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The importance of dietary HUFA for meagre larvae (*Argyrosomus regius*; Asso, 1801) and its relation with antioxidant vitamins E and C

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Abstract

Despite the interest of meagre (*Argyrosomus regius*) as a fast-growing candidate for Mediterranean aquaculture diversification, there is a lack of information on nutrition along larval development. Importance of highly unsaturated fatty acids (HUFA) and the antioxidant vitamins E and vitamin C has not been investigated yet in this species. Six diets with two levels of HUFA (0.4% and 3% dw), two of vitamin E (1500 and 3000 mg kg^{-1}) and two of vitamin C (1800 and 3600 mg kg^{-1}) were fed to 15 dah meagre larvae. Larval growth in total length and dry body weight was significantly lowest in larvae fed diet 0.4/150/180 and showed few lipid droplets in enterocytes and hepatocytes and lower HUFA contents than the initial larvae. Increase in dietary HUFA up to 3%, significantly improved larval growth and lipid absorption and deposition. Besides, among fish fed 3% HUFA, increase in vitamin E and vitamin C significantly improved body weight, as well as total lipid, 22:6n-3 and n-3 fatty acids contents in the larvae. Thus, the results showed that 0.4% dietary HUFA is not enough to cover the essential fatty acid requirements of larval meagre and a high HUFA requirement in weaning diets is foreseen for this species. Besides, the results also pointed out the importance of dietary vitamin E and C to protect these essential fatty acids from oxidation, increase their contents in the larvae and promote growth, suggesting high vitamin E and C requirements in meagre larvae (higher than 1500 and 1800 mg kg^{-1} for vitamin E and vitamin C respectively).

Keywords: n-3 HUFA, requirements, antioxidant, *Argyrosomus regius*, fish larvae, vitamin E, vitamin C

Introduction

Meagre (*Argyrosomus regius*) is one of the fast-growing species proposed as a candidate for marine finfish diversification on commercial aquaculture in Mediterranean and Eastern Atlantic coasts (Queméner 2002; Mateos 2007; Gil, Grau, Basilone, Ferreri & Palmer 2013). At present, optimized spawning induction protocols produce high quality eggs and larvae from this highly fertile species denoting the excellent aquaculture potential of this species (Fernández-Palacios, Schuchardt, Roo, Hernández-Cruz & Duncan 2009; Mylonas, Mitrizakis, Sigelaki & Papadaki 2011; Duncan, Estévez, Porta, Carazo, Norambuena, Aguilera, Gairin, Bucci, Vallés & Mylonas 2012). Besides, larval development (Fernández-Palacios, Schuchardt, Roo, Borrero, Hernández-Cruz & Socorro 2007; Hernández-Cruz, Schuchardt, Roo, Borrero & Fernández-Palacios 2007; Jiménez, Rodríguez la Rúa, Sánchez & Cárdenas 2007; Pastor & Cárdenas 2007; Abreu, Socorro, Betancor, Caballero, Fernández-Placios, Hernández-Cruz, Roo & Schuchardt 2009; Cardiera, Vallés, Dionisio, Estévez, Gisbert, Pousao-Ferreira, Cancela & Gavaia 2012), as well as larval rearing techniques (Estévez, Treviño & Gisbert 2007; Roo, Hernández-Cruz, Borrero, Schuchardt & Fernández-Palacios 2010; Vallés & Estévez 2013) have been extensively

studied. Despite weaning to dry diets remains to be an important bottleneck for this species, there is a lack of information on meagre nutrition along larval development.

One of the most important nutritional factors for marine fish larvae is the dietary content on highly unsaturated fatty acids with 20 or more carbon atoms (HUFA) (Watanabe 1982). Namely, the n-3 series HUFA docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), and the n-6 series HUFA arachidonic acid (ARA, 20:4n-6), play very important roles in marine fish larval development (Izquierdo & Koven 2011). However, they are synthesized in very small quantities from their precursors linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) (Izquierdo, Robaina, Juárez-Carrillo, Oliva, Hernández-Cruz & Afonso 2008), due to the limited activity of Δ-5 and Δ-6 desaturases and elongases, and are considered essential (Sargent, Bell, Bell, Henderson & Tocher 1995; Izquierdo 1996). Therefore, they must be included in live preys and weaning diets to fulfil the requirements for growth, organ and tissue development and functioning, stress resistance and survival (Izquierdo, Tandler, Salhi & Kolkovski 2001). The n-3 HUFA requirements have been extensively studied in fish larvae such as red seabream (*Pagrus major*) (Izquierdo, Watanabe, Takeuchi, Arakawa & Kitajima 1989; Tandler, Watanabe, Satoh & Fukusho 1989), plaice (*Pleuronectes platessa*) (Rainuzzo, Farestveit & Jorgensen 1993), gilthead seabream (*Sparus aurata*) (Rodríguez, Pérez, Izquierdo, Mora, Lorenzo & Fernández-Palacios 1993; Rodríguez, Pérez, Lorenzo, Izquierdo & Cejas 1994; Izquierdo, Socorro, Arantzamendi & Hernández-Cruz 2000; Izquierdo 2005; Benítez-Santana, Masuda, Juárez-Carrillo, Ganuza, Valencia, Hernández-Cruz & Izquierdo 2007), Japanese flounder (*Paralichtys olivaceus*) (Furuita, Takeuchi & Uematsu 1998), atlantic halibut (*Hippoglossus hippoglossus*) (Shields, Bell, Luizi, Gara, Bromage & Sargent 1999). However, these fatty acids, particularly DHA, are very prone to oxidation and more exposed in formulated diets for marine fish larvae (Moren, Waagbø & Hamre 2011; Izquierdo, Scolamachia, Betancor, Roo, Caballero, Terova & Witten 2013). Moreover, at a physiological level, oxidative risk is particularly high in the fast growing larvae, due to the high metabolic rate, oxygen consumption and water content in the larval tissues (Betancor, Caballero, Terova, Cora, Saleh, Benítez-Santana, Bell, Hernández-Cruz & Izquierdo 2012). Thus, dietary inclusion of adequate levels of anti-oxidant nutrients is required to avoid *in vivo* lipid peroxidation and the determination excessive of HUFA requirements.

