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Lipid classes and their content of *n*-3 highly unsaturated fatty acids (HUFA) in *Artemia franciscana* after hatching, HUFA-enrichment and subsequent starvation

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Abstract The distribution of *n*-3 highly unsaturated fatty acids (HUFA) over the major neutral and polar lipid classes was determined for two predominant types of live food used in the larviculture of marine fish and shrimp, i.e. freshly hatched and HUFA-enriched *Artemia*, and compared with data reported in the literature for wild copepods, representing the natural diet of these larvae. Lipid class composition and their content of *n*-3 HUFA, particularly eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3), were assessed in freshly hatched, HUFA-enriched and subsequently starved *Artemia franciscana*. The *n*-3 HUFA enrichment was based on feeding *Artemia* a lipid emulsion in which either fatty acid ethyl esters (EE, diluted with olive oil) or triacylglycerol (TAG) provided a level of 30% *n*-3 HUFA. Enrichment of *Artemia* with either type of the lipid emulsions resulted in an increase of total lipid content from 20.0 to 28.2–28.7% of dry matter mainly due to the accumulation of neutral lipid, primarily TAG (from 82 to 158 mg g^{-1} dry wt in freshly hatched and 24-h enriched *Artemia*). Enriched brine shrimp utilized up to 27–30% of their TAG content during 72 h of starvation at 12 °C. The absolute tissue concentrations of polar lipids remained constant at 71 to 79 mg g^{-1} dry wt throughout the enrichment and subsequent starvation. The level of *n*-3 HUFA increased drastically during enrichment from 6.3% of total fatty acids (8.2 mg g^{-1} dry wt) in freshly hatched nauplii to between 20.4 and 21.8% (40.4 to 43.2 mg g^{-1} dry wt) in 24-h enriched *Artemia* and was not significantly affected by the source of *n*-3 HUFA. During starvation, 18:0, 20:4*n*-6 and 20:5*n*-3 were retained, whereas 18:4*n*-3, 22:5*n*-3 and 22:6*n*-3 were specifically catabolized. The major polar lipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), of freshly hatched *Artemia* showed very low levels of DHA (< 0.1% of total fatty acids) and carried about 45% of the total EPA present. Enrichment with either of the emulsions resulted in an increase of the neutral lipid fraction which concentrated > 64% of the EPA and > 91% of the total DHA present. This is in sharp contrast with the high levels of *n*-3 HUFA, in particular DHA, in the polar lipid fraction reported for wild copepods. The contrasting distribution of DHA in the neutral and polar lipid fractions of enriched brine shrimp compared to the natural diet may influence the efficacy of this essential fatty acid for marine fish larvae in aquaculture systems.

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Introduction

Dietary lipid is required by all animals for providing metabolic energy, the synthesis of cell membranes, and as a source of precursors for eicosanoids (Sargent et al. 1988). Although lipid is a quantitatively major nutrient for fish growth, lipid requirements, particularly in larval stages of marine species, remain poorly understood. The larval requirements for *n*-3 highly unsaturated fatty acids (HUFA), primarily eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3), have been demonstrated for various species of marine fish (Izquierdo 1996; Sargent et al. 1997). A greatly reduced activity of the Δ^5 fatty acid desaturase, which is needed for the conversion of dietary 18:3*n*-3 to *n*-3 HUFA, has been found to be the background of this requirement (Sargent et al. 1993). Recent studies with various species of marine fish have revealed that DHA, above all, plays an important role in various physiological functions, including survival, growth, and pigmentation success (Kanazawa 1993a; Watanabe 1993;

Reitan et al. 1994). The essentiality of DHA is most critical during the early development of fish larvae when the normal development of neural and visual functions requires an increased dietary supply of DHA (Mourente and Tocher 1992; Bell and Dick 1993; Bell et al. 1995). Nevertheless, major controversies continue to exist over what constitutes the optimal quantitative requirements, the dietary balances between various essential fatty acids (EFA) and the relative importance of various lipid classes in supplying these fatty acids (Sargent et al. 1993, 1997). The beneficial effects of dietary phospholipids on the performance of larval and juvenile stages have been demonstrated for a number of marine fish species, although the mechanisms behind this requirement remain unclear (Kanazawa 1993b; Coutteau et al. 1997). Various authors have suggested that phospholipids may be superior to neutral lipids as an EFA source for larval stages, the digestive capacity of which may not be fully developed (Olsen et al. 1991; Koven et al. 1993; Sargent et al. 1993).

Due to the limited acceptance of artificial diets at start-feeding in many marine fish species, most studies determining larval nutritional requirements for EFA have been carried out using enrichment techniques to manipulate the composition of live feeds such as rotifers (*Brachionus plicatilis*: Rainuzzo et al. 1994b) and brine shrimp (*Artemia* sp.: Sorgeloos and Léger 1992). Rotifers and *Artemia* are naturally deficient in *n*-3 HUFA and particularly DHA (Léger et al. 1986; Navarro et al. 1992a; Rainuzzo et al. 1994b). This can be corrected prior to offering them to the fish larvae by prefeeding the live preys with *n*-3 HUFA-rich products, such as microalgae, yeast-based diets, microparticulate diets, microcapsules, or emulsions (Léger et al. 1986; Coutteau and Sorgeloos 1997). However, the lipid composition of live preys can only be manipulated to a limited extent, and is not completely defined, which strongly limits the research possibilities. Thus, it is difficult to incorporate a suitable and stable range of concentrations of DHA and phospholipids in live preys, particularly *Artemia* (Rainuzzo et al. 1994a; McEvoy et al. 1996; Evjemo et al. 1997).

