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The effect of live food enrichment with docosahexaenoic acid (22:6n-3) rich emulsions on growth, survival and fatty acid composition of meagre (*Argyrosomus regius*) larvae

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ABSTRACT

While spawning induction and larval rearing of meagre (*Argyrosomus regius*) have advanced as forcing factors to move this finfish species into the commercial aquaculture sector, larval nutrition still has unanswered issues to address, specifically in regard to live prey enrichment and fatty acid composition. In this study, two experimental trials for larval rearing of meagre utilizing different methods of live prey enrichment with emulsions of different specialist oils having distinct fatty acid composition, have demonstrated that docosahexaenoic acid (DHA) requirements may be species-specific, with a DHA supplement of 12–15% in live prey enrichment diets yielding optimum larval growth. Cannabilism in early life stages (post 20 dph) in this species also remains a challenge and requires stocks to be managed accordingly. Further, we found evidence suggesting meagre larvae are not capable of elongation or desaturation of fatty acids when precursors such as LA, GLA, LNA or SDA are offered in the live prey.

1. Introduction

The understanding of nutrition and feeding during early development is a major prerequisite to counter the challenges of marine fish larvae culture. The success of larval rearing is greatly influenced by first feeding regimes and the nutritional quality of starter diets, with dietary lipids being recognized as one of the most important nutritional factors affecting larval growth and survival (Watanabe, 1993).

Marine lipids are rich in saturated and monounsaturated fatty acids, which are a vital source of metabolic energy for the rapidly developing and growing fish larvae. In addition, they supply polyunsaturated fatty acids (PUFA) which are considered essential fatty acids (EFA) for marine fish since they cannot be biosynthesized and hence must be provided in the diet. Three long chain PUFA (LC-PUFA), namely docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) have a variety of vital functions in fish species, as in most vertebrates, being the main components of membranes and precursors of bioactive metabolites such as eicosanoids (Tocher, 2010). DHA plays an important role during larval development as it is incorporated into nervous and retina tissue (Mourente and Tocher, 1992; Bell et al., 1996), and when absent from the larval diet, leads to poor growth and high mortality as well as to several behavioral, physiological and morphological alterations (Lingenfelser et al., 1995; Tocher, 2010).

Meagre, *Argyrosomus regius*, is a valuable new finfish species for European aquaculture desired for its rapid growth during ongrowing and the quality of its meat (Poli et al., 2003; Grigorakis et al., 2011). At present, spawning induction (Duncan et al., 2012), larval development (Jimenez et al., 2007; Cardeira et al., 2012) and larval rearing protocols (Roo et al., 2010; Vallés and Estevez, 2013; Campoverde et al., in press) have already been established. However, nutritional requirements of the larva, including LC-PUFA, have not been established yet, although some preliminary information, using commercial enrichment products or microdiets for larval feeding, already exist (Vallés and Estevez, 2015; El Kertaoui et al., 2017). According to Monroig et al. (2013) they express at least one fatty acyl desaturase (Fads2) and one elongase (Elovl5) involved in the endogenous production of LC-PUFA.

Hemp seed oil has been dubbed “Nature's most perfectly balanced oil” (Callaway, 2004), due to the fact that it contains the perfectly balanced 3:1 ratio of Omega 6 (18:2n-6, linoleic, LA) to Omega 3 (18:3n-3, α-linolenic, LNA) essential fatty acids, determined to be the optimum requirement for long-term healthy human nutrition. In addition, it also contains smaller amounts of three other PUFA such as γ-linolenic acid (18:3n-6, GLA), oleic acid (18:1n-9, OA) and stearidonic acid (18:4n-3, SDA). This fatty acid combination is unique among edible oil seeds and can be used to check the capacity of marine

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Table 1
Formulation (g/100 g) and fatty acid composition (% weight of total fatty acids –TFA–) of the emulsions containing high (DHA-H), medium (DHA-M) and low (DHA-L) DHA concentrations, hemp oil and commercial product (Red Pepper) used in Experiments 1 (left) and 2 (right).

	Exp 1				Exp 2			
Ingredients (g)	DHA-H	DHA-M	DHA-L	Hemp	DHA-H	DHA-M	Hemp	
Cod liver oil[a]	10.7	45.3	71.4	5.7				
Incromega 500TG[b]	66.7	33.3	7.1	–	75.8	–	–	
Incromega 3322TG[b]					–	75.8	–	
Olive oil[e]	8.0	6.7	5.7	7.1	7.6	7.6	7.6	
Hemp oil[c]	–	–	–	71.4	–	–	75.8	
Soybean lecithin[f]	9.3	9.3	10.0	10.0	10.6	10.6	10.6	
α-Tocopherol[f]	5.3	5.3	5.7	5.7	6.1	6.1	6.1	
	DHA-H	DHA-M	DHA-L	Hemp	DHA-H	DHA-M	Hemp	Red Pepper[d]
Total lipids (mg/g DW)	705.2	650.8	703.5	680.3	703.6	641.7	672.9	359.8
Total FA (mg/g lipids)	644.7	686.5	711.4	712.0	684.8	789.8	849.5	821.3
Fatty acids (%TFA)								
14:0	3.1	2.9	2.6	2.1	0.1	0.1	0.1	3.6
16:0	3.2	7.2	11.1	2.9	3.6	6.0	6.7	32.3
18:0	3.5	3.3	3.2	2.6	3.1	2.8	2.6	1.3
Total SFA	10.0	13.6	17.1	7.8	6.7	9.0	9.4	37.4
16:1n-7	1.7	3.3	4.8	0.7	0.4	0.4	0.2	0.6
18:1n-9	18.5	21.5	24.5	19.1	12.3	14.2	14.6	2.3
18:1n-7	1.1	3.3	5.4	0.8	1.2	1.3	1.3	0.6
20:1n-9	2.8	6.3	9.5	0.9	1.8	1.0	0.0	0.2
Total MUFA	25.1	39.1	52.3	22.0	15.7	16.9	16.1	3.7
18:2n-6 (LA)	6.6	7.5	8.9	45.2	4.6	30.9	52.7	4.2
18:3n-6 (GLA)	0.1	0.1	0.0	2.9	0.2	2.1	3.3	0.2
20:4n-6 (ARA)	1.2	0.7	0.2	0.0	2.2	0.9	0.1	1.0
22:5n-6	2.2	1.2	0.3	0.0	3.0	0.2	0.0	12.2
Total n-6 PUFA	10.1	9.5	9.4	48.1	10.2	34.2	56.2	17.9
18:3n-3 (LNA)	0.2	0.4	0.6	12.5	0.5	9.5	16.2	0.3
18:4n-3 (SDA)	0.8	1.4	2.0	1.7	1.1	2.1	1.4	0.5
20:5n-3 (EPA)	8.2	7.2	6.1	0.9	11.5	13.5	0.0	2.0
22:5n-3	2.2	4.2	6.1	0.4	2.3	1.8	0.0	0.4
22:6n-3 (DHA)	40.2	22.8	5.9	1.2	50.7	11.9	0.7	37.2
Total n-3PUFA	52.1	36.4	20.9	16.7	67.5	40.0	18.3	41.0
Total PUFA	62.3	45.9	30.2	64.8	77.6	74.2	74.5	58.9
n-3/n-6	5.2	3.8	2.2	0.4	6.6	1.2	0.3	2.3
DHA/EPA	4.9	3.2	1.0	1.3	4.4	0.9	–	18.3