Vitamin E is recognized as the major hydrophobic chain-breaking antioxidant that prevents the propagation of free radical reactions in membranes and lipoproteins (Izquierdo & Betancor 2015). The specific location of vitamin E as a structural component of cell membranes confers this vitamin a particular role in the control of peroxidation of HUFA (Izquierdo & Betancor 2015). Early nutritional studies have showed that vitamin E is essential for marine fish larvae (Watanabe, Takashima, Ogino & Hibiya 1970; Murai & Andrews 1974; González, Izquierdo, Salhi, Hernández-Cruz & Fernández-Palacios 1995). Moreover, dietary vitamin E must be raised when dietary HUFA are high as it is found in carp (*Cyprinus carpio*) (Watanabe, Takeuchi & Wade 1981; Schwarz, Kirchgessner, Steinhart & Runge 1988), tilapia (*Oreochromis niloticus*) (Satoh, Takeuchi & WAatanabe 1987), Atlantic salmon (*Salmo salar* L.) (Hamre & Lie 1995), grouper (*Epinephelus malabaricus*) (Lin & Shiau 2005), gilthead seabream (Atalah, Hernández-Cruz, Ganga, Ganuza, Benítez-Santana, Roo, Fernández-Palacios & Izquierdo 2012; Izquierdo *et al.* 2013) or European seabass (*Dicentrarchus labrax* L.) (Betancor, Atalah, Caballero, Benítez-Santana, Roo, Montero & Izquierdo 2011). Thus, elevation of dietary PUFA causes a reduction in vitamin E contents in the liver of Atlantic salmon (Waagbø, Sandnes, Torrissen, Sandvin & Lie 1993) or African catfish (*Clarias gariepinus*) (Lim, Boey & Ng 2001). Recently, it has been shown that high levels of dietary vitamin E (3000 mg kg^{-1}) are required in diets for European seabass larvae (Atalah *et al.* 2012), particularly to promote health and stress resistance (Betancor *et al.* 2011). Nevertheless, vitamin E requirements depend on the interactions of this vitamin with other nutrients (Hamre 2011). The high vitamin E requirements of fish larvae have been associated to the high HUFA needs during larval stages (Atalah *et al.* 2012). For instance, increase in dietary vitamin E supplementation in high-DHA feeds protected this fatty acid from oxidation and reduced the occurrence of chondroid bones anomalies (Izquierdo *et al.* 2013). Deficient symptoms of vitamin E in fish larvae include accumulation of lipid oxidation products, muscle dystrophy and reduced growth and survival (Betancor *et al.* 2011; Moren *et al.* 2011; Izquierdo & Betancor 2015).

Unless it is regenerated, after neutralizing free radicals vitamin E must be replenished through the diet or from reserves elsewhere (Burton & Traber 1990). Thus, the vitamin E radical produced can probably be regenerated to vitamin E by vitamin C in the interface between water and lipid (Tappel 1962; Packer, Slater & Wilson 1979). Ascorbic acid seems to play a significant role in α–tocopherol metabolism, reducing α-tocopheroxyl radicals and regenerating them to α-tocopherol (Niki, Kawakami, Yamamoto & Kamiya 1985). Consequently, optimum dietary vitamin E levels may be also determined by the levels of vitamin C (Hamre, Waagbo, Berge & Lie 1997; Sealey & Gatlin 2002; Shiau & Hsu 2002; Chen, Lochmann, Goodwin, Praveen, Dabrowski & Lee 2004; Atalah, Hernández-Cruz, Ganuza, Benítez-Santana, Ganga, Roo, Fernández-Palacios & Izquierdo 2010). For instance, elevation of dietary vitamin C from 1800 to 3600 mg kg^{-1} during weaning of European seabass increases tissue contents in α–tocopherol and reduces the occurrence of muscular dystrophy and tissue TBARs, denoting its sparing effect over dietary vitamin E (Betancor *et al.* 2012).

At present, there is no information about the requirements of HUFA, vitamin E or vitamin C of meagre larvae during weaning, and therefore, this study was conducted to determine the importance of these nutrients and their relation in weaning diets for the fast-growing larvae of meagre.