Bioencapsulation of *Artemia* is often considered as a simple filling of the gut with little biotransformation of the enrichment product in the digestive system of the brine shrimp (Tocher et al. 1997). As a result, novel enrichment diets are evaluated mostly based on their capability to increase the content of *n*-3 HUFA in live preys following a standard enrichment technique (e.g. Dhert et al. 1993; Southgate and Lou 1995; McEvoy et al. 1996). However, HUFA-enrichment in filter-feeding organisms involves a complex chain of biological processes, including particle filtration, ingestion, digestion, assimilation and metabolism (Omori and Ikeda 1984). Despite the wide application of *n*-3 HUFA enriched *Artemia* and rotifers for feeding marine fish larvae in commercial hatcheries, the mechanisms whereby ingested lipids are bioencapsulated in the live preys and the form in which the EFA are available for the fish larvae as compared to their natural diet are poorly documented. On the other hand, the lipid class and fatty acid composition of wild copepods, which are an important component in the natural diet of marine fish larvae (Kinne 1977), have been described in several studies of marine ecosystems (Tande and Henderson 1988; Fraser et al. 1989; Norrbin et al. 1990; Lokman 1993). Therefore, the present study aimed at determining the distribution of *n*-3 HUFA over the major lipid classes in two predominant types of live food used in marine larviculture, i.e. freshly hatched and HUFA-enriched *Artemia*. These results were compared with data reported in the literature for HUFA levels in polar and neutral lipid classes of wild copepods, representing the natural diet of marine fish larvae. Also, the stability of *n*-3 HUFA in *Artemia* was followed by evaluating the effect of prolonged storage at reduced temperature on the levels and distribution of *n*-3 HUFA. Furthermore, fatty acid ethyl esters (EE) and triacylglycerols (TAG) were compared with regards to their efficacy as sources of *n*-3 HUFA in enrichment emulsions containing similar levels of *n*-3 HUFA.

Materials and methods

Hatching, enrichment and starvation of *Artemia*

Artemia franciscana cysts (Great Salt Lake, Utah, USA; INVE Aquaculture N.V., Belgium; ARC No. 1311) were disinfected and hatched in seawater at 28 °C under continuous illumination and aeration following Van Stappen (1997). After 24 h of incubation, freshly hatched nauplii were transferred to 1-liter cylindroconical tubes (triplicate samples per treatment) filled with 0.45-µm filtered seawater at a density of 200 ml^{-1} for enrichment over 24 h at 28 °C with aeration giving in excess of 4 ppm oxygen. The enrichment diets were added at the start and after 12 h of enrichment. Following enrichment, *Artemia* were rinsed on a sieve under tapwater to remove all residual emulsion, and transferred to cylindroconical tubes at a density of 50 ml^{-1} for up to 72-h starvation at 12 °C under continuous aeration. Triplicate samples were taken after 24-h hatching, 24-h enrichment, 24-h and 72-h starvation, rinsed on a sieve under tapwater, dipped on absorbing paper till dry in appearance and frozen (−22 °C) under nitrogen atmosphere until analysis.

The enrichment diets consisted of experimental oil emulsions made available within the framework of the International Council for the Exploration of the Sea – Working Group on the Mass Rearing of Juvenile Fish (ICES 1994). ICES emulsions were isolipidic and contained 30% of total *n*-3 HUFA with a DHA/EPA ratio of 4 either as TAG or EE (ICES 30/4/C/3 and ICES 30/4/C EE, respectively). The ethyl ester concentrate (50% *n*-3 HUFA) was diluted with olive oil to obtain similar levels of *n*-3 HUFA in both emulsions, which resulted in a higher proportion of oleic acid in ICES 30/4/C/EE and the presence of non-HUFA containing TAG (Table 1).

Lipid and fatty acid analysis

Lipids were extracted from preweighed samples of *Artemia* by homogenization in chloroform/methanol (2:1, v/v), containing 0.01% butylated hydroxytoluene (BHT) as an antioxidant, according to Folch et al. (1957). Solvent was evaporated under a stream of nitrogen and lipid extracts desiccated overnight in vacuum before their mass was determined gravimetrically. Lipid extracts were redissolved in chloroform/methanol (2:1, v/v; containing 0.01% BHT) at a concentration of 10 mg ml^{-1} and stored under nitrogen atmosphere at −20 °C until analysis.

Table 1 Lipid class and fatty acid composition of the experimental ICES (International Council for Exploration of the Sea) emulsions containing *n*-3 HUFA either as triacylglycerols (ICES 30/4/C/3) or fatty acid ethyl esters (ICES 30/4/C/EE) (*PUFA* polyunsaturated fatty acids; *HUFA* highly unsaturated fatty acids; *DHA* docosahexaenoic acid; *EPA* eicosapentaenoic acid)

	ICES 30/4/C/3	ICES 30/4/C/EE
Total lipid (% wet weight)	62	62
Lipid class composition (% of total lipid)		
Free fatty acids	3.4	5.9
Triacylglycerol	78.9	42.2
Fatty acid ethyl esters	–	33.1
Other (sterols, pigments, non-identified polar lipids)	17.7	18.8
Fatty acid composition (% of total fatty acids)		
14:0	3.4	0.2
15:0	1.0	–[1]
16:0	19.6	7.5
16:1*n*-7	4.3	0.8
18:0	5.4	3.0
18:1*n*-9	13.6	45.0
18:1*n*-7	2.2	1.4
18:2*n*-6	4.9	6.6
18:3*n*-6	0.2	0.4
19:1*n*-9	0.1	0.6
18:3*n*-3	1.1	0.8
18:4*n*-3	0.7	0.4
20:1*n*-9	0.6	0.4
20:4*n*-6	1.7	0.3
20:5*n*-3	5.7	5.2
21:5*n*-3	0.2	0.5
22:5*n*-6	1.8	0.5
22:4*n*-3	–	0.5
22:5*n*-3	1.1	1.7
22:6*n*-3	24.6	22.1
Σsaturates[2]	30.5	10.9
Σmonoenes	21.8	48.6
Σ*n*-6 PUFA	9.0	7.8
Σ*n*-3 PUFA	33.8	31.3
Σ*n*-3 HUFA[3]	32.0	30.1
DHA/EPA	4.3	4.2

[1] Not identified
[2] Sums include minor fatty acids (≤0.3%) not mentioned in table
[3] ≥20:3*n*-3

Lipid classes were separated by high-performance thin-layer liquid chromatography (HPTLC) using a one-dimensional double development (Tocher and Harvie 1988). Lipids were separated on HPTLC plates (10 × 10 cm × 0.15 mm, silica gel 60 without fluorescent indicator, Merck, Germany) using methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, v/v) and hexane/diethyl ether/acetic acid (80:20:2, v/v) as a developing solvent mixture for polar and neutral lipid classes, respectively. Separated lipid classes were charred for 20 min at 160 °C, after spraying the plate with 3% (w/v) cupric acetate in 8% (v/v) phosphoric acid, and quantified by calibrated scanning densitometry using a Shimadzu CS-9001PC dual wavelength flying spot scanner (Olsen and Henderson 1989). The results were expressed as milligrams of each lipid class per gram of dry weight.

