Aquaculture 412–413 (2013) 173–178

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Enriched on-grown *Artemia* metanauplii actively metabolise highly unsaturated fatty acid-rich phospholipids

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ARTICLE INFO

Article history:
Received 16 April 2013
Received in revised form 16 July 2013
Accepted 19 July 2013
Available online 26 July 2013

Keywords:
Docosahexaenoic acid
Fatty acids
Lipid conversions
On-grown *Artemia*
Polar lipids

ABSTRACT

On-grown (metanaupliar) stages of *Artemia* have been regarded as more adequate preys for early life-cycle stages of cephalopods, crustaceans, and a variety of fish species. In recent studies, we obtained successful enhancements of highly unsaturated fatty acids (HUFA) and polar lipids (PL) in enriched *Artemia* metanauplii using either a combination of a commercial, neutral lipid (NL)-based HUFA-rich emulsion and Soya lecithin, or HUFA-rich phospholipids. The present study aimed at exploring the molecular form under which dietary HUFA are actually deposited in the metanaupliar lipids. Thus, we analysed the fatty acid (FA) composition of the PL and NL fractions from enriched metanauplii, with special emphasis on the fate of docosahexaenoic acid (DHA) within *Artemia* lipids. The results show that on-grown *Artemia* actively translocated ingested FA contained from PL to NL classes including triacylglycerides.

1. Introduction

Highly unsaturated fatty acids (HUFA) and polar lipids (PL) are regarded as essential nutrients for marine species and beneficial aspects derived from the dietary intake of these compounds have been reported on survival, growth, normal development and stress tolerance (Cahu et al., 2009; Glencross, 2009; Kanazawa, 1997; Sargent et al., 1997; Tocher, 2010; Tocher et al., 2008). For example, dietary intake of the HUFA docosahexaenoic acid (22:6n−3, DHA) is required for early life-cycle stages of marine finfish, since they have an apparently limited ability for endogenous biosynthesis of this essential nutrient (Bell et al., 2003; Tocher, 2010). On the other hand, phospholipids, a predominant fraction among PL, have emulsifying properties (Koven et al., 1993; Olsen et al., 1991) that may facilitate lipid absorption and increase the tolerance of stress conditions (Kanazawa, 1997). Importantly, HUFA delivered as PL have more beneficial effects than those delivered as neutral lipids (NL) (Cahu et al., 2003, 2009; Gisbert et al., 2005; Rainuzzo et al., 1994). Additionally, live preys as copepods naturally contain high levels of essential HUFA, like eicosapentaenoic acid (20:5n−3, EPA) and DHA, predominantly esterified into phospholipids (Bell et al., 2003).

Enrichment protocols have been developed to enhance the nutritional quality of live preys used in aquatic larviculture (Conceição et al., 2010). *Artemia*, particularly its newly hatched naupliar stages, is arguably the most commonly used live prey in marine finfish and crustacean larviculture (Conceição et al., 2010; Sorgeloos et al., 2001). However, their suitability as a diet for marine larvae has been often questioned due to their relatively low HUFA and PL contents in comparison with natural preys such as copepods (Bell et al., 2003). Extensive investigations have been carried out on this subject, but it is still difficult to enrich live preys such *Artemia* with adequate levels of essential HUFA, particularly DHA (Tocher, 2010). DHA contents of enriched *Artemia* have been reported as unstable by different authors (Evjemo et al., 1997; Triantaphyllidis et al., 1995), since undesired metabolic conversions of lipid classes containing DHA (Harel et al., 1999; McEvoy et al., 1996; Rainuzzo et al., 1994) and from DHA to other FA (Navarro et al., 1999) may occur. Consequently, establishing optimised protocols for the simultaneous bioencapsulation of HUFA-rich PL into *Artemia* is a challenge that needs to be urgently achieved.

On-grown stages of *Artemia*, namely metanauplii, are live preys less commonly used than nauplii, but regarded as having more adequate size for feeding early life-cycle stages of some organisms like cephalopods (Domingues et al., 2001; Iglesias et al., 2006), crustaceans (Ritar et al., 2002) and a variety of fish species (Lim et al., 2003; Woods, 2003; Zaki and Saad, 2010). Information about the use of metanauplii or *Artemia* biomass as live preys is scarce as compared to that on the use of newly hatched nauplii. In a recent study, we succeeded in the simultaneous enhancement of HUFA and PL contents of *Artemia* metanauplii (Guinot et al., 2013). Two different enrichment diets were used: 1) a combination of Soya lecithin and the commercial emulsion Easy DHA Selco (containing HUFA-rich NL); and 2) the commercial product Marine lecithin LC60 (ML), a HUFA-rich PL-based product with great potential as enrichment diet (Guinot et al., 2013). Beyond the goal of this former approach aiming at establishing optimised enrichment protocols, the present study focused on exploring the molecular form in which dietary HUFA were actually deposited in the metanaupliar lipids. We hereby show the FA compositions of the PL

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http://dx.doi.org/10.1016/j.aquaculture.2013.07.030

and NL fractions of the enriched metanauplii, with special emphasis on the fate of DHA within *Artemia* lipids.