Totals include some minor components not shown.
[a] Cod liver oil.
[b] Incromega oil, Croda Iberica, Spain.
[c] Hemp oil, Hempoil Canada Inc., Canada.
[d] Red Pepper, Bernaqua, Belgium.
[e] Olive oil.
[f] Supplements: Soy lecithin, Laboratories Korott, Spain; α-tocopherol, Sigma-Aldrich, Spain.

fish larvae to elongate or desaturate fatty acids from precursors.

Thus, the objectives of the present study were (1) to examine the effect on growth, survival and fatty acid composition of the larvae of different enrichment emulsions for live prey based on specialist oils with different DHA content and DHA/EPA ratio and (2) to check the ability of meagre larvae to elongate or desaturate fatty acids from precursors using an enrichment emulsion formulated with hemp oil.

2. Materials and methods

2.1. Meagre larviculture and sampling

Newly hatched meagre (*Argyrosomus regius*) larvae were obtained after hormonal induction (Duncan et al., 2012) in the Centro de Aquicultura, Institut de Recerca i Tecnologia Agroalimentaries (IRTA, San Carles de la Rapita, Spain). Floating eggs were incubated in 35 L cylindrical PVC containers provided with air-lift systems and high aeration supply at 20 °C. Newly hatched larvae were distributed into sixteen 100 L tanks at a density of 50 larvae L^{-1}, in three replicated tanks per treatment. The tanks were connected to a recirculation unit (IRTAmar™). Water temperature was controlled every day, increased from 20 to 23 °C at 1 °C day^{-1} and maintained at 23.0 ± 1.7 °C, salinity at 35.82 ± 0.33 ppt, dissolved oxygen at 7.2 ± 1.0 mg L^{-1} whereas pH (7.97 ± 0.06) nitrities (0.02 ± 0.02 mg L^{-1}) and amonia (0.1 ± 0.05 mg L^{-1}) were checked 2 times per week (Hach Colorimeter DR/890, USA). Photoperiod was kept at 16 h light: 8 h darkness and light intensity was maintained at 500 lx at water surface following the recommendations of Vallés and Estevez (2013). At mouth opening, 2 days post-hatching (dph), larvae started being fed enriched rotifers until 14 dph twice daily (10:00 and 16:00 h) at a density of 10 rotifers mL^{-1}. Freshly enriched *Artemia* metanauplii were introduced at 8 dph and fed until 30 dph, in quantities ranging from 0.5 to 6 metanauplii mL^{-1}, and adjusted based on the increase of larval weight, in two to three daily rations (9:00–13:00–17:00 h). Larvae were fed metanauplii until the end of the experiment.

Samples of 10 larvae were collected from each tank at 0, 7, 16 and 30 dph in Experiment 1 and at 0, 14, 27 and 30 dph in Experiment 2 and killed with an overdose of anesthetic MS222 (1000 mg L^{-1}). Standard length (SL) was measured using a dissecting microscope and an image analyser (Analysis, SIS Gmbh, Germany). The same larvae were then washed on a mesh with distilled water, dry blotted to remove excess water and pooled onto pre-weighted coverslips. They were then oven-dried at 60 °C for 24 h and weighed to determine dry weight (DW) on a Mettler A-20 microbalance (Mettler Toledo, Columbus, OH, USA) to the nearest ± 1 μg. Specific growth rate (SGR) was calculated at the end of the trials using the formula:

$$SGR = (\ln Wf - \ln Wi \times 100)/t \ (\%day^{-1}).$$

where lnWf = the natural logarithm of the final weight; lnWi = the natural logarithm of the initial weight and t = time (days) between lnWf and lnWi.

At the end of the experiment, all the larvae remaining in the tanks were counted and, to evaluate survival, both these and the larvae sampled during the experiment were taken into account, using the formula developed by Buckley et al. (1984).

Two trials were carried out with meagre larvae in consecutive years using spawns from the same broodstock and under the same hormonal induction protocol. Briefly, in Experiment 1 three different levels of DHA were used to formulate the enrichment emulsions and compared to an emulsion formulated with hemp oil, whereas in Experiment 2 only two DHA levels, without any addition of cod liver oil, using only specialist oils, were assayed and the results were also compared to a commercial enricher (Red Pepper, Bernaqua, Belgium) generally used in meagre hatcheries (J. Carrillo, Pers. Com., 2016).

Samples of meagre larvae at the beginning and at the end of the trials and of the enriched live prey (at least 3 samples of each live prey throughout the duration of the experiments) were taken for lipid and fatty acid analysis. The larvae were previously anesthetized with MS-222, concentrated in a mesh sieve and washed with distilled water before freezing at − 20 °C for later analysis of lipids and fatty acids.

2.2. Experimental live prey enrichment emulsions

The emulsions to enrich the live prey used in the trials (Table 1), were prepared following the methodology described in Villalta et al. (2005) and Estevez & Gimenez (in press), mixing cod liver oil (Sigma-Aldrich, Madrid, Spain), Incromega DHA500 and Incromega DHA3322TG oil (Croda Iberica, Spain), high oleic olive oil (Borges, Lleida, Spain) and hemp oil (Hempoil Canada Inc., Ste. Agathe, Canada). The different oil mixtures were emulsified with warm (50 °C) distilled water, soy lecithin (Laboratorios Korott, Alicante, Spain) and α-tocopherol (Sigma-Aldrich, Madrid, Spain) by homogenising with an Ultra-turrax T25 at high speed for 60–90 s. The emulsions were then transferred to plastic syringes, the air removed, and kept refrigerated (4 °C) in an upright position until used for enriching the live prey.