Fatty acid composition of total lipid was determined following the method of Christie (1989). Prior to fatty acid analysis, predominant polar (PC, PE) and neutral lipids (TAG, FFA) were separated by thin-layer chromatography (TLC) on plates (20 × 20 cm × 0.25 mm, silica gel 60 without fluorescent indicator, Merck, Germany) using the polar and neutral solvent mixture (see above), respectively. After spraying the plate with 2′,7′-dichlorofluorescein (1% in 98% methanol) isolated spots were visualized under ultraviolet light, scrapped off and put into 11 ml quick fit test tubes for transesterification. Fatty acids were transesterified for 16 h at 50 °C using a mixture of sulfuric acid and methanol (1:100 by volume), and nonadecanoic acid (19:0) as internal standard. Fatty acid methyl esters (FAME) were extracted with hexane, dissolved in iso-octane and determined quantitatively with a Chrompack CP9001 gas chromatograph equipped with an autosampler. Injection was done on column into a very polar 50 m capillary column, BPX70, with a diameter of 0.32 mm and a layer thickness of 0.25 μm connected to a 2.5 m methyl deactivated precolumn. The carrier gas was H_2 and the detection mode flame ionization (FID). The oven was programmed to raise the initial temperature from 85 to 150 °C at a rate of 20 °C min^{-1}, from 150 to 152 °C at 0.1 °C min^{-1}, from 152 to 174 °C at 0.7 °C min^{-1}, from 174 to 180 °C at 10 °C min^{-1} and to stay at 180 °C for 2 min. Identification was based on a standard reference mixture (GLC 68B, NU-Chech Prep). Integration and calculation were done with the software program "Maestro" (Chrompack). FAME results were expressed either as percent of total FAME or as milligrams fatty acid per gram of dry weight.

Statistical analysis

Data represent means of triplicate analyses, except for the fatty acid composition of individual lipid classes (see Table 4; $n = 1$), and were subjected to one-way analysis of variance (ANOVA) followed, when pertinent, by a Tukey's multiple range test to detect significant differences among means ($P \leq 0.05$). Data were checked for homoscedasticity by the Bartlett test. Prior to statistical analysis, percentage compositions were arcsin transformed (Sokal and Rohlf 1995).

Results

Enrichment of *Artemia* with the lipid emulsions resulted in a significant increase of total lipid content from 20.0 to 28.2–28.7% of dry matter (Table 2). This was due to a significant accumulation of neutral lipid, mainly as TAG and to a lesser extent as free fatty acids (FFA), which was independent of the use of either pure TAG or a 56:44% mixture of TAG/ethyl esters as a lipid source in the emulsion (ICES 30/4/C/3 and 30/4/C/EE, respectively). In agreement with the higher level of FFA in the EE-based emulsion (Table 1), *Artemia* enriched with ethyl esters showed a higher increase of FFA content than brine shrimp fed the TAG-based emulsion (respectively, 66% and 26% increase compared with the level in freshly hatched nauplii; Table 2).

Total lipid decreased again during starvation at 12 °C, although significantly lower levels were observed only after 72 h of starvation (Table 2). Starvation induced a selective utilization of the TAG reserves, which proceeded initially slower for *Artemia* enriched with the ethyl ester-based emulsion (nonsignificant 3% decrease during the first 24 h of starvation versus significant 12% for TAG-enriched *Artemia*; Table 2). At the end of the 72-h starvation period, *Artemia* had lost 27 to 30% of the TAG reserves that it contained after enrichment. The increased FFA content, which was built up during enrichment, disappeared again during starvation. The absolute tissue concentrations of polar lipids remained constant in *Artemia* throughout enrichment and subse-

Table 2 Lipid content and lipid class composition (mg g^{-1} dry wt) of freshly hatched *Artemia franciscana*, after 24-h enrichment with *n*-3 HUFA presented either as triacylglycerol or ethyl esters, and subsequent starvation for 24 and 72 h at 12 °C. Data represent means ± SD (n = 3). Different superscripts in the same row denote significant differences ($P < 0.05$)

	Freshly hatched	Triacylglycerol enrichment			Ethyl ester enrichment		
		24-h enriched	24-h starved	72-h starved	24-h enriched	24-h starved	72-h starved
Total lipid	200.4 ± 0.5^{d}	281.7 ± 13.6^{ab}	267.6 ± 0.2^{abc}	239.4 ± 2.3^{c}	286.9 ± 12.4^{a}	283.4 ± 3.5^{ab}	249.9 ± 14.1^{bc}
Total polar lipid	71.1 ± 2.3	74.5 ± 4.9	79.3 ± 8.1	74.1 ± 5.6	72.0 ± 5.9	78.3 ± 5.5	74.9 ± 1.9
Total neutral lipid	129.3 ± 2.3^{d}	207.2 ± 4.9^{a}	188.3 ± 8.1^{b}	165.3 ± 5.6^{c}	214.9 ± 5.9^{a}	205.0 ± 5.5^{a}	175.0 ± 1.9^{bc}
Polar lipids							
Lyso phosphatidylcholine	0.7 ± 0.1	1.0 ± 0.4	0.6 ± 0.0	1.0 ± 0.3	0.7 ± 0.3	0.9 ± 0.4	1.0 ± 0.2
Sphingomyelin	1.0 ± 0.1	1.7 ± 0.7	1.5 ± 0.6	1.0 ± 0.2	1.1 ± 0.5	1.1 ± 0.3	0.9 ± 0.2
Phosphatidylcholine	25.7 ± 1.5	26.0 ± 2.3	27.2 ± 2.2	26.5 ± 1.8	25.8 ± 1.4	28.6 ± 3.2	25.3 ± 1.6
Phosphatidylserine	4.3 ± 0.2	5.1 ± 1.2	6.3 ± 1.4	6.9 ± 1.2	4.3 ± 1.1	5.9 ± 0.9	6.5 ± 0.8
Phosphatidylinositol	6.3 ± 0.5	6.7 ± 0.9	7.2 ± 0.5	6.2 ± 0.6	6.3 ± 1.1	6.5 ± 0.4	6.4 ± 0.5
Phosphatidic acid + cardiolipin	7.0 ± 0.4^{a}	6.5 ± 0.5^{ab}	6.3 ± 0.4^{ab}	5.8 ± 0.1^{b}	6.4 ± 0.1^{ab}	6.4 ± 0.4^{ab}	6.3 ± 0.3^{ab}
Phosphatidylethanolamine	19.3 ± 0.6^{b}	21.7 ± 0.9^{ab}	23.0 ± 2.5^{a}	19.4 ± 1.1^{ab}	20.1 ± 0.9^{ab}	21.5 ± 1.2^{ab}	20.1 ± 0.6^{ab}
Unknown	6.9 ± 1.1	5.9 ± 1.6	7.2 ± 0.8	7.3 ± 0.6	7.3 ± 0.5	7.4 ± 1.0	8.5 ± 1.3
Neutral lipids							
Pigment	8.1 ± 1.1^{a}	4.8 ± 0.7^{bc}	3.5 ± 0.2^{c}	5.8 ± 0.6^{abc}	6.0 ± 1.2^{abc}	6.5 ± 1.4^{ab}	7.8 ± 0.6^{a}
Free sterol	17.0 ± 0.8^{b}	19.3 ± 1.9^{ab}	20.8 ± 0.9^{ab}	22.1 ± 1.7^{ab}	20.9 ± 2.8^{ab}	21.9 ± 1.0^{ab}	23.1 ± 3.0^{a}
Free fatty acid	11.2 ± 0.8^{c}	14.1 ± 1.1^{b}	12.1 ± 1.4^{bc}	12.2 ± 0.4^{bc}	18.6 ± 2.3^{a}	10.9 ± 0.4^{c}	12.6 ± 0.4^{bc}
Triacylglycerol[1]	82.1 ± 2.2^{d}	158.2 ± 3.8^{a}	139.8 ± 7.6^{b}	110.5 ± 4.9^{c}	157.7[1] ± 6.0^{a}	152.8[1] ± 3.6^{a}	115.0[1] ± 1.0^{c}
Sterol ester	10.8 ± 1.7	10.7 ± 1.4	12.1 ± 1.3	14.8 ± 3.2	11.7 ± 0.6	12.9 ± 3.3	16.6 ± 1.6