2. Materials and methods

2.1. Artemia hatching and culture

Artemia franciscana metanauplii were obtained from the hatching of Great Salt Lake *Artemia* cysts (INVE Aquaculture Nutrition, Dendermonde, Belgium). Cysts were incubated during 24 h in 1 L cylindrical–conical glass tubes containing seawater (37 g L^{-1} salinity) at 28 °C, continuous light and vigorous aeration. After hatching, Instar I nauplii were placed in seawater at room temperature in 90 L cylindrical methacrylate containers, at a density of 4000 individuals L^{-1}. Nauplii were fed daily with microalgae *Tetraselmis suecica* at densities around 200,000 cells mL^{-1}. *Artemia* metanauplii were grown for 5 days, attaining a mean length of 1.5 mm and subsequently used in the different enrichments procedures.

2.2. Composition of products used in enrichment diets

Soya lecithin (SL, Korot SL, Alcoy, Spain) contained 74% total lipids, mainly (80%) as PL, 52% of total FA as linoleic acid (18:2n−6, LA) and lacked EPA and DHA. Easy DHA Selco (SS) contained 18% DHA of total FA presented largely as NL. Marine lecithin LC60 (ML, Phosphotech Laboratories, St. Herblain, France) contained 68% total lipids (~50% of TL being PL), with 13% and 33% of total FA as EPA and DHA, respectively. Soya and marine lecithins were dispersed in seawater with a domestic blender and Easy DHA Selco was self-dispersed following supplier's instructions. FA of total lipids, PL and NL of Soya lecithin, Easy DHA Selco and Marine lecithin LC60 are shown in Table 1.

2.3. Artemia metanauplii enrichments

Two (triplicated) enrichment treatments were established: Treatment 1 (termed as 'Treatment 3C' by Guinot et al. (2013)) consisted of a mixture of dispersed Soya lecithin (0.3 g L^{-1}) and Easy DHA Selco (0.3 g L^{-1}); Treatment 2 consisted of a dispersion of Marine lecithin LC60 at 0.6 g L^{-1} (termed as 'Treatment 3A' by Guinot et al. (2013)). The enrichment diets were dispensed at the beginning of the incubation and maintained for 4 h. The enrichment experiments were carried out by placing ~30,000 five day old metanauplii in 0.5 L of filtered seawater at 28 °C in vigorous aeration and continuous light. Metanauplii samples were collected and immediately stored at −80 °C for further analyses.

Table 1
Selected fatty acid contents (percentage of total fatty acids) of total lipids, polar lipids (PL) and neutral lipids (NL) of products (Soya lecithin, Easy DHA Selco and Marine lecithin LC60) used in enrichment diet preparation.

Fatty acid	Soya lecithin			Easy DHA Selco			Marine lecithin LC60		
	Total lipids	PL	NL	Total lipids	PL	NL	Total lipids	PL	NL
14:0	0.1	0.1	0.6	3.2	11.2	4.6	1.5	1.4	3.7
15:0	0.1	0.1	0.4	0.5	0.5	0.7	0.6	0.6	0.9
16:0	21.7	22.8	15.9	12.4	21.8	17.7	31.2	33.8	22.4
16:1n−7	0.1	0.1	ND	4.5	4.5	6.7	0.5	0.1	2.8
16:2	0.1	0.2	2.0	0.2	ND	0.3	0.9	0.9	2.1
18:0	3.7	3.8	5.8	3.8	5.4	4.2	4.1	4.1	7.1
18:1	15.2	15.6	28.4	13.8	14.9	16.7	2.1	3.8	27.1
18:2n−6	52.0	51.5	31.6	4.7	19.0	5.1	0.5	0.2	4.5
18:3n−3	4.1	4.1	2.9	1.1	2.4	1.2	ND	0.1	3.4
18:4n−3	ND	0.0	ND	1.6	1.4	1.5	0.1	0.1	1.2
20:0	0.1	0.2	0.5	0.5	0.9	0.2	ND	0.1	ND
20:1n−9	ND	0.1	ND	2.5	ND	2.0	4.9	4.5	4.9
20:4n−6	ND	ND	ND	1.5	0.7	1.4	1.8	1.9	0.8
20:5n−3	ND	ND	ND	17.2	5.7	9.4	13.7	13.4	6.8
22:0	0.4	0.4	ND	0.1	0.3	0.1	ND	0.1	ND
22:1n−11	ND	ND	ND	1.3	ND	1.1	0.7	0.8	0.7
22:6n−3	ND	0.0	ND	18.3	4.3	16.1	33.0	31.8	8.6