Materials and methods

Fish and rearing

Meagre larvae were obtained from an induced spawning broodstock from the GIA facilities (Grupo de Investigación en Acuicultura) at University of Las Palmas de Gran Canaria (ULPG) (Canary Islands, Spain) where the experiment was carried out. A trial was conducted to test six microdiets in triplicates. Larvae were previously fed enriched rotifers (DHA Protein Selco; INVE, Dendermonde, Belgium) until they reached 14 days after hatching (dah). Meagre larvae (initial total length 4.07 ± 0.26 mm, mean ± SD; dry body weight 0.06 ± 0.01 mg) were randomly distributed in 18 experimental tanks at a density of 2500 larvae per tank and were fed one of the experimental diets tested in triplicates for 14 days, at an average water temperature of 23.21 ± 0.20°C. All tanks (200 L fibreglass cylinder tanks with conical bottom and painted a light grey colour) were supplied with filtered seawater (37 mg L^{-1} salinity) at an increasing rate of 8% h^{-1} to guarantee good water quality during the trial. Water entered from the tank bottom and exited from the top to ensure water renewal and maintain high water quality, a daily check out was respected. Water was continuously aerated (125 mL min^{-1}). Average water dissolved O_2 reach 5.26 ± 0.28 mg. Therefore, water quality in terms of temperature, dissolved O_2 and pH were appropriate for this species. Photoperiod was kept at 12 h light: 12 h dark by fluorescent lights. Fish larvae were manually fed 14 times per day each 45 min from 8:00 to 18:00 hours. Daily feed supplied was 1.5 and 2 g per tank during the first and second week of feeding respectively.

To avoid the nutritional contribution of *Artemia* with essential fatty acids and vitamins, this live prey was not added to the rearing tanks. Despite this complete weaning from the beginning of the study could reduce growth or survival, it was required to determine more accurately the effect of the levels of essential fatty acids and antioxidant vitamins in the weaning diets.

Experimental diets

Six isonitrogenous and isolipidic experimental microdiets (pellet size <250 μm & 250–500 μm) were formulated using fish oil (Peruvian anchovy) as source of high n-3 HUFA contents only for diets containing 3% n-3 HUFA (Table 1). The desired lipid content was completed with a non-essential fatty acid source, oleic acid (Oleic acid vegetable; Merck, Darmstadt, Germany). The protein source used (squid meal) was defatted (three consecutive times with a chloroform: squid meal ratio of 3:1) to allow a better control of the fatty acid profile of the microdiet. Two different dietary levels of n-3 HUFAs were formulated: 0.4% (low) and 3% (high) combined with three combined levels of vitamin E+C (Vitamin E: DL-α-tocopherol acetate; Sigma-Aldrich, Madrid, Spain. Vitamin C: ROVIMIX Stay-C-35), levels vitamin E/vitamin C: 1500/1800, 3000/1800 and 3000/3600 mg kg^{-1} (Table 1). Therefore, six experimental diets (0.4/150/180, 0.4/300/180, 0.4/300/360, 3/150/180, 3/300/180, 3/300/360) were tested according to HUFA, vitamin E and vitamin C levels respectively. The microdiet was prepared in the following manner: the squid powder was carefully mixed with the

other water-soluble ingredients (attractants, minerals and water-soluble vitamins) in a mortar. In a separated mixture, oils and fat-soluble vitamins were combined to obtain a homogeneous mix, which blended together with the powder mixture. Gelatine was then dissolved in warm water and, when its temperature was lower than 35°C, added to the rest of the previously mixed ingredients.

The paste was compressed pelleted (Severin, Suderm, Germany) and dried in an oven (Ako, Barcelona, Spain) at 38°C for 24 h. Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain the desired particle size. Diets were analysed for proximate composition and fatty acids levels (Tables 1 and 2). Diets were kept at −20°C until each feeding.

After feeding, larvae were observed under the binocular microscope to determine feed acceptance. Final survival was calculated by individually counting all the larvae alive at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight and total length (Profile Projector; Nikon V-12A, Tokyo, Japan) of 30 fish per tank at the beginning, at 24 (dah) and 20 fish per tank at the end of the trial.

At the end of the trial, the remaining larvae in each tank were starved for 12 h then washed with distilled water, sampled and kept at −80°C for biochemical composition.

Sample analysis

Moisture (AOAC 1995), crude protein (AOAC 1995) and crude lipid (Folch, Lees & Sloane-Stanley 1957) contents of larvae and diets were

Table 1 Ingredients and proximate composition of early weaning diets containing several n-3 HUFA, vitamin E and vitamin C levels fed to meagre (*Argyrosomus regius*) larvae from 14 to 28 dah

	Diets					
	0.4/150/180	**0.4/300/180**	**0.4/300/360**	**3/150/180**	**3/300/180**	**3/300/360**
Ingredients						
Peruvian anchovy oil	0.0	0.0	0.0	10.0	10.0	10.0
Oleic acid (%)*	10.0	10.0	10.0	0.0	0.0	0.0
Deffated Squid (g/100 g)†	69.0	69.0	69.0	69.0	69.0	69.0
Soy lecithin‡	4.5	4.5	4.5	4.5	4.5	4.5
Gelatin§	3.0	3.0	3.0	3.0	3.0	3.0
Min Px¶	4.5	4.5	4.5	4.5	4.5	4.5
Vit Px**	6.0	6.0	6.0	6.0	6.0	6.0
Attractants††	3.0	3.0	3.0	3.0	3.0	3.0
Vitamin E‡‡	150.0	300.0	300.0	150.0	300.0	300.0
Vitamin C‡‡	180.0	180.0	360.0	180.0	180.0	360.0
Proximate composition (%)						
Crude lipids	16.0 ± 0.3	17.1 ± 0.2	17.1 ± 0.4	17.2 ± 0.7	17.3 ± 0.2	17.4 ± 0.5
Crude protein	65.1 ± 0.6	64.7 ± 0.4	65.0 ± 0.5	65.4 ± 0.6	65.5 ± 0.7	64.9 ± 0.2
Moisture	10.3 ± 0.2	10.6 ± 0.1	9.4 ± 0.1	9.7 ± 0.1	9.4 ± 0.0	9.4 ± 0.1
Ash	5.5 ± 0.1	5.6 ± 0.0	5.7 ± 0.0	5.9 ± 0.0	5.7 ± 0.1	5.8 ± 0.1

*Merck, Darmstadt, Germany.