[1] Separate neutral HPTLC development detected fatty acid ethyl esters in the TAG band at a level of < 4% and < 2% of the reported TAG value for the EE-enriched and subsequently starved *Artemia*, respectively

quent starvation. Due to the accumulation of neutral lipid during enrichment, the proportion of total polar lipid decreased from 35.5% of total lipid in freshly hatched nauplii to 26.4–25.1% in enriched *Artemia*.

The level of *n*-3 HUFA increased drastically during enrichment from 6.3% of total fatty acids (8.2 mg g^{-1} dry wt) in freshly hatched nauplii to between 20.4 and 21.8% (40.4 to 43.2 mg g^{-1} dry wt) in 24-h enriched *Artemia* (Table 3). The level of *n*-3 HUFA in the enriched *Artemia* was not significantly affected by the source of *n*-3 HUFA, whereas the different levels of the other fatty acids in the emulsions were reflected significantly in the fatty acid profile of the enriched *Artemia*. A higher proportion of 18:1*n*-9 and lower content of saturated fatty acids, 18:3*n*-3 and 20:4*n*-6, were thus observed in the *Artemia* fed the ethyl ester-based emulsion.

The effect of the 72-h starvation period on the fatty acid profile of enriched *Artemia* revealed a similar evolution in both types of enriched *Artemia*. The proportion of 18:0, 20:4*n*-6 and 20:5*n*-3 increased during 72 h of starvation compared to the level after enrichment (respectively, +19/22%, +31/21%, and +40/30% for TAG/EE-enriched *Artemia*; Table 3). In contrast, the level of 18:4*n*-3, 22:5*n*-3 and 22:6*n*-3 decreased significantly during starvation (respectively, −37/43%, −44/19%, and −48/52% for TAG/EE-enriched *Artemia*).

The major phospholipids of freshly hatched *Artemia* carried insignificant amounts of DHA (< 0.1% of total fatty acids in PE and PC; Table 4). As a result, minimum 91% of the 0.2 mg g^{-1} dry wt of DHA found in *Artemia* nauplii was present primarily in neutral lipid classes such as TAG and FFA (Fig. 1). In contrast, the level of EPA was over twofold higher in PE and PC than that in TAG and FFA; and minimum 45% of the total amount of EPA (7.1 mg g^{-1} dry wt) in the freshly hatched brine shrimp was thus present in phospholipids. Enrichment resulted in an increased proportion of EPA in all studied lipid classes, except in PE (Table 4). However, as a result of the high load of TAG in enriched brine shrimp, 64% of the total EPA was incorporated as TAG. The TAG fraction showed a 63- to 72-fold increase of the DHA proportion compared to the level in the freshly hatched nauplii, and contained over 90% of the total DHA present in enriched *Artemia* (Fig. 1). During starvation, the proportion of EPA tended to increase or remain stable in all studied lipid classes, except in FFA. By contrast, DHA levels decreased in particular in the TAG fraction. The absolute amount of DHA presented as TAG thus decreased with 70% over 72 h of starvation (Table 4).

Discussion

Few studies have dealt with the lipid class composition of *Artemia* and even less regarding their variations due to enrichment procedures to enhance its *n*-3 HUFA content for marine larviculture. Literature data on the lipid level of decapsulated cysts and freshly hatched nauplii show a high variability due to differences between various populations and harvests of brine shrimp (Léger et al. 1986; Navarro et al. 1992b) as well as analytical methodology (Léger et al. 1989; Coutteau and Sorgeloos 1995). Lipid class composition of *Artemia* found in the present study differed clearly from those presented by other authors using different analytical techniques (Takeuchi et al. 1992). On the other hand, the proportions of total polar lipid in *Artemia* enriched with EE- or TAG-based emulsions are close to the values reported for similarly treated brine shrimp by Rainuzzo et al. (1994a) using the TLC-FID iatroscan technique (27.4 to 27.9% versus 25.1 to 26.4% in the present study). An increase of total lipid due to the expansion of the TAG reserves has been observed during enrichment of *Artemia* (Takeuchi et al. 1992; McEvoy et al. 1996) as well as rotifers (Rainuzzo et al. 1994a, b), despite large differences in initial and final lipid contents reported by various authors. For example, McEvoy et al. (1996) obtained after 18 h of enrichment a total lipid content in *Artemia* of 19.7% of which 12.5% (on a dry weight basis) was TAG. However, these authors started their enrichment with 6-h-old brine shrimp that contained only 12.3% of total lipid and 7.2% of TAG. Conversely, Takeuchi et al. (1992) reported an increase of lipid content in *Artemia* from 33% up to 45% after 24 h of enrichment with EE- or TAG-based emulsions.