2.3. Live food enrichment

2.3.1. Rotifers

Rotifers (*Brachionus* sp.) used for enrichment were cultured in 100 L conical fibreglass tanks at a salinity of 26 ppt, water temperature of 26 °C and 8 mg L^{-1} dissolved oxygen. The rotifer culture was aerated and daily fed microalgae (*Tetraselmis chuii)* at 4 × 10^5 cell mL^{-1} and yeast (Mauripan, Spain) at 0.7 g/million rotifers. The daily ration of rotifers was harvested and divided into four oxygenated 10 L containers filled with UV filtered seawater and enriched for 12 h with one of the four experimental emulsions at a density of 500 rotifers mL^{-1} and 28 ± 1 °C, using 0.6 g L^{-1} of each emulsion. After 12 h the rotifers were gently filtered and washed using a 40 μm mesh with UV filtered seawater and disinfected by further rinsing for 1 min with freshwater, before feeding to the larvae.

2.3.2. Artemia

Brine shrimp *Artemia* cysts (2 g L^{-1}, EG type; INVE, Belgium) originating from Great Salt Lake, USA, were incubated during 24 h in 100 L cylindrical-conical tanks and hatched at 28 °C in highly aerated seawater. The newly hatched nauplii were harvested and washed using a 150 μm mesh, transferred to clean seawater and their concentration was determined by counting the nauplii in 1 mL samples under a binocular microscope. *Artemia* nauplii were then enriched in 20 L containers, using 0.6 g L^{-1} of the emulsions for 12 h. The oil emulsions were blended with water (1:1) in a high-speed blender. Enriched metanauplii were harvested, washed and disinfected with hydrogen peroxide at 8000 ppm for 5 min, thoroughly washed for 15 min on 150 μm plankton nets with UV filtered seawater and counted.

2.4. Lipid and fatty acid analysis

Total lipids were extracted in chloroform:methanol (2:1, v:v) using the method of Folch et al. (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by vacuum desiccation overnight. Total lipids were stored in chloroform:methanol (2:1, 20 mg mL^{-1}) containing 0.01% butylated hydroxytoluene (BHT) at − 20 °C prior to analysis. Acid catalyzed transmethylation was carried out using the method of Christie (1982). Methyl esters were extracted twice using isohexane diethyl ether (1:1, v:v), purified on TLC plates (Silica gel 60, VWR, Lutterworth, UK) and analyzed by gas–liquid chromatography on a Thermo TraceGC (Thermo Fisher, Spain) fitted with a BPX70 capillary column (30 m × 0.25 mm id; SGE, UK), using a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C min^{-1} and holding at 250 °C after ramping at 2 °C min^{-1}. Helium (1.2 mL·min^{-1} constant flow rate) as the carrier gas and on-column injection and flame ionization detection at 250 °C were used. Peaks of each fatty acid were identified by comparison with known standards (Supelco Inc., Spain) and a well characterized fish oil, and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrom-card for Windows (TraceGC, Thermo Fisher, Spain). Results of total lipids, total fatty acids and fatty acid content in the enrichment emulsions, enriched rotifers and enriched *Artemia* metanauplii for each treatment are shown in Table 2 and expressed as percentage of total fatty acids (TFA).

2.5. Statistics

Results in terms of larval DW, SL and survival of the larvae and the fatty acid profile of the larvae and live prey were compared by one way analysis of variance (ANOVA). Percentage values were arcsine transformed and the assumption of homogeneity of variances was checked using the Shapiro-Wilk test. Data was analyzed at a significance level of 0.05. When significant differences were found, the Tukey HSD multiple range test was performed, using SigmaPlot 12.0 software. All data is given as mean values and standard deviations (± SD).

3. Results

Tables 1, 2 and 3 show the formulation, lipid and fatty acid composition of the emulsions (Table 1) used for live prey enrichment and the fatty acid profile of the enriched live prey (Tables 2 and 3), used in both experiments. The main difference in the formulation of the emulsions was the use of cod liver oil mixed with Incromega 500TG oil to have the desired levels of DHA (Low –L–, Medium –M– and High –H–) in Experiment 1, whereas two different Incromega oils (500TG and 3322TG) with different content of DHA and EPA were used in Experiment 2 for the same purpose. Cod liver oil provided the emulsions with a lower content of EPA, and consequently a higher DHA/EPA ratio, and a higher content of oleic acid (18:1n-9), and consequently higher content of monounsaturated fatty acids (MUFA). The use of hemp oil in the emulsions resulted in a higher content of LA, GLA, total n-6 PUFA and LNA and very low content in n-3 PUFA and EPA and DHA.

The fatty acid composition of live prey (Tables 2 and 3, only mean values are shown for clarity) reflected the composition of the emulsions. Thus, rotifers enriched with DHA-H had the highest content of DHA 34.54% in Exp 1 and 42.59% in Exp 2 and DHA/EPA ratio of 7.36 in Exp 1 and 6.17 in Exp 2, whereas those enriched with DHA-L had the lowest DHA content and DHA/EPA ratio (8.7% and 1.44, respectively).

Table 2
Fatty acid composition (%TFA) of the enriched live prey enriched on experimental emulsions used in Experiment 1. Different letters indicate significant differences (ANOVA, $P < 0.05$).

	Rotifer				*Artemia*			
	DHA-H	DHA-M	DHA-L	Hemp	DHA-H	DHA-M	DHA-L	Hemp
Total lipids (mg/g DW)	138.3	122.2	149.6	167.5	191.3	169.3	123.8	221.9
Total fatty acids (mg/g lipids)	605.8	596.9	619.8	620.0	689.3	616.0	649.4	644.4
Fatty acid composition (% TFA)								
14:0	0.8	2.0	2.6	0.6	0.2	0.5	0.6	0.6
16:0	5.3	7.8	9.8	8.1	7.7	10.4	10.0	9.4
18:0	3.9	3.6	33.4	3.5	5.3	6.4	5.2	5.9
Total saturated	10.0c	13.4b	15.8a	12.2b	13.4	17.6	15.9	15.4
16:1n-7	5.4b	13.3a	12.2a	5.8b	0.6	2.0	2.1	0.6
18:1n-9	20.5	23.8	26.2	22.5	19.0	22.2	24.5	21.2
18:1n-7	7.8	7.3	4.4	2.3	8.6	6.3	7.6	3.9
20:1n-9	2.9	5.7	8.6	2.0	1.1	2.2	3.6	0.9
Total monounsaturated	38.1b	52.8a	56.3a	32.8b	29.7a	33.8ab	40.1b	26.8a
18:2n-6	5.0b	3.9b	5.7b	40.9a	6.0b	5.9b	7.5b	29.6a
18:3n-6	0.0b	0.0b	0.0b	2.0a	0.6b	0.8b	0.7b	1.5a
20:4n-6	1.0a	0.9a	0.4b	0.1c	1.2a	1.2a	0.6b	0.2c
Total n-6 PUFA	8.2b	6.6b	7.1b	43.4a	8.8b	8.7b	8.9b	21.4a
18:3n-3	0.6	0.6	0.9	9.45	21.7	22.3	19.1	22.2
18:4n-3	0.3	0.7	1.3	0.6	2.7	3.4	2.9	2.4
20:5n-3	4.7b	5.2ab	6.0a	0.7c	6.0a	5.1b	5.9a	1.0c
22:5n-3	1.3	1.0	0.8	0.1	0.6	0.5	0.5	0.0
22:6n-3	34.5a	16.1b	8.7c	0.7d	16.2a	7.6b	5.6c	0.5d
Total n-3 PUFA	42.1a	24.3b	18.5c	11.6d	48.0a	40.0b	35.0c	26.5d
Total PUFA	50.4a	31.0b	25.6b	55.0a	56.8a	48.6b	44.0b	57.9a
n-3/n-6	5.1a	3.7b	2.6c	0.3d	5.4a	4.6b	3.9c	1.2d
DHA/EPA	7.4a	3.1b	1.4c	1.0d	2.7a	1.5b	1.0c	0.5d

