fin, snout cartilage, head and jaw, gill support cartilage and bones as collagen-forming areas in the fish. Scorbutic fish has been shown to exhibit abnormal support cartilage in the gills (Halver, 1989). Dabrowski et al. (1990) reported that inadequate intake of dietary ascorbic acid results in de-calcification, which lead to twisted gill filaments in rainbow trout. Cleft branchiostegal membrane, an opercular abnormality commonly observed among milkfish fry and juveniles, has been reported to be associated with deformity or absence of branchiostegal rays (Hilomen-Garcia, 1997). Ascorbate deficiency in the diet could thus affect the development of the gill support structures such as the branchiostegal rays. In the study, HUFA and ascorbate supplementation alleviated incidence of opercular deformity in milkfish (Fig. 5), possibly indicating that the syndrome may be an ascorbate deficiency-related case. Distortion of gill filament cartilages and short opercules were observed in scorbutic fish (Lim and Lovell, 1978; Soliman et al., 1986). Resorbed operculae observed among salmonids (Halver, 1957, 1989) and the Mexican native cichlid have been traced to vitamin C deficiency (Chavez de Martinez, 1990). The high incidence of opercular deformity in milkfish given unenriched live food (control), despite relative high ascorbate levels (rotifers cultured on *Chlorella* sp. had high vitamin C content, Fig. 2), is difficult to explain. It may be possible that the effect of vitamin C was rendered ineffective or masked by the effect of sub-optimal HUFA diet. Note that the control fish had the lowest DHA:EPA ratio (probably lower than what is required for normal growth, Table 3) among the treatment groups. Although the HUFA-treated fish was given moderate amounts of ascorbic acid, its DHA:EPA ratio was relatively high, comparable with that of the HUFA + vitamin C-treated fish. Both treatment groups exhibited better growth, resistance to stress and had lower incidence of deformity.

In the current investigation, HUFA-treated and HUFA + vitamin C-treated 25-day-old milkfish were more resistant to salinity stress, i.e., fish were dying less slowly, compared with the control fish. The lowest slope value consistently occurring in fish treated with HUFA + vitamin C may indicate that ascorbic acid supplementation enhanced resistance to stress. When subjected to salinity stress test, 20-day-old *Clarias gariepinus* larvae fed ascorbate-supplemented diet exhibited significantly low mortality than those larvae fed an ascorbate-free diet (Merchie et al., 1995). It was also observed that both the HUFA-treated and HUFA + vitamin C-treated milkfish exhibited better growth than the untreated fish. Although it is possible that HUFA alone may have improved growth performance in milkfish (as reported in other species), the synergistic effect of vitamin C cannot be discounted. Better growth was observed among tilapia fingerlings (Anadu et al., 1990) and plaice (Rosenlund et al., 1990) fed diets supplemented with ascorbic acid.

The preceding studies attest the importance of EFA and/or vitamin C in fish growth and development. Optimum requirements of these nutrients in milkfish, however, are not yet known. Further studies on the etiology of opercular deformity in milkfish are recommended to determine if such deformity is ascorbate deficiency-related.

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