†Riber and Son, Bergen, Norway.

‡Acrofarma, Barcelona, Spain.

§Panreac, Barcelona, Spain.

¶Mineral premix supplied g per 100 g diet: NaCl 215.133 mg, $MgSO_4$ $7H_2O$ 677.545 mg, NaH_2PO_4 H_2O 381.453 mg, $Ca(H_2PO_4)$ $2H_2O$ 671.610 mg, $FeC_6H_5O_7$ 146.884 mg, $C_3H_5O_3$ 1/2Ca 1,617.210 mg, $Al_2(SO_4)_3$ $6H_2O$ 0.693 mg, $ZnSO_4$ $7H_20$ 14.837 mg, $CuSO_4$ $5H_2O$ 1.247 mg, $MnSO_4$ H_2O 2.998 mg, $CoSO_4$ $7H_2O$ 10.706 mg.

**Vitamin premix supplied per 100 g diet: cyanocobalamine 0.03 mg, astaxanthin 5.0 mg, folic acid 5.4 mg, pyridoxine-HCI 17.3 mg, thiamine 21.7 mg, riboflavin 72.5 mg, calcium-pantothenate 101.5 mg, p-aminobenzoic acid 145.0 mg, nicotinic acid 290.1 mg, myo-inositol 1450.9 mg, menadione 17.3 mg.

††Attractant premix supplied per 100 g diet: inosine-5-monophosphate 500.0 mg, betaine 660.0 mg, L-serine 170.0 mg, L-phenylalanine 250.0 mg, DL-alanine 500.0 mg, L-sodium aspartate 330.0 mg, L-valine 250.0 mg, glycine 170.0 mg.

‡‡mg 100 g^{-1}, Vitamin E: α-tocopheryl acetate [Sigma-Aldrich (Madrid, Spain)], Vitamin C: Ascorbyl monophosphate ROVIMIX Stay-C-35 (Roche, Paris, France).

Table 2 Main fatty acid composition (% dw) of the early weaning diets containing several n-3 HUFA, vitamin E and vitamin C levels used to fed larval meagre (*Argyrosomus regius*) from 14 to 28 dah

	Diets					
	0.4/150/180	0.4/300/180	0.4/300/360	3/150/180	3/300/180	3/300/360
14:0	0.1	0.1	0.1	0.6	0.6	0.6
15:0	0.0	0.0	0.0	0.1	0.1	0.1
16:0	2.4	2.2	2.0	3.3	3.8	3.3
16:1n-7	0.0	0.0	0.0	0.7	0.6	0.8
16:1n-5	0.0	0.0	0.0	0.0	0.0	0.0
16:2n-4	0.0	0.0	0.0	0.1	0.1	0.1
16:3n-1	0.0	0.0	0.0	0.0	0.0	0.0
16:4n-3	0.0	0.0	0.0	0.1	0.1	0.1
18:0	0.3	0.6	0.5	0.8	1.0	0.8
18:1n-9	9.9	10.1	10.1	3.4	3.2	3.3
18:1n-7	0.1	0.2	0.2	0.4	0.4	0.5
18:1n-5	0.0	0.0	0.0	0.0	0.0	0.0
18:2n-9	0.0	0.0	0.0	0.0	0.0	0.0
18:2n-6	2.5	3.2	3.3	2.6	2.3	2.7
18:3n-6	0.0	0.0	0.0	0.1	0.1	0.1
18:3n-3	0.1	0.2	0.2	0.4	0.4	0.4
18:4n-3	0.0	0.0	0.0	0.2	0.1	0.2
20:0	0.0	0.0	0.0	0.1	0.1	0.1
20:1n-9	0.0	0.0	0.0	0.1	0.1	0.1
20:1n-7	0.1	0.1	0.1	0.5	0.7	0.5
20:1n-5	0.0	0.0	0.0	0.0	0.0	0.0
20:2n-9	0.0	0.0	0.0	0.0	0.0	0.0
20:2n-6	0.0	0.0	0.0	0.1	0.1	0.1
20:3n-6	0.0	0.0	0.0	0.0	0.0	0.0
20:4n-6	0.0	0.0	0.0	0.1	0.1	0.2
20:3n-3	0.0	0.0	0.0	0.0	0.0	0.0
20:4n-3	0.0	0.0	0.0	0.1	0.1	0.1
20:5n-3	0.1	0.1	0.1	1.0	0.9	1.0
22:1n-11	0.0	0.0	0.0	0.3	0.6	0.4
22:1n-9	0.0	0.0	0.0	0.1	0.1	0.1
22:4n-6	0.0	0.0	0.0	0.0	0.0	0.0
22:5n-6	ND	0.0	0.0	0.1	0.1	0.1
22:5n-3	0.0	0.0	0.0	0.2	0.2	0.2
22:6n-3	0.2	0.2	0.3	1.6	1.5	1.7
Saturated	2.8	2.9	2.7	4.9	5.6	4.9
Monoenoic	10.2	10.4	10.5	5.6	5.7	5.7
n-3	0.5	0.5	0.6	3.5	3.2	3.6
n-6	2.5	3.2	3.3	3.0	2.6	3.1
n-9	10.0	10.1	10.1	3.6	3.3	3.4
n-3HUFA	0.3	0.3	0.4	2.9	2.6	3.0
n-6HUFA	0.0	0.1	0.0	0.3	0.3	0.3
(n-3 + n-6)HUFA	0.3	0.4	0.4	3.2	2.9	3.3
n-3/n-6	0.2	0.2	0.2	1.2	1.2	1.2
EPA/ARA	5.0	5.0	5.0	10	9	5
DHA/EPA	2	2	3	1.6	1.7	1.7