Despite the vast amount of literature on *n*-3 HUFA enrichment of *Artemia*, comparison of the present enrichment results with data reported previously by other authors is difficult because of major differences in the enrichment protocol (species and initial stage of the *Artemia*; duration, dosage, concentration and type of the enrichment diet; physical and chemical conditions) as well as the lipid content and *n*-3 HUFA profile of the enrichment diet. Comparisons may be facilitated through a better standardization of the enrichment diets used in nutritional studies with larval marine fish. In this regard, a series of experimental emulsions were made available through the International Council for the Exploration of the Sea – Working Group on Mass Rearing of Juvenile Fish which are a better alternative for commercial enrichment diets as they have a stable formulation between production batches and offer a specific range of (*n*-3) HUFA profiles (ICES 1994). The present enrichment results are comparable with those reported recently for *Artemia* enriched with an experimental diet, consisting of tuna orbit oil emulsified with 12% herring roe polar lipid, with a similar fatty acid profile as the TAG-based emulsion used in this study (7.0% EPA and 24.2% DHA; McEvoy et al. 1996). In the latter study, levels of EPA and DHA increased from 3.9 and 0 to 10.8 and 19.4 mg g^{-1} dry wt, respectively, over a 18-h enrichment period starting from 6-h-old *Artemia* nauplii (versus from 5.5 and 0.2 to 16.8 and 21.0, respectively, over a 24-h enrichment in the present study). The higher incorporation of DHA in proportion to that of EPA observed by McEvoy and coworkers was thought to be due to the better availability of DHA from the herring

Table 3 Fatty acid composition of total lipids (% of total fatty acids and mg g^{-1} dry wt) of freshly hatched *Artemia franciscana*, after 24-h enrichment with *n*-3 HUFA presented either as triacylglycerol or ethyl esters, and subsequent starvation for 24 and 72 h at 12 °C. Data represent means ± SD ($n = 3$). Different superscripts in the same row for each type of data denote significant differences ($P < 0.05$) (0.0, trace < 0.1%)

	% of total fatty acids							mg g^{-1} dry weight						
	Freshly hatched	Triacylglycerol enrichment			Ethyl ester enrichment			Freshly hatched	Triacylglycerol enrichment			Ethyl ester enrichment		
		24-h enriched	24-h starved	72-h starved	24-h enriched	24-h starved	72-h starved		24-h enriched	24-h starved	72-h starved	24-h enriched	24-h starved	72-h starved
14:0	0.9^{a}	0.9^{a}	0.8^{a}	0.8^{a}	0.5^{b}	0.4^{b}	0.5^{b}	1.2^{bc}	1.6^{a}	1.4^{ab}	1.2^{bc}	1.0^{cd}	0.8^{d}	0.8^{d}
14:1*n*-5	0.9^{a}	0.5^{ab}	0.5^{ab}	0.4^{b}	0.4^{ab}	0.4^{ab}	0.3^{b}	1.2^{a}	1.0^{ab}	0.9^{ab}	0.6^{b}	0.8^{ab}	0.8^{ab}	0.5^{b}
16:0	12.9^{a}	12.0^{b}	11.9^{b}	11.9^{b}	8.3^{c}	8.2^{c}	8.4^{c}	16.8^{c}	22.1^{a}	19.8^{ab}	17.5^{bc}	17.6^{bc}	16.4^{c}	13.3^{d}
16:1*n*-7	4.8^{a}	3.9^{ab}	3.8^{b}	3.8^{b}	2.3^{c}	2.3^{c}	2.1^{c}	6.2^{ab}	7.2^{a}	6.4^{ab}	5.6^{bc}	4.9^{cd}	4.5^{d}	3.4^{e}
17:0	0.7	0.8	0.9	0.8	0.3	0.4	0.5	0.9	1.4	1.5	1.1	0.7	0.8	0.9
17:1*n*-7	1.1^{a}	0.9^{ab}	0.8^{ab}	0.9^{a}	0.4^{c}	0.5^{c}	0.6^{bc}	1.4^{ab}	1.6^{a}	1.4^{ab}	1.3^{abc}	0.8^{c}	1.0^{abc}	0.9^{bc}
18:0	4.8^{bc}	4.6^{bc}	5.0^{ab}	5.5^{a}	3.6^{e}	3.8^{de}	4.4^{cd}	6.2^{c}	8.5^{a}	8.4^{a}	8.2^{ab}	7.6^{ab}	7.6^{ab}	6.9^{bc}
18:1*n*-9	19.5^{b}	18.1^{b}	18.6^{b}	19.3^{b}	33.6^{a}	34.7^{a}	35.4^{a}	25.2^{e}	33.5^{c}	31.2^{cd}	28.5^{de}	71.1^{a}	69.3^{a}	55.8^{b}
18:1*n*-7	9.8	8.4	9.1	9.6	7.9	8.9	8.9	12.8	15.7	15.4	14.2	16.8	18.1	14.0
18:2*n*-6	5.5^{b}	5.3^{bc}	5.2^{c}	5.3^{bc}	5.8^{a}	5.9^{a}	5.8^{a}	7.2^{d}	9.9^{b}	8.8^{bcd}	7.9^{cd}	12.3^{a}	11.9^{a}	9.2^{bc}
18:3*n*-3	26.7^{a}	16.5^{b}	16.5^{b}	16.9^{b}	13.0^{c}	13.5^{c}	13.3^{c}	34.7^{a}	30.5^{ab}	27.6^{bc}	25.0^{cd}	27.5^{bc}	27.1^{bc}	21.0^{d}
18:4*n*-3	3.6^{a}	1.6^{b}	1.4^{bc}	1.0^{d}	1.2^{cd}	1.1^{cd}	0.7^{e}	4.7^{a}	2.9^{b}	2.3^{b}	1.5^{cd}	2.5^{b}	2.2^{bc}	1.0^{d}
20:1*n*-9	0.3^{ab}	0.5^{ab}	0.4^{ab}	0.5^{a}	0.3^{b}	0.3^{b}	0.4^{ab}	0.4^{c}	0.8^{a}	0.7^{ab}	0.8^{ab}	0.6^{abc}	0.6^{abc}	0.6^{bc}
20:4*n*-6	0.8^{c}	1.8^{b}	2.1^{ab}	2.4^{a}	0.6^{c}	0.7^{c}	0.8^{c}	1.0^{b}	3.4^{a}	3.4^{a}	3.5^{a}	1.3^{b}	1.4^{b}	1.2^{b}
20:5*n*-3	5.5^{e}	9.0^{cd}	10.3^{bc}	12.6^{a}	8.4^{d}	8.7^{cd}	10.9^{ab}	7.1^{b}	16.8^{a}	17.2^{a}	18.6^{a}	17.8^{a}	17.3^{a}	17.3^{a}
22:5*n*-6	0.0^{c}	0.8^{a}	0.7^{a}	0.3^{ab}	0.0^{bc}	0.0^{c}	0.2^{bc}	0.0^{c}	1.4^{a}	1.1^{ab}	0.5^{bc}	0.0^{c}	0.0^{c}	0.4^{c}
22:5*n*-3	0.0^{b}	0.8^{b}	0.6^{b}	0.4^{b}	1.0^{b}	0.9^{b}	0.8^{b}	0.0^{c}	1.4^{ab}	1.0^{abc}	0.7^{bc}	2.0^{a}	1.8^{a}	1.2^{ab}
22:6*n*-3	0.2^{e}	11.3^{a}	9.0^{ab}	5.9^{cd}	10.5^{a}	7.5^{bc}	5.0^{d}	0.2^{d}	21.0^{a}	15.2^{b}	8.7^{c}	22.3^{a}	15.1^{b}	7.9^{c}
Σ saturated[1]	19.7^{a}	18.8^{a}	19.2^{a}	19.4^{a}	13.1^{b}	13.2^{b}	14.2^{b}	25.5^{cd}	34.8^{a}	32.0^{ab}	28.7^{bc}	27.8^{bc}	26.4^{cd}	22.4^{d}
Σmonoenes	36.9^{c}	32.9^{e}	34.0^{de}	35.1^{d}	45.4^{b}	47.7^{a}	47.9^{a}	47.9^{c}	61.0^{c}	57.0^{c}	51.7^{c}	96.2^{a}	95.5^{a}	75.6^{b}
Σ*n*-6 PUFA	6.7^{b}	8.4^{a}	8.4^{a}	8.3^{a}	6.8^{b}	6.9^{b}	6.9^{b}	8.7^{d}	15.5^{a}	14.0^{ab}	12.2^{bc}	14.5^{ab}	13.7^{ab}	10.9^{cd}
Σ*n*-3 PUFA	36.7^{bc}	39.8^{a}	38.5^{ab}	37.3^{b}	34.6^{c}	32.3^{d}	31.0^{d}	47.5^{c}	73.8^{a}	64.6^{ab}	55.0^{bc}	73.2^{a}	64.6^{ab}	49.0^{c}
Σ*n*-3 HUFA[2]	6.3^{e}	21.8^{a}	20.7^{ab}	19.4^{bc}	20.4^{ab}	17.7^{cd}	17.1^{d}	8.2^{e}	40.4^{ab}	34.7^{bc}	28.6^{cd}	43.2^{a}	35.3^{b}	26.9^{d}