Rotifers enriched with the emulsion formulated with hemp oil had the highest levels of LA, GLA and LNA in both trials.

A similar trend was also observed in enriched *Artemia*, with DHA levels ranging from 16.22% to 5.61% when enriched with DHA-H and DHA-L, respectively, whereas *Artemia* enriched with hemp oil emulsion had the highest content of LA, GLA and LNA fatty acids. In experiment 2 the use of Incromega 3322TG gave as a result the highest EPA content in both live prey whereas the use of the commercial enricher, Red Pepper, produced rotifers and *Artemia* metanauplii with intermediate levels of DHA (23.83% and 6.95%, respectively) and the highest DHA/EPA ratio in the rotifers.

Larval growth is presented in Fig. 1. In experiment 1 larvae fed DHA-H enriched live prey had a statistically significant (ANOVA $P < 0.001$) better growth than the rest of the treatments, reaching

Table 3
Fatty acid composition (%TFA) of the enriched live prey enriched on experimental emulsions used in Experiment 2. Different letters indicate significant differences (ANOVA, $P < 0.05$).

	Rotifer				*Artemia*			
	DHA-H	DHA-M	Red Pepper	Hemp	DHA-H	DHA-M	Red Pepper	Hemp
Total lipids (mg/g DW)	180.4	194.3	170.9	196.0	122.4	138.3	113.2	130.6
Total FA (mg/g lipids)	574.5	535.2	524.4	693.9	678.3	723.0	687.7	720.1
Fatty acid composition (% TFA)								
14:0	0.2	0.2	1.9	0.2	0.4	0.4	0.7	0.4
16:0	4.1b	4.7b	18.6a	7.0b	9.0b	8.4b	14.7a	9.2b
18:0	3.5	3.7	3.1	3.2	5.9	5.7	7.0	5.1
Total saturated	7.8b	8.6b	23.9a	10.4b	15.8b	15.1b	23.2a	15.1b
16:1n-7	3.0b	3.4b	6.3a	2.4b	1.5	1.5	1.5	1.0
18:1n-9	16.2	17.3	12.4	17.8	22.2	21.7	19.1	20.6
18:1n-7	2.2	2.8	2.4	1.6	7.0	7.0	8.0	4.0
20:1n-9	2.4	2.9	1.8	1.0	1.3	1.5	0.7	0.7
Total monounsaturated	23.8	26.5	22.9	22.8	32.3	32.1	29.3	26.3
18:2n-6	5.7b	4.8b	7.1b	44.3a	7.2b	6.0b	6.2b	36.9a
18:3n-6	0.3b	0.3b	0.4b	3.1a	0.2b	0.2b	0.0b	1.6a
20:3n-6	0.3	0.5	0.6	0.0	0.1	0.1	0.2	0.0
20:4n-6	1.9a	2.1a	1.2b	0.5c	1.7a	1.6a	2.4a	0.4b
Total n-6 PUFA	11.2bc	8.6c	16.7b	48.1a	10.2b	8.2b	11.4b	38.9a
18:3n-3	1.0b	1.1b	1.3b	13.7a	18.2	16.8	20.5	16.8
18:4n-3	1.0b	2.0a	0.7b	1.8a	2.2	2.0	2.4	1.7
20:4n-3	1.2a	1.9a	1.9a	0.4b	0.4	0.9	0.5	0.0
20:5n-3	6.9b	25.6a	3.3c	0.6d	6.7b	16.3a	5.4b	0.9c
22:5n-3	1.8b	3.7a	0.7c	0.0d	0.8b	1.4a	0.2c	0.0d
22:6n-3	42.6a	18.5c	23.8b	0.9d	13.4a	6.8b	7.0b	0.4c
Total n-3 PUFA	55.1a	54.3a	32.4b	17.7c	41.8a	44.6a	36.0b	19.8c
Total PUFA	66.4a	62.9a	49.1b	65.8a	51.9a	52.8a	47.5b	58.6a
n-3/n-6	4.9b	6.3a	2.0c	0.4d	4.1b	5.4a	3.2c	0.5d
DHA/EPA	6.2a	0.7c	7.3a	1.5b	2.0a	0.4	1.3b	0.5c