ND, not detected.

analysed. Fatty acid methyl esters were obtained by transmethylation of total lipids as described by Christie (1982) separated using gas liquid chromatography (GLC), quantified using flame ionization detection (GC-14A; Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo, Watanabe, Takeuchi, Arakawa and Kitajima (1990) and identified by comparison to previously characterized standards and gas–liquid chromatography-mass spectrometry.

Ten larvae from each tank were collected at the beginning and the end of the feeding trial, fixed in 10% buffered formalin for 1 or 2 days, dehydrated through graded alcohols, then xylene and finally embedded in paraffin wax. Two paraffin blocks containing five larvae per tank (six blocks per diet) were sectioned at 5 μm, and sections were stained with haematoxylin and eosin (H&E) for histopathological evaluation (Martoja & Martoja-Pearson 1970).

Statistical analysis

Data were treated using the STATGRAPHICS (version 5.1 Plus for Windows; Graphic Software Systems, Inc., Warrenton, VA, USA) and SPSS software (SPSS for Windows 11.5; SPSS, Chicago, IL, USA). *S*tudent's *t*-test was used to compare 2 samples. Survival and growth data were tested using one-way ANOVA. Means were compared by Duncan's test ($P < 0.05$). For analysis of one-way ANOVA the following general linear model was used:

$$Y_{ij} = m + D_i + e_{ij}$$

where Y_{ij} is the mean value of the tank, m is the mean population, D_i is the fixed effect of the diet and e_{ij} is the residual error.

To verify the results, two-way ANOVA was applied on growth data (weight, length), normality and homogeneity of variance were checked, and the following general liner model was used:

$$Y_{ijk} = \mu + \alpha_i + \delta_j + (\alpha\delta)_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} is the mean value of the tank, μ is the mean population, α_i is the fixed effect of the first factor (vitamin E or C for example), δ_j the fixed effect of the second factor (HUFA for example), $(\alpha\delta)_{ij}$ the interaction between fixed effects, and ε_{ijk} is the residual error.

Results

Larval performance

At the end of the feeding trial (28 dah), larval survival was not significantly different among the different groups of larvae, being in average 13.45 ± 3.08% (mean ± SD) (Table 3). Daily weight gain in this study was ranging between 17.48 ± 2.57% (treatment: 0.4/300/360) and

Table 3 Total length (mm), dry weight (mg), and survival of meagre *Argyrosomus regius* larvae fed early weaning diets containing two levels of n-3 HUFA, vitamin E and vitamin C from 14 dah (initial total length 4.07 ± 0.26 mm and dry body weight 0.06 ± 0.01 mg)

		Diets									
		0.4/150/180	0.4/300/180	0.4/300/360	3/150/180	3/300/180	3/300/360	HUFA	VIT E	VIT C	HUFA VIT E	HUFA VIT C
Total length	24 dah	4.75 ± 0.44^{b}	5.00 ± 0.39^{a}	4.91 ± 0.40^{ab}	4.96 ± 0.45^{a}	4.96 ± 0.48^{a}	5.06 ± 0.38^{a}	*	NS	NS	NS	NS
	28 dah	5.16 ± 0.46^{ab}	5.20 ± 0.43^{ab}	5.14 ± 0.51^{ab}	5.29 ± 0.44^{a}	4.97 ± 0.31^{b}	5.34 ± 0.59^{a}	NS	*	*	**	**
Body weight	24 dah	0.19 ± 0.04^{c}	0.21 ± 0.02^{bc}	0.20 ± 0.03^{bc}	0.21 ± 0.02^{bc}	0.22 ± 0.02^{ab}	0.24 ± 0.03^{a}	*	NS	NS	NS	NS
	28 dah	0.23 ± 0.02	0.21 ± 0.04	0.21 ± 0.03	0.27 ± 0.05	0.23 ± 0.05	0.24 ± 0.04	*	NS	NS	NS	NS
Survival (%)		12.09 ± 4.96	8.04 ± 5.20	15.12 ± 4.14	14.16 ± 8.29	16.68 ± 3.45	15.16 ± 7.67					

Values (mean ± SD) with the same superscripts are not significantly different. One-way ANOVA, $P_{Length} < 0.01$; $P_{Weight} < 0.05$. Asterisks denote significant differences as $*P < 0.05$; $**P < 0.01$, NS, non-significant differences. 0.4/150/180 0.4% n-3 HUFA with 1500 mg kg^{-1} vitamin E and 1800 mg kg^{-1} vitamin C diet; 0.4/300/180 0.4% n-3 HUFA with 3000 mg kg^{-1} vitamin E and 1800 mg kg^{-1} vitamin C diet; 0.4/300/360 0.4% n-3 HUFA with 3000 mg kg^{-1} vitamin E and 3,600 mg kg^{-1} vitamin C diet; 3/150/180 3% n-3 HUFA with 1500 mg kg^{-1} vitamin E and 1800 mg kg^{-1} vitamin C diet; 3/300/180 3% n-3 HUFA with 3000 mg kg^{-1} vitamin E and 1800 mg kg^{-1} vitamin C diet; 3/300/360 3% n-3 HUFA with 3000 mg kg^{-1} vitamin E and 3600 mg kg^{-1} vitamin C diet.