[1] Sums include minor fatty acids not shown in table
[2] ≥20:3*n*-3

Table 4 Proportion (% of total fatty acids) and content (mg g^{-1} dry wt) of EPA, DHA and total *n*-3 HUFA in the two major polar (*PC* phosphatidylcholine; *PE* phosphatidylethanolamine) and neutral (*TAG* triacylglycerol; *FFA* free fatty acid) lipid classes in freshly hatched *Artemia franciscana*, after 24-h enrichment with *n*-3 HUFA presented either as triacylglycerol or ethyl esters, and subsequent starvation for 72 h at 12 °C. Data are based on one analysis ($n = 1$)

	% of total fatty acids					mg g^{-1} dry weight				
	Freshly hatched	Triacylglycerol enrichment		Ethyl ester enrichment		Freshly hatched	Triacylglycerol enrichment		Ethyl ester enrichment	
		24-h enriched	72-h starved	24-h enriched	72-h starved		24-h enriched	72-h starved	24-h enriched	72-h starved
PC										
20:5(*n*-3)	7.9	11.9	14.6	13.2	14.9	1.92	2.61	3.08	3.61	3.71
22:6(*n*-3)	0.0	2.6	1.4	1.9	1.4	0.00	0.57	0.30	0.51	0.34
Σ*n*-3 HUFA	8.7	15.4	16.3	15.8	16.7	2.10	3.37	3.45	4.30	4.16
DHA/EPA	0.0	0.2	0.1	0.1	0.1	0.00	0.22	0.10	0.14	0.09
PE										
20:5(*n*-3)	8.0	8.5	13.2	8.9	9.7	1.25	1.48	1.83	1.45	1.21
22:6(*n*-3)	0.1	2.0	1.0	2.0	0.9	0.02	0.35	0.14	0.32	0.11
Σ*n*-3 HUFA	8.5	13.2	14.5	13.7	10.8	1.34	2.30	2.00	2.24	1.35
DHA/EPA	0.0	0.2	0.1	0.2	0.1	0.02	0.24	0.08	0.22	0.09
TAG										
20:5(*n*-3)	2.7	8.3	9.7	6.7	8.7	2.09	10.58	8.48	11.19	9.17
22:6(*n*-3)	0.2	14.4	6.2	12.5	5.6	0.16	18.40	5.42	20.78	5.95
Σ*n*-3 HUFA	3.4	24.0	17.4	21.5	15.8	2.66	30.77	15.16	35.59	16.71
DHA/EPA	0.1	1.7	0.6	1.9	0.6	0.08	1.74	0.64	1.86	0.65
FFA										
20:5(*n*-3)	3.7	5.1	10.4	4.5	7.9	0.10	0.16	0.45	0.20	0.26
22:6(*n*-3)	1.9	1.2	8.2	5.6	4.0	0.05	0.04	0.36	0.25	0.13
Σ*n*-3 HUFA	6.9	8.8	21.0	13.0	13.6	0.18	0.28	0.91	0.57	0.45
DHA/EPA	0.5	0.2	0.8	1.2	0.5	0.53	0.24	0.79	1.24	0.50