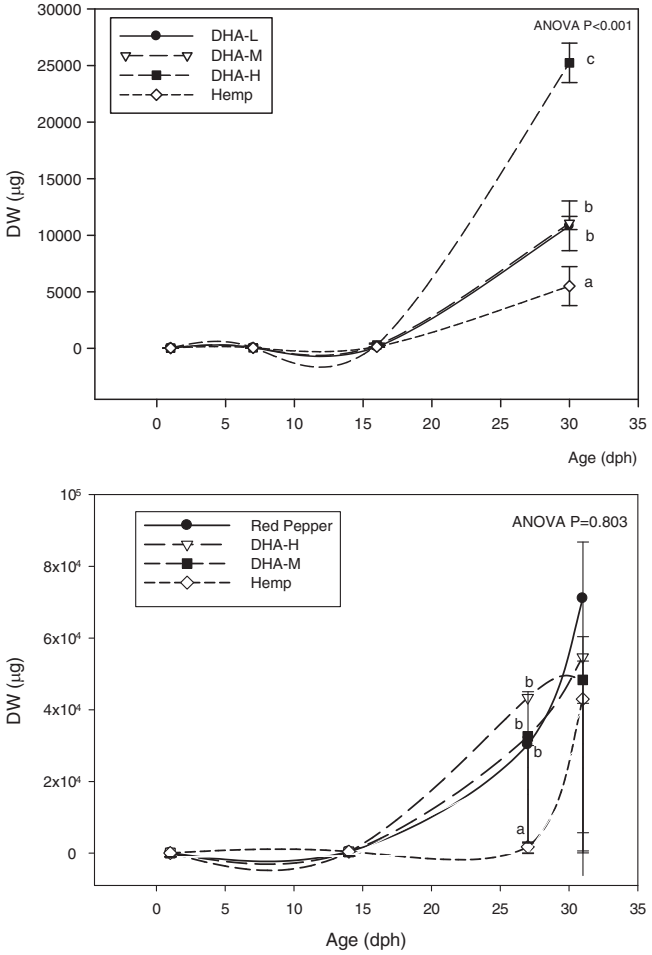

Fig. 1. Growth (dry weight, DW, µg) of the larvae in Experiments 1 and 2. Letters indicate significant differences (ANOVA, $P < 0.05$).

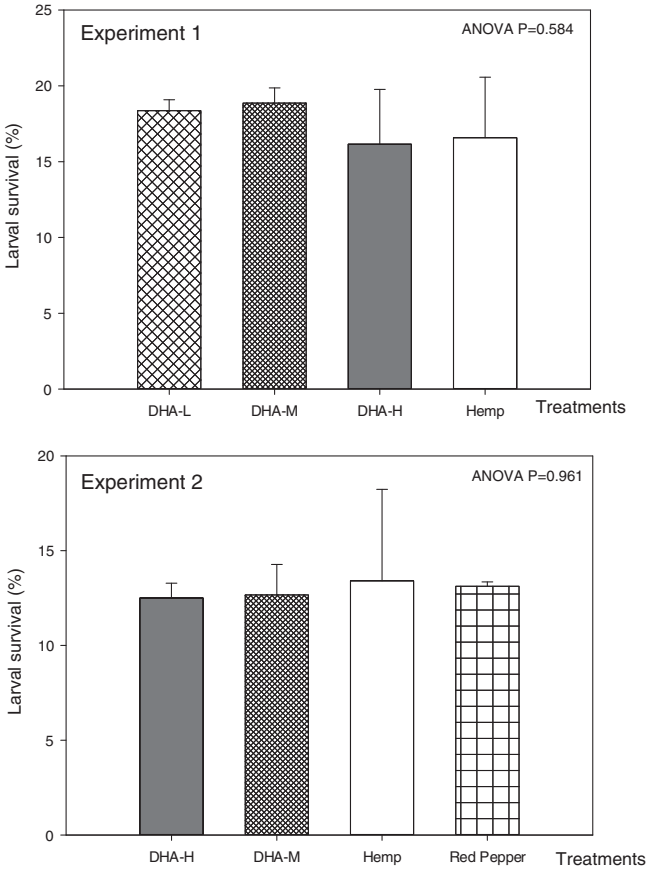

Fig. 2. Larval survival rate (%) obtained after 30 days feeding live prey enriched with the experimental emulsions. a) Experiment 1 and b) Experiment 2.

25.24 mg of dry weight at 30 dph and a specific growth rate (SGR) of 22.31% day^{-1} (Table 4). In experiment 2 no significant differences could be detected among the 4 groups of larvae due to the high variability of sizes and weights found among the larvae of each group. However, in this experiment larvae fed DHA-H enriched live prey attained 54.70 mg dry weight and SGR of 24.24% day^{-1} at 30 dph. One of the problems of experiment 2 was the high incidence of cannibalistic behaviour observed from 20 dph onwards that has already been described in the rearing of meagre larvae (Roo et al., 2010; Campoverde et al., in press) and in a closely related species *Argyrosomus japonicus* (O'Sullivan and Ryan, 2001; Timmer and Magellan, 2011). Having in mind this cannibalistic behaviour, at the end of Experiment 2, large (50 to 210 mg DW and 2.4 to 2.9 cm SL) and small (1.5 to 25 mg DW and 1.3 to 1.5 cm SL) larvae were separated to carry out the fatty acid analyses and detect possible differences in the fatty acid profile between them.

Larval survival rate is shown in Fig. 2, no significant differences could be found among the four larval groups in any of the experiments, with the survival rate being relatively higher (around 18%) in Experiment 1 compared to Experiment 2 (around 14%).

The fatty acid composition of the larvae at the end of the trials is shown in Tables 5, 6 and 7. Fatty acid composition of the larvae reflected the composition of the live prey. In both experiments larvae

Table 4
Dry weight (µg, mean ± SD) and specific growth rate (SGR. % day^{-1}) values obtained in Experiments 1 and 2.

Experiment 1					
	0 dph	7 dhp	16 dhp	30 dph	SGR (%)
DHA-H	31.3 ± 0.4	49.1 ± 6.5b	294.4 ± 172.4b	25,240.5 ± 1745.8c	22.3c
DHA-M	31.3 ± 0.4	63.8 ± 4.6d	259.0 ± 116.2b	11,089.8 ± 582.8b	19.6b
DHA-L	31.3 ± 0.4	57.8 ± 11.9c	109.9 ± 9.0a	10,838.0 ± 2199.4b	19.5b
Hemp	31.3 ± 0.4	41.5 ± 6.3[a]	138.0 ± 0.0a	5499.8 ± 1722.8a	17.2a
ANOVA *P*	< 0.001		< 0.001	< 0.001	< 0.001
Experiment 2					
	0 dph	14 dhp	27 dhp	30 dph	SGR (%)
DHA-H	29.8 ± 2.1	352.7 ± 115.9	32,586.8 ± 25,366.7b	54,702.5 ± 51,813.7	24.2
DHA-M	29.8 ± 2.1	314.4 ± 91.0	43,378.9 ± 36,515.7b	48,333.3 ± 46,421.0	23.8
Hemp	29.8 ± 2.1	388.5 ± 76.2	1755.9 ± 175.8a	42,980.0 ± 31,302.3	23.5
Red pepper	29.8 ± 2.1	424.2 ± 107.7	30,263.0 ± 23,595.1b	71,025.0 ± 81,238.5	25.1
ANOVA *P*	0.595 NS		0.03	0.803 NS	0.857 NS

Table 5
Fatty acid composition (%TFA, mean ± standard deviation) of meagre larvae at the beginning and end of the Experiment 1. Different letters indicate significant differences (ANOVA, $P < 0.05$).