24.64 ± 2.62% (treatment: 3/150/180); being higher in larvae fed 3% n-3 HUFA (22.43 ± 2.01%) compared to those fed 0.4% n-3 HUFA (18.80 ± 1.60%). However, after only 10 days of feeding (24 dah), growth in terms of total length and dry body weight was significantly lowest in larvae fed diet 0.4/150/180 (Table 3), which contained the lowest HUFA, vitamin E and vitamin C levels. Larger growth was obtained in meagre fed diets 3/150/180, 3/300/180 and 3/300/360 (Table 3). Thus, regardless the dietary vitamin E and vitamin C levels, the increase in dietary HUFA from 0.4% to 3%, significantly ($P < 0.01$) improved larval growth in terms of total length (4.89 ± 0.42 and 5.00 ± 0.43 mm for 0.4 and 3% HUFA respectively) and dry weight (0.20 ± 0.03 and 0.22 ± 0.03 mg for 0.4 and 3% HUFA respectively). Among fish fed 0.4% HUFA, elevation of dietary vitamin E from 1500 to 3000 mg kg^{-1} significantly improved total length in 24 dah larvae ($P < 0.01$; Table 3). Among fish fed 3% HUFA, increase in both vitamin E and vitamin C significantly improved body weight ($P < 0.05$; Table 3) and a significant positive linear correlation was found between dry body weight and dietary vitamin E+ vitamin C levels ($y = 9E\text{-}05x + 0.18$ $R^2 = 0.995$). At 28 dah, the two-way ANOVA analysis showed that the combination of vitamin (either E or C) and HUFA induced a significant ($P < 0.05$) effect on body weight (Table 3).

The increase of dietary HUFA induced a significant ($P < 0.05$) increase of larvae length, whereas there were no effects of vitamin (either E or C) inclusion or the combination of vitamins and HUFAs in diet (Table 3).

Biochemical analysis

The diets without fish oil and containing oleic acid (0.4/150/180, 0.4/300/180 and 0.4/300/360) were characterized by a high level of monoenoic and n-9 fatty acids, particularly oleic acid (OA: 18:1n-9), as well as n-6 fatty acids such as LA (Table 2). Accordingly, a high ratio OA/n-3 HUFA was obtained in these diets (Table 2). On the contrary, diets containing fish oil (3/150/180, 3/300/180 and 3/300/360) were high on saturated fatty acids, specially lauric (14:0), palmitic (16:0) and stearic (18:0) acids, as well as on n-3 fatty acids, including ALA, eicosatetraenoic (20:4n-3), EPA, n-3 docosapentaenoic (DPA, 22:5n-3) and DHA acids. Fish oil inclusion also raised the levels of n-6 HUFA, such as 20:2n-6, 20:6n-6, 22:5n-6 and 20:4n-6 ARA, but in a lower extend than n-3 fatty acids, and subsequently the n-3/n-6 ratio was high. All the diets kept constant proportions of the ratios among the essential fatty acids EPA/ARA and DHA/EPA (Table 2).

Despite dietary lipids levels were similar among diets, elevation of dietary HUFA tended to increase larval total lipid contents (Table 4). Moreover, lipid contents in larvae fed 3% HUFA were increased by dietary vitamin E + vitamin C levels, and a significant positive correlation was found among the two parameters ($y = 0.00151x + 13.26$, $R^2 = 0.91$) (Fig. 1).

The n-3 HUFA contents in larvae fed diets with the low HUFA levels were even lower than those of the initial larvae, whereas feeding the high HUFA levels increased larval n-3 HUFA even over the initial levels (Table 4). Student's *t*-test showed that larval contents of n-3 HUFA were significantly ($P < 0.05$) higher in larvae fed high n-3 HUFA. Accordingly, higher contents of DHA, EPA and ARA ($P < 0.05$) were found in larvae fed high dietary n-3 HUFA. However, the total amount of saturated fatty acids was similar among larvae fed the different diets regardless dietary contents. Besides, only slightly higher values were found in larvae fed 0.4% HUFA for monounsaturated and n-6 polyunsaturated fatty acids. Despite EPA/ARA and DHA/EPA ratios were similar among the different diets, their values were higher ($P < 0.05$) in larvae fed fish oil, particularly when vitamin E or vitamin E+ vitamin C were increased in the diet.