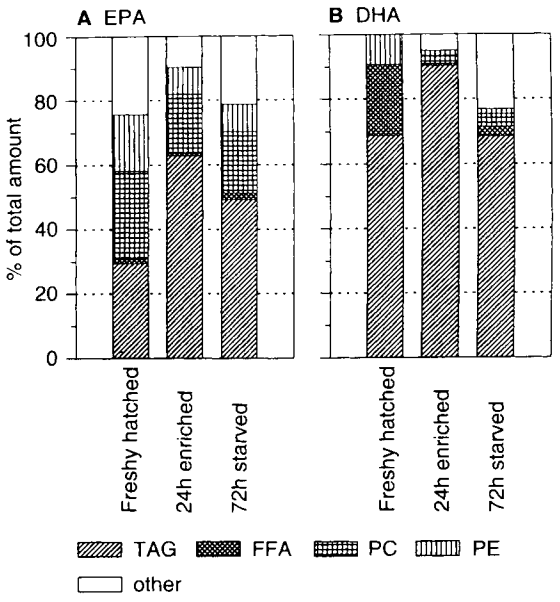

Fig. 1 *Artemia franciscana*. Percent distribution of **A** EPA and **B** DHA in the major lipid classes of *A. franciscana* after hatching, after 24-h enrichment and subsequent 72-h starvation at 12 °C. Data represent the amount of each fatty acid (mg g^{-1} dry wt) recovered from each of the lipid fractions (TAG, FFA, PC, PE, mg g^{-1} dry wt; Table 4) expressed as a percentage of the amount found in total lipids (mg g^{-1} dry wt; Table 3). Average values are presented for the enrichment with triacylglycerol and ethyl esters

roe polar lipid as they did not observe this when Tween 80 was used to emulsify the tuna orbit oil.

Fatty acid ethyl ester concentrates are commonly used in enrichment diets for live food to reach higher concentrations of *n*-3 HUFA and DHA/EPA ratios than is possible with TAG fish oils. The low levels of EE recovered from the *Artemia* after 24 h of enrichment with the emulsion containing EE demonstrated an efficient metabolic conversion into TAG. This confirms earlier findings that ethyl esters are effectively assimilated and incorporated into the TAG fraction of rotifers (Rainuzzo et al. 1994a) as well as *Artemia* (Takeuchi et al. 1992). Nevertheless, so far few studies have evaluated the relative efficacy of ethyl esters compared to TAG for HUFA boosting of live food. Rainuzzo et al. (1994a) obtained a more efficient enrichment in *Artemia* with an emulsion containing ethyl esters compared to an emulsion based on TAG (8.9 and 6.9% *n*-3 HUFA of *Artemia* dry weight, respectively), but interference from the simultaneous variation of total *n*-3 HUFA content (81 and 68%, respectively) and DHA/EPA ratio (5.8 and 1.3, respectively) in the emulsions could not be excluded. The present results showed that EE and TAG are equivalent sources of *n*-3 HUFA for *Artemia* enrichment using ICES emulsions. This appears to be in contradiction with Takeuchi et al. (1992) who reported a higher incorporation of *n*-3 HUFA but a similar increase of total lipid content in *Artemia* enriched for 24 h with emulsified ethyl esters compared to an emulsion based on TAG with the same fatty acid profile (27% EPA, 13% DHA, 43% *n*-3 HUFA). It should be noted that enrichment studies only evaluate the end result of a chain of biological processes, including ingestion, digestion and metabolic conversion. As each of these may be influenced in a different way by the choice of the *n*-3 HUFA source, it is not possible to conclude on the relative digestibility of EE and TAG from the present study. The use of free fatty acids in egg yolk-based emulsions resulted in poor enrichment results due to the poor stability of the emulsions (Takeuchi et al. 1992). Although no differences were observed in physical appearance of the emulsions used in this study, it is not known to what extent different oil sources may cause small shifts in particle size distribution and/or stability of emulsions which may in turn affect the filtering efficiency of *Artemia*.

A decrease of the DHA level in enriched *Artemia* during storage at low temperatures has been observed for various species, including *A. franciscana* (Léger et al. 1987; Triantaphyllidis et al. 1995; Evjemo et al. 1997). A similar specific utilization of 18:4*n*-3 and 22:5*n*-3 was observed during starvation, whereas 18:0, 20:4*n*-6 and 20:5*n*-3 were retained. Most species of brine shrimp appear to lack a requirement for DHA and even in eye phospholipids of adult *Artemia* grown on the alga *Dunaliella*, EPA was the major PUFA (7.1 and 14.1% in PC and PE, respectively) whereas DHA occurred only in trace amounts (Navarro et al. 1992c). The amount of DHA present in TAG decreased about twice as fast as the TAG fraction itself during starvation, which indicated that DHA was exchanged for other fatty acids in the TAG prior to being metabolized. It is not known through which mechanism the DHA is degraded. Retroconversion of DHA into EPA via 22:5*n*-3 was suggested by Watanabe (1993) as one of the possible reasons why the incorporation of DHA in *Artemia* is less efficient than that of EPA. Further research using purified DHA is required to unravel the metabolism of this particular fatty acid in brine shrimp.

The live preys commonly used in larviculture, *Artemia* nauplii and rotifers, are in terms of HUFA nutrition intrinsically unsuitable feeds for marine fish larvae as they are naturally deficient in these essential fatty acids (Sargent et al. 1997). This has been largely resolved by the development of bioencapsulation techniques which allow propagators to "tailor" the total *n*-3 HUFA content of *Artemia* and rotifers to the nutritional requirements of the larvae of any particular species of marine fish (Léger et al. 1986; Sorgeloos and Léger 1992; Izquierdo 1996; Rainuzzo et al. 1997). The importance of the DHA content as well as the ratio of DHA to EPA in nutrition of marine fish larvae is well documented (Watanabe 1993; Kanazawa 1993a; Reitan et al. 1994). This is further corroborated by the fact that wild copepods, the natural prey for marine fish larvae (Kinne 1977), are generally rich in *n*-3 HUFA with a DHA EPA ratio of 1.5 to 2 (Fraser et al. 1989; Lokman 1993; Naess et al. 1995; Sargent et al. 1997). However, major difficulties are encountered with most species of *Artemia*,

Table 5 Comparison of the proportion of EPA and DHA in major polar (*PL*) and neutral lipid classes (*NL*) obtained in the present study for *Artemia* with data reported in literature for copepods