	Day 0	Day 30			
		DHA-H	DHA-M	DHA-L	HEMP
Total lipids (mg/g DW)	86.0 ± 3.6	97.2 ± 16.9	75.3 ± 20.2	82.2 ± 4.1	109.0 ± 7.7
Total Fatty acids (mg/g lipids)	474.7 ± 1.5	455.7 ± 27.2	467.8 ± 38.9	476.3 ± 42.4	478.5 ± 21.5
Fatty acids (% TFA)					
14:0	1.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
16:0	19.8 ± 0.3	12.3 ± 0.4	13.4 ± 0.5	14.1 ± 1.4	12.5 ± 0.2
18:0	7.9 ± 0.3	10.2 ± 0.4	10.4 ± 0.8	11.0 ± 1.7	10.6 ± 0.3
Total saturated	28.7 ± 0.6	22.9 ± 0.7	24.4 ± 1.3	25.5 ± 2.9	23.6 ± 0.3
16:1n-7	5.8 ± 0.5	0.7 ± 0.3b	0.9 ± 0.3b	2.2 ± 0.7a	0.6 ± 0.2b
18:1n-9	14.2 ± 0.1	20.9 ± 1.8	21.7 ± 1.0	21.1 ± 5.1	21.7 ± 1.2
18:1n-7	1.9 ± 0.0	4.6 ± 0.4	5.1 ± 0.7	2.7 ± 3.8	4.5 ± 0.5
20:1n-9	0.7 ± 0.1	1.0 ± 0.2	1.6 ± 0.3	2.0 ± 0.5	0.7 ± 0.6
Total monounsaturated	22.5 ± 0.4	27. ± 2.4	29.3 ± 1.3	28.0 ± 7.8	27.4 ± 0.9
18:2n-6	4.6 ± 0.0	5.8 ± 0.4a	5.6 ± 0.2a	7.7 ± 1.0b	17.8 ± 0.7c
18:3n-6	1.5 ± 0.4	0.6 ± 0.1b	0.5 ± 0.1b	0.5 ± 0.1b	1.2 ± 0.2a
20:4n-6	1.9 ± 0.1	2.3 ± 0.1	2.3 ± 0.2	1.9 ± 0.4	1.2 ± 0.1
22:5n-6	0.4 ± 0.0	1.2 ± 0.3	0.9 ± 0.2	0.5 ± 0.1	0.1 ± 0.1
Total n-6 PUFA	8.8 ± 0.3	10.0 ± 0.4a	9.6 ± 0.3a	10.8 ± 1.3a	20.8 ± 0.4b
18:3n-3	1.3 ± 0.1	13.4 ± 1.5	12.7 ± 1.5	13.2 ± 0.1	14.4 ± 1.6
18:4n-3	0.1 ± 0.2	1.6 ± 0.1	1.4 ± 0.2	1.1 ± 0.3	1.8 ± 0.2
20:4n-3	0.1 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.2 ± 0.4
20:5n-3	4.5 ± 0.2	6.4 ± 0.8b	7.0 ± 0.4a	7.9 ± 1.5a	4.3 ± 0.4b
22:5n-3	1.6 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	1.6 ± 0.8	0.5 ± 0.2
22:6n-3	27.7 ± 0.4	15.7 ± 4.4a	12.5 ± 1.7ab	7.5 ± 0.3bc	5.0 ± 2.6c
Total n-3 PUFA	35.4 ± 0.3	38.8 ± 2.2b	35.4 ± 0.2b	32.0 ± 1.2b	27.0 ± 1.3a
Total PUFA	44.2 ± 0.6	48.8 ± 1.8	45.0 ± 0.3	43.3 ± 1.5	47.8 ± 1.1
n-3/n-6	4.0 ± 0.1	3.9 ± 0.4b	3.7 ± 0.1b	3.2 ± 0.1b	1.3 ± 0.1a
DHA/EPA	6.2 ± 0.4	2.5 ± 1.0a	1.8 ± 0.3b	1.0 ± 0.8c	1.1 ± 0.5c

fed DHA-H enriched live prey had a significantly higher content of DHA, whereas larvae fed hemp oil enriched prey showed the lowest DHA content and the highest levels of LA and GLA. EPA levels were similar in the larvae reared in Experiment 1, whereas in Experiment 2 larvae fed DHA-M enriched live prey showed the highest EPA content, especially in the case of small larvae (Table 6).

Fig. 3 shows the differences found in the fatty acid profile between small and large larvae of Experiment 2. Large larvae had in all the cases

Table 6
Fatty acid composition of meagre larvae (% TFA, mean ± S D) at the beginning and at the end of Experiment 2 showing the fatty acid profile of the larvae classified as small. Different letters indicate significant differences (ANOVA, $P < 0.05$).

	Day 0	Small larvae day 30			
		DHA-H	DHA-M	Red Pepper	Hemp
Total lipids (mg/g DW)	112.4	108.0 ± 16.6	95.0 ± 2.6	93.2 ± 11.8	101.8 ± 10.5
Total fatty acids (mg/g lipids)	437.3	546.8 ± 34.4a	476.4 ± 24.8	504.2 ± 15.7ab	454.6 ± 20.0b
Fatty acids (% total FA)					
14:0	2.2	0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.0
16:0	20.0	13.3 ± 0.2d	15.4 ± 0.8b	17.1 ± 0.2a	14.2 ± 0.3c
18:0	4.0	8.2 ± 0.1	10.0 ± 0.5	10.1 ± 0.4	9.9 ± 0.0
Total saturated	26.7	22.3 ± 0.4d	26.6 ± 1.2b	28.4 ± 0.5a	25.2 ± 0.3c
16:1n-7	7.7	1.6 ± 0.1	1.9 ± 0.2	1.6 ± 0.2	1.4 ± 0.2
18:1n-9	16.7	16.8 ± 0.3bc	17.5 ± 0.4b	14.9 ± 0.3d	18.0 ± 0.5ab
18:1n-7	3.6	7.4 ± 0.4	7.9 ± 0.4	7.2 ± 0.3	6.6 ± 0.1
20:01	1.1	0.9 ± 0.1	0.9 ± 0.0	0.7 ± 0.0	1.1 ± 0.1
Total monounsaturated	29.2	26.7 ± 0.1bc	28.3 ± 0.8b	24.4 ± 0.7d	27.2 ± 0.2ab
18:2n-6	6.4	5.8 ± 0.2b	5.1 ± 0.1c	4.6 ± 0.1d	23.6 ± 0.0a
18:3n-6	1.2	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	1.0 ± 0.0
20:4n-6	1.3	3.2 ± 0.2b	3.4 ± 0.2b	4.1 ± 0.3a	2.3 ± 0.1c
22:5n-6	0.2	1.3 ± 0.0	0.3 ± 0.1	4.4 ± 0.3	0.1 ± 0.0
Total n-6 PUFA	9.4	10.6 ± 0.4c	9.3 ± 0.2d	13.5 ± 0.6b	27.4 ± 0.0a
18:3n-3	2.1	10.2 ± 0.7b	10.8 ± 1.2ab	6.0 ± 0.8b	12.5 ± 0.6a
18:4n-3	0.9	1.2 ± 0.1	1.1 ± 0.2	1.1 ± 0.1	1.4 ± 0.0
20:4n-3	0.3	0.4 ± 0.1	0.6 ± 0.4	0.5 ± 0.0	0.5 ± 0.0
20:5n-3	4.0	5.9 ± 0.5b	12.8 ± 0.6a	4.3 ± 0.2c	3.2 ± 0.1d
22:5n-3	1.3	1.1 ± 0.1b	2.2 ± 0.3a	0.7 ± 0.1c	0.4 ± 0.0d
22:6n-3	20.4	20.5 ± 1.1a	6.6 ± 0.7c	16.6 ± 0.8b	1.0 ± 0.2d
Total n-3 PUFA	29.3	39.4 ± 0.4a	34.3 ± 0.8b	32.1 ± 1.0c	18.9 ± 0.3d
Total PUFA	38.7	50.0 ± 0.4a	43.6 ± 0.6c	45.6 ± 0.7b	46.3 ± 0.3b
n-3/n-6	3.1	3.7 ± 0.2a	3.7 ± 0.2a	2.4 ± 0.2b	0.7 ± 0.0c
DHA/EPA	5.1	3.5 ± 0.4a	0.5 ± 0.1b	3.9 ± 0.3a	0.3 ± 0.0b