In larvae fed 3% n-3 HUFA, inclusion of vitamin E increased LA, ARA, EPA, 22:4n-6, 22:5n-6, DPA, DHA and, accordingly, n-3, n-3 HUFA, n-6 and n-3/n-6 ratios (Table 4), regardless similar levels were found in the respective diets (Table 2). Particularly, increase in dietary vitamin E+ vitamin C levels lead to a significant linear increase in the DHA ($y = 0.008x - 0.45$, $R^2 = 0.97$) and n-3 fatty acid ($y = 0.0009x + 0.082$, $R^2 = 0.99$, Fig. 1) contents in the larvae. In larvae fed either 0.4 or 3% HUFA diets, the combined elevation of vitamin E and vitamin C, tended to raise larval lipid contents by increasing 14:0, 15:0, 16:0, 16:1n-7, 16:1n-5, 18:0, 18:1n-7, 20:0, 20:1n-7 and 22:1n-11, end-products of non-essential fatty acid synthesis in marine fish, as well as the levels of 20:2n-6, 20:3n-3, EPA, DPA and DHA, suggesting the antioxidant protection of these vitamins (Table 4).

Table 4 Total lipid content and fatty acid composition (% dw) of whole body meagre, *Argyrosomus regius* larvae, after 14 days of feeding several n-3 HUFA, vitamin E and C dietary contents