Species	TL (% dry wt)	PL (% total lipid)	TAG (% total lipid)	WE (% total lipid)	EPA (% of total FAME)			Ratio[1] EPA in PL/ EPA in NL	DHA (% of total FAME)			Ratio[1] DHA in PL/ DHA in NL	Source
					PL[2]	TAG	WE		PL[2]	TAG	WE		
Copepods													
Calanus glacialis (CIV-June)	19.0	5.0	9.9	74.2	34.4	14.9	16.8	0.2	28.8	2.4	0.7	2.2	Tande and Henderson (1988)
Calanus glacialis (CIII-July)	47.9	6.4	1.5	81.4	24.6	13.5	8.4	2.9	34.2	4.9	0.9	3.7	
Pseudocalanus sp. (May)	NA[3]	30.4	11.1	50.6	22.1	11.2	9.6	1.4	31.8	9.2	5.9	2.9	Fraser et al. (1989)
Temora longicornis (May)	NA	44.3	31.5	4.0	18.4	14.6	21.2	1.3	31.9	12.4	6.7	2.7	
Calanus finmarchicus (May)	NA	21.0	3.1	73.8	23.1	7.4	6.3	1.4	30.9	5.1	2.2	4.9	
Pseudocalanus acuspes (June)	NA	x[4]	0.6x	2.8x	31.6	23.3	19.8	0.6	25.8	3.2	1.9	4.3	Norrbin et al. (1990)
Pseudocalanus acuspes (Sept)	NA	x	1.2x	8x	18.6	12.4	10.2	0.3	20.3	8.1	4.7	0.5	
Acartia longiremis (June)	NA	x	0.15x	0.08x	13.2	6.6	13.7	6.6	14.6	6.2	2.6	0.9	
Acartia longiremis (Sept)	NA	x	2x	0.1x	26.3	16.2	7.0	0.6	27.0	9.6	2.0	1.1	
Average					23.6	13.3	12.6	1.7	27.3	6.8	3.1	2.6	
Artemia franciscana													
freshly hatched	20.0	35.5	41.0	–	7.9/8.0	2.7	–	1.5	0.0/0.1	0.2	–	0.1	Present study
24-h enriched with TAG	28.2	26.4	56.2	–	11.9/8.5	8.3	–	0.4	2.6/2.0	14.4	–	0.05	
24-h enriched with EE	28.7	25.1	55.0	–	13.2/8.9	6.7	–	0.4	1.9/2.0	12.5	–	0.04	

[1] For copepods this ratio was calculated based on the proportion of the major neutral lipid classes [wax esters (WE) + TAG] and total polar lipids, their fatty acid profile and approximate fatty acid content (PL: 74%, TAG: 95%, WE: 50%; R.J. Henderson personal communication). For *Artemia*, the ratio was based on the mg g^{-1} DW data for EPA and DHA presented in Table 4 with TAG + FFA and PC + PE as major neutral and polar lipid classes, respectively

[2] Level of EPA and DHA reported for total polar lipids (copepods) or PC/PE (*Artemia*)

[3] Not reported by author

[4] Norrbin et al. (1990) reported lipid classes as proportion of PL (= x).

including the most commonly used species *A. franciscana*, when trying to obtain a high DHA/EPA ratio, due to the specific break-down of DHA in brine shrimp during enrichment (Dhert et al. 1993). Also, unlike the DHA in copepods, DHA incorporated in enriched *Artemia* is rapidly catabolized during starvation in the fish tank prior to being ingested by the larvae (Evjemo et al. 1997). The present study furthermore showed that even if the EFA profile of copepods could be mimicked in *Artemia* by enrichment, still there would be an important difference in the source of the *n*-3 HUFA between these two types of live preys. It was shown that the *n*-3 HUFA boosting of *Artemia* resulted in an expansion of the neutral lipid fraction which concentrated >64% of the EPA and >91% of the DHA present in enriched *Artemia*. Despite the successful enrichment in terms of the EFA profile of total lipid, in the phospholipid fraction, the proportion of DHA remained <3% and the DHA/EPA ratio was only 0.2. In contrast, the polar lipid fraction in various species of wild copepods showed average levels of EPA and DHA of 23.6 and 27.3%, respectively (Table 5). The proportion of these fatty acids was considerably lower in the dominant neutral lipid classes, i.e. 1.8- and 1.9-fold for EPA and 4.0- and 8.8-fold for DHA in TAG and wax esters (WE), respectively. The distribution of *n*-3 HUFA over polar and neutral lipid fluctuated in copepods with the amount and type of neutral lipid reserves, which in turn depended on the species and the season (Table 5). Nevertheless, an approximate ratio between the DHA and EPA present in polar lipid (PL) and that occurring in neutral lipid (NL) was calculated based on the proportion of the major lipid classes in total lipid (WE, TAG, PL), their fatty acid profile and approximate fatty acid content (PL: 74%, TAG: 95%, WE: 50%; R.J. Henderson, personal communication; Table 5). The average ratio EPA in PL/EPA in NL was 1.7 in copepods, which corresponded well with the ratio determined analytically in the present study for freshly hatched *Artemia* nauplii (1.5; Table 5). Enrichment increased the absolute level of EPA in *Artemia*, though it simultaneously reduced the proportion of EPA presented as phospholipid down to 0.4. The situation for DHA was even more extreme with an average ratio of DHA in PL/DHA in NL of 2.6 in copepods versus ≤0.1 in *Artemia*, irrespective of HUFA enrichment (Table 5).

Wild zooplankton often remains a superior diet compared to the common aquaculture food chain of rotifers and enriched *Artemia* (Naess et al. 1995). The importance of the different source of DHA in the major live food fed in marine fish hatcheries and the natural diet of marine fish larvae remains to be investigated. Polar lipids may be superior to neutral lipids as an EFA source for larval stages whose digestive capacity may not be fully developed. PL are more polar and may be more easily emulsified and thus less susceptible to a hypothetical bile salt limitation for their assimilation (Koven et al. 1993; Sargent et al. 1993). In this way, Olsen et al. (1991) suggested that larval and early juvenile cod may have an absolute need for polar lipids, both for the supply of energy and for essential fatty acids, because of the limited digestibility of neutral lipids. On the other hand, various studies have demonstrated that phospholipids enhance the efficiency of dietary HUFA presented as neutral lipid in artificial diets in postlarval fish and shrimp (Geurden et al. 1997; Kontara et al. 1997). The high polar lipid content in live feed (7 to 8% on a dry matter basis in *Artemia*) may thus enhance the utilization of the *n*-3 HUFA present as TAG, representing the bulk of *n*-3 HUFA in enriched *Artemia*.

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