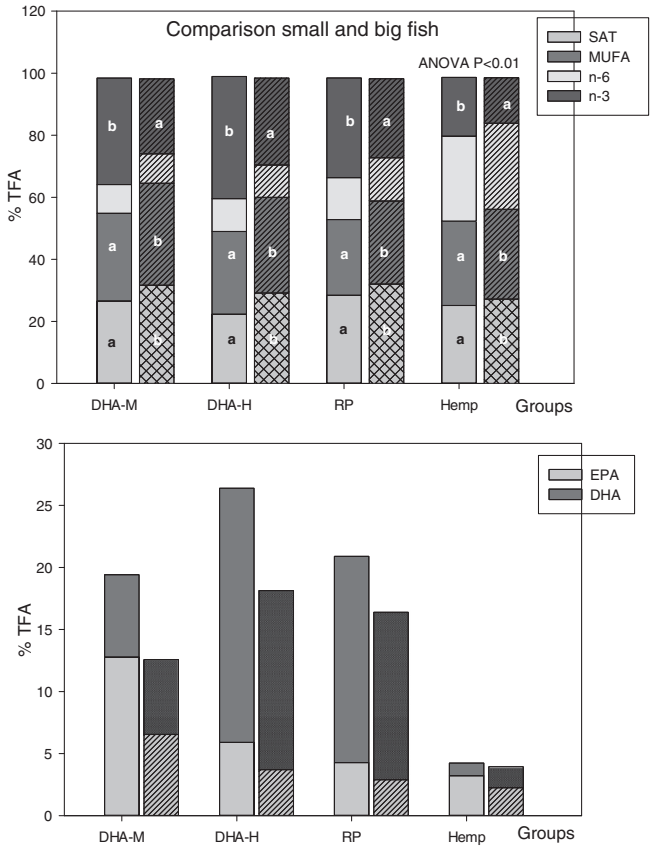

Fig. 3. Differences in the fatty acid profile between small (left hand bars without lines) and big (right hand bars with lines) larvae. Different letters indicate significant differences (ANOVA $P < 0.01$). Big larvae = 50–210 mg DW, 2.4–2.9 cm SL. Small larvae = 1.5–25 mg DW, 1.3–1.5 cm SL.

a significantly higher content of saturated (SAT, ANOVA $P = 0.022$) and monounsaturated (MUFA, ANOVA $P = 0.018$) fatty acids and a significantly lower content of n-3 PUFA (ANOVA, $P = 0.0015$) than small larvae. DHA and EPA content did not show statistically significant differences between small and large larvae, although in the case of EPA significant differences can be detected between the larvae fed diets DHA-H and Hemp (see Table 7). DHA levels were more conserved and similar between both larval sizes.

4. Discussion

The fatty acid composition of enriched live prey as well as the larvae reflected the profile obtained in the emulsions, as already published in previous reports (Dhert et al., 1993; Rainuzzo et al., 1994; Villalta et al., 2005).

Although differences exist between the two experiments carried out, there is a clear effect of the DHA level and DHA/EPA ratio in the live prey and emulsions on larval growth. Thus, the lower growth was obtained when the larvae were fed live prey with low levels of DHA and DHA/EPA ratio and n-3 PUFA content (Hemp and DHA-L groups) as already observed by El Kertaoui et al. (2017) in meagre larvae and in other marine fish (reviewed by Izquierdo and Koen, 2011). In the second experiment the differences in growth due to DHA content and DHA/EPA ratio in the live prey were clear until 27 dph, but not at the end of the trial due to the high cannibalistic behaviour of the larvae and the high differences and variability observed in size and weight between large and small larvae (see Table 4). Cannibalistic behaviour was also observed in experiment 1 but in this case this behaviour was observed later (day 28–29) than in experiment 2 due to the lower growth of the larvae (see Table 4 dry weight at 30 dph in Exp 1 is lower than the dry weight observed in 27 dph larvae in Exp 2) and lower differences in size and weight among the larvae. These differences in growth observed between the two trials were not related to differences in the rearing environment because the same protocols in terms of environmental parameters or live prey density were used for both.

Table 7
Fatty acid composition (% TFA, mean ± SD) of meagre larvae at the end of Experiment 2 showing the fatty acid profile of larvae classified as big. Different letters indicate significant differences (ANOVA, $P < 0.05$).