ND, not detected.

2.4. Analysis of fatty acids from Artemia total, polar and neutral lipids

Total lipids from lyophilised *Artemia* samples were extracted according to the method of Folch et al. (1957) with the modifications described by Monroig et al. (2006). Two milligrammes of total lipids were applied onto 20 × 20 cm Silica Gel 20 (Merck, Darmstadt, Germany) thin-layer chromatography (TLC) plates and subsequently eluted with a mixture of n-hexane:diethyl ether:acetic acid (85:15:1.5, v/v/v). One single PL fraction and two distinct NL fractions corresponding to triacylglycerides (TAG), and the combination of monoacylglycerides, diacylglycerides and free fatty acids (hereafter referred to as 'combined fraction', CF) were scraped from the plate after identification and quantification (Olsen and Henderson, 1989) with known standards. FA methyl esters (FAME) from total, polar and neutral (TAG and CF) lipids were prepared by direct acid transmethylation following the protocols described in Christie (2003). FAME were analysed with a Thermo gas chromatograph (Thermo Trace GC Ultra, Thermo Electron Corporation, Waltham, MA, USA) fitted with an on-column injection system and a FID detector. Analytical temperature was programmed from 50 °C to 220 °C. Chromatograms were integrated and analysed with Azur Datlys (St Martin d'Heres, France) software. FAs were identified by comparison of retention times of each peak with those of well characterised standards.

2.5. Statistical analysis

Statistical analyses were performed with the SPSS for Windows 15.0 statistical package (SPSS Inc., Chicago, IL, USA). Data are expressed as means ± standard deviations (n = 3). The FA profiles obtained were integrated chemometrically in a principal component analysis (PCA) model. The score plot obtained after the generation of the two principal components was used to identify patterns of distribution of FA among treatments and lipid classes.

3. Results

Total lipids from Treatment 1 metanauplii accounted for 16.5 ± 1.0 mg g dw $^{-1}$. Quantification of the different lipid fractions prepared from *Artemia* metanauplii samples showed that PL accounted for 30% of total lipids, whereas NL accounted for 70% (25% TAG and 16% CF) of total lipids in both treatments. FA composition of total lipids, PL and both NL fractions (TAG and CF) from Treatment 1 *Artemia* is shown in Table 2. The main FA found in total lipids included 16:0, 18:0, 18:1, 18:2n−6, 18:3n−3, 22:5n−3 and 22:6n−3. DHA (22:6n−3), supplied in the enrichment diet of Treatment 1 mainly as NL (Table 1), reached up to 5.8% in the total lipid fraction of metanauplii. While DHA represented only 0.8% of total FA in PL, DHA contents of 12.7% in TAG and 10.4% in CF were found, indicating that it was mainly deposited into *Artemia* NL. Importantly, LA (18:2n−6), the main FA of Soya lecithin (Table 1), accounted for 6.8% of total FA in PL, 10.9% as TAG and 14.1% in CF.

In Treatment 2, *Artemia* metanauplii contained 17.6 ± 0.1 mg g dw $^{-1}$ total lipids. FA composition of total lipids, PL and both NL fractions from Treatment 2 metanauplii are presented in Table 3. The main FA found in the total lipid fraction were 16:0, 18:0, 18:1, 18:3n−3, 20:5n−3 and DHA. Content of DHA, presented basically as PL in the enrichment diet Marine lecithin LC60 (Table 1), reached a value of 13.1% in the total lipid fraction of *Artemia* metanauplii. Interestingly, only 1.9% of total FA in the metanaupliar PL was DHA, while the contents of this fatty acid attained 26.0% of total FA in TAG and 22.2% in CF. Clearly, this result showed again a preferential deposition of DHA in the NL of enriched *Artemia*.