	Big larvae day 30			
	DHA-H	DHA-M	Red Pepper	Hemp
Total lipids (mg DW)	84.6 ± 9.0	97.8 ± 5.7	87.7 ± 8.2	98.5 ± 9.5
Total Fatty acids (mg)	441.7 ± 31.1	463.4 ± 43.6	453.6 ± 69.6	537.8 ± 28.4
Fatty acids (% total FA)				
14:0	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.0
16:0	17.5 ± 1.7ab	19.5 ± 0.2a	19.7 ± 1.0a	15.6 ± 1.0b
18:0	10.8 ± 0.7	11.1 ± 0.8	11.3 ± 1.1	10.9 ± 0.5
Total saturated	29.2 ± 2.4ab	31.8 ± 0.8a	32.1 ± 1.2a	27.3 ± 1.4b
16:1n-7	2.1 ± 0.3	2.3 ± 0.3	1.8 ± 0.2	1.9 ± 0.1
18:1n-9	19.3 ± 1.0a	19.8 ± 0.1a	16.5 ± 1.1b	18.8 ± 0.6a
18:1n-7	8.4 ± 0.4	9.8 ± 1.6	7.7 ± 0.2	7.2 ± 0.1
20:01	1.0 ± 0.2	1.0 ± 0.0	0.8 ± 0.1	1.0 ± 0.0
Total monounsaturated	30.8 ± 1.0ab	32.9 ± 1.7a	26.8 ± 1.1c	29.0 ± 0.6bc
18:2n-6	5.7 ± 0.1bc	5.1 ± 0.2b	4.7 ± 0.1c	23.8 ± 0.8a
18:3n-6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.5
20:4n-6	3.3 ± 0.2b	3.3 ± 0.2b	4.4 ± 0.2a	2.6 ± 0.3c
22:5n-6	1.1 ± 0.1	0.7 ± 0.8	4.1 ± 0.1	0.2 ± 0.0
Total n-6 PUFA	10.4 ± 0.2c	9.5 ± 1.1c	13.9 ± 0.3b	27.7 ± 0.8a
18:3n-3	7.6 ± 0.9	8.3 ± 0.4	7.1 ± 0.2	8.7 ± 1.1
18:4n-3	0.9 ± 0.0	0.9 ± 0.1	0.8 ± 0.0	0.9 ± 0.1
20:4n-3	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
20:5n-3	3.7 ± 0.6b	6.5 ± 1.5a	2.9 ± 0.2b	2.3 ± 0.2b
22:5n-3	1.0 ± 0.1b	1.9 ± 0.3a	0.8 ± 0.0b	0.7 ± 0.1b
22:6n-3	14.4 ± 1.8a	6.0 ± 1.2b	13.5 ± 2.1a	1.7 ± 0.1c
Total n-3 PUFA	28.0 ± 3.5a	24.2 ± 1.5a	25.5 ± 2.5a	14.7 ± 1.0b
Total PUFA	38.5 ± 3.6ab	33.6 ± 1.4b	39.4 ± 2.3ab	42.4 ± 1.8a
n-3/n-6	2.7 ± 0.3a	2.6 ± 0.4a	1.8 ± 0.3b	0.5 ± 0.0c
DHA/EPA	3.9 ± 0.3b	1.0 ± 0.4c	4.7 ± 0.3a	0.7 ± 0.0c

DHA levels found in this study are similar to those reported by Vallés and Estevez (2015) using commercial enrichers and considered the most adequate for the proper growth and development of meagre larvae. DHA levels around 12–15%TFA in *Artemia* metanauplii, that correspond to around 40–50% of TFA in the emulsion, and around 260–300 mg of DHA per g of lipids in the *Artemia* can be considered as the required levels for this species. Similar levels of DHA are cited for gilthead sea bream (Salhi et al., 1994) and red porgy (Roo et al., 2009) for better growth and for preventing skeletal deformations in marine fish larvae.

Although there is a clear effect of DHA on larval growth, no effects on survival rate could be detected. In previous trials carried out with meagre, an effect of DHA and DHA/EPA ratio on survival was observed (Vallés and Estevez, 2015), although in that case commercial enrichers with other additives of unknown composition were used. Similar results not linking DHA levels with survival were observed in other species such as cod (Park et al., 2006; Garcia et al., 2008; Copeman and Laurel, 2010) or striped trumpeter (Bransden et al., 2005). These authors found that the length and dry weight of larvae at the end of the experiment were directly related to dietary DHA, whereas survival rate was not influenced by dietary DHA.

Other studies carried out with marine fish larvae suggested that higher levels of DHA (or n-3 PUFA) might reduce larval survival (Planas and Cunha, 1999). Izquierdo et al. (1992) showed that, in larval Japanese flounder (*Paralichthys olivaceus*), lower (or higher) DHA content in *Artemia* metanuplii did not affect survival rate, but larvae were significantly larger when fed *Artemia* containing a higher percentage of DHA (up to 3.5%). Thus, it seems that the requirement of dietary DHA levels of marine finfish larvae is species dependent as Sargent et al. (1999) suggested.

In experiment 2 large and small larvae were analyzed separately and a clear effect of the diet is observed in the case of the small larvae that show a fatty acid profile similar to the enriched *Artemia* metanauplii (and the emulsion used for enrichment), whereas the large larvae showed a similar composition among them, independent of the diet used, with a higher content of saturated and monounsaturated fatty acids, that are metabolic energy reserves (Tocher, 2003). This suggests that the fatty acid profile of large larvae is due to their cannibalistic behaviour where consumption of their smaller siblings provides another mechanism for accumulation of energy reserves, rather than the effect of live food enrichment, or the composition of the enrichment emulsions.

Having in mind the diadromy of meagre, that migrates from deep waters of the North Atlantic and Mediterranean Sea to coastal areas to spawn in river estuaries, similarly to Atlantic salmon, Monroig et al. (2013) studied the fatty acyl desaturase and elongase capacity of this species and found that meagre can express at least one fatty acyl desaturase with homology to Fads2 and one elongase, Elov15. According to these authors meagre is able to elongate a range of C_{18} PUFA to C_{20} PUFA and even towards C_{22} PUFA. However the results of the present study show that the use of hemp oil rich in 18:2n-6, 18;3n-6, 18:3n-3 and 18:4n-3 (the precursors and first desaturation product), as the main component of enrichment had a negative effect on the growth of the larvae, with the larvae reared using live prey enriched with hemp oil being the smallest and with the lowest content of n-6 and n-3 PUFA. A clear indication of the low or non-existent elongation or desaturation capacity of meagre larvae.

5. Conclusions

1.-It is advisable to include between 12 and 15% of DHA (260–300 mg/g lipids) in live prey enrichment diets used for meagre larval rearing to obtain optimal larval growth.

2.-Survival rate in meagre was not affected by the diet used for larval feeding, but it was affected by the cannibalistic behaviour of the larvae from 20 dph onwards. Cannibalistic behaviour should be avoided, either by reducing light intensity, increasing feeding frequency, or grading the larvae, to allow better survival rates. DHA levels did not show any effect on survival rate.

3.-Meagre larvae are not capable of elongation or desaturation of fatty acids when precursors such as LA, GLA, LNA or SDA are offered in the live prey.

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