		28 dah					
	14 dah	0.4/150/180	0.4/300/180	0.4/300/360	3/150/180	3/300/180	3/300/360
Lipids	19.5 ± 2.8	17.5 ± 2.1^{b}	17.6 ± 3.8^{b}	21.1 ± 1.1^{ab}	18.7 ± 1.7^{b}	19.6 ± 0.1^{ab}	23.6 ± 0.9^{a}
14:0	0.2 ± 0.0	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.2 ± 0.1^{abc}	0.2 ± 0.0^{bc}	0.1 ± 0.0^{b}	0.1 ± 0.0^{c}
15:0	0.1 ± 0.0	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{abcd}	0.1 ± 0.0^{b}	0.1 ± 0.0^{c}	0.1 ± 0.0^{d}
16:0	4.9 ± 0.0	3.8 ± 0.0^{a}	3.9 ± 0.0^{ac}	4.8 ± 0.6^{abc}	5.4 ± 0.1^{b}	4.3 ± 0.1^{ac}	4.8 ± 0.1^{c}
16:1n-7	1.1 ± 0.0	0.1 ± 0.0^{a}	0.1 ± 0.0^{b}	0.2 ± 0.1^{abcd}	0.3 ± 0.0 ^{cd}	0.3 ± 0.0^{c}	0.3 ± 0.0^{d}
16:1n-5	0.1 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.1 ± 0.0^{b}	0.1 ± 0.0^{ab}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
16:2n-4	0.2 ± 0.1	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.2 ± 0.0^{ac}	0.2 ± 0.0^{b}	0.2 ± 0.0^{c}
16:3n-1	0.5 ± 0.0	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.3 ± 0.1^{ab}	0.4 ± 0.0^{ab}	0.3 ± 0.0^{a}	0.4 ± 0.0^{b}
16:4n-3	0.1 ± 0.0	0.1 ± 0.0^{ac}	0.1 ± 0.0^{a}	0.2 ± 0.2^{abc}	0.1 ± 0.0^{ac}	0.1 ± 0.0^{b}	0.1 ± 0.0^{c}
18:0	1.8 ± 0.0	2.5 ± 0.0^{a}	2.4 ± 0.0^{a}	3.0 ± 0.3^{ab}	2.5 ± 0.6^{ab}	2.5 ± 0.0^{ab}	2.9 ± 0.1^{b}
18:1n-9	3.9 ± 0.1	4.9 ± 0.1^{a}	5.0 ± 0.0^{a}	5.5 ± 0.1^{b}	3.4 ± 0.1 ^{cd}	2.6 ± 0.0^{c}	3.2 ± 0.1^{d}
18:1n-7	0.7 ± 0.0	0.4 ± 0.0^{a}	0.4 ± 0.0^{ab}	0.5 ± 0.0^{ac}	0.6 ± 0.0 ^{cd}	0.5 ± 0.0^{bcd}	0.6 ± 0.0^{d}
18:1n-5	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{ab}	0.0 ± 0.0^{abc}	0.0 ± 0.0^{ac}	0.0 ± 0.0^{bc}	0.0 ± 0.0^{c}
18:2n-9	0.2 ± 0.0	0.0 ± 0.0^{ab}	0.0 ± 0.0^{ab}	0.0 ± 0.0^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{b}
18:2n-6	1.5 ± 0.0	2.5 ± 0.0^{a}	2.6 ± 0.0^{b}	2.7 ± 0.2^{abd}	1.9 ± 0.1^{c}	1.9 ± 0.0^{d}	2.4 ± 0.0^{a}
18:3n-6	0.1 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
18:3n-3	0.2 ± 0.0	0.1 ± 0.0^{a}	0.1 ± 0.0^{b}	0.3 ± 0.1^{abc}	0.1 ± 0.0^{ad}	0.1 ± 0.0^{bd}	0.2 ± 0.0^{c}
18:4n-3	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.2 ± 0.1^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
20:0	0.1 ± 0.0	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.1 ± 0.1^{a}	0.1 ± 0.0^{a}
20:1n-9	0.0 ± 0.0	0.0 ± 0.0^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{ab}	0.0 ± 0.0^{b}	0.2 ± 0.2^{ab}	0.0 ± 0.0^{b}
20:1n-7	0.4 ± 0.0	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}	0.5 ± 0.1^{a}	0.4 ± 0.0^{a}	0.2 ± 0.2^{a}	0.4 ± 0.0^{a}
20:1n-5	0.1 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{ab}	0.1 ± 0.0^{ab}	0.0 ± 0.0^{ab}	0.1 ± 0.0^{b}
20:2n-9	0.1 ± 0.0	0.0 ± 0.0^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{ab}	0.0 ± 0.0^{b}	0.1 ± 0.1^{ab}	0.0 ± 0.0^{b}
20:2n-6	0.1 ± 0.0	0.1 ± 0.0^{ab}	0.1 ± 0.0^{a}	0.3 ± 0.1^{ab}	0.1 ± 0.0^{ab}	0.1 ± 0.1^{ab}	0.2 ± 0.0^{b}
20:3n-6	0.1 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{ab}	0.1 ± 0.0^{b}
20:4n-6	0.5 ± 0.0	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.3 ± 0.1^{abc}	0.4 ± 0.0^{b}	0.6 ± 0.0 ^{cd}	0.7 ± 0.0^{d}
20:3n-3	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{ac}	0.0 ± 0.0^{abc}	0.0 ± 0.0^{b}	0.0 ± 0.0^{c}	0.0 ± 0.0^{bc}
20:4n-3	0.1 ± 0.0	0.0 ± 0.0^{ab}	0.0 ± 0.0^{a}	0.1 ± 0.1^{ab}	0.0 ± 0.0^{ab}	0.0 ± 0.0^{ab}	0.1 ± 0.0^{b}
20:5n-3	0.3 ± 0.0	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.2 ± 0.0^{ab}	0.3 ± 0.0^{b}	0.6 ± 0.0^{c}	0.7 ± 0.0^{d}
22:1n-11	0.0 ± 0.1	0.1 ± 0.2^{a}	0.1 ± 0.1^{a}	0.0 ± 0.0^{a}	0.2 ± 0.1^{a}	0.2 ± 0.2^{a}	0.2 ± 0.1^{a}
22:1n-9	0.1 ± 0.1	0.1 ± 0.2^{a}	0.1 ± 0.2^{a}	0.2 ± 0.0^{a}	0.1 ± 0.2^{a}	0.2 ± 0.2^{a}	0.1 ± 0.2^{a}
22:4n-6	0.0 ± 0.0	0.0 ± 0.0^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{b}	0.1 ± 0.0^{c}
22:5n-6	0.1 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{ab}	0.1 ± 0.0^{b}	0.1 ± 0.0^{c}	0.2 ± 0.0^{c}
22:5n-3	0.1 ± 0.0	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{ab}	0.1 ± 0.0^{b}	0.3 ± 0.0^{c}	0.4 ± 0.0^{c}
22:6n-3	1.4 ± 0.1	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	0.7 ± 0.0^{a}	1.1 ± 0.1^{b}	3.5 ± 0.0^{c}	4.3 ± 0.3^{c}
Saturated	7.1 ± 0.0	6.7 ± 0.0^{a}	6.6 ± 0.0^{a}	8.4 ± 0.9^{abc}	8.3 ± 0.5^{abc}	7.1 ± 0.0^{b}	8.1 ± 0.1^{c}
Monoenoic	6.5 ± 0.0	6.2 ± 0.1^{a}	6.3 ± 0.1^{a}	7.0 ± 0.1^{b}	5.2 ± 0.2^{acd}	4.2 ± 0.0^{c}	5.1 ± 0.1^{d}
n-3	2.3 ± 0.1	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}	1.8 ± 0.5^{abc}	1.8 ± 0.1^{b}	4.9 ± 0.2^{c}	6.2 ± 0.0^{c}
n-6	2.4 ± 0.1	3.0 ± 0.0^{ac}	3.1 ± 0.0^{ac}	3.4 ± 0.3^{ab}	2.6 ± 0.1^{ab}	2.8 ± 0.0^{bc}	3.7 ± 0.2^{c}
n-9	4.2 ± 0.0	5.1 ± 0.1^{ab}	5.2 ± 0.1^{b}	5.8 ± 0.1^{c}	3.6 ± 0.3^{ad}	3.0 ± 0.1^{d}	3.4 ± 0.1^{d}
n-3HUFA	1.9 ± 0.0	0.9 ± 0.1^{a}	0.9 ± 0.1^{a}	1.1 ± 0.2^{ab}	1.6 ± 0.1^{b}	4.5 ± 0.0^{c}	5.6 ± 0.4^{c}
n-6HUFA	0.9 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	1.0 ± 0.1	1.3 ± 0.2
(n-3 + n-6)HUFA	2.8 ± 0.0	1.4 ± 0.1^{a}	1.3 ± 0.1^{a}	1.6 ± 0.3^{ab}	2.2 ± 0.1^{b}	5.5 ± 0.1^{c}	6.9 ± 0.2^{d}
n-3/n-6	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
EPA/ARA	0.6 ± 0.0	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.8 ± 0.3^{abc}	0.8 ± 0.0^{b}	1.2 ± 0.0^{c}	1.2 ± 0.0^{c}
DHA/EPA	4.7 ± 0.0	4.4 ± 0.4^{ab}	4.6 ± 0.3^{ab}	3.8 ± 1.0^{ab}	4.2 ± 0.1^{a}	5.5 ± 0.0^{b}	5.0 ± 0.4^{ab}

Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Histological study

Histological study of larval foregut showed that larvae fed 0.4% HUFA presented condensed enterocytes with scarce accumulation of lipid vacuoles (Fig. 2a and 2b). However, larvae fed higher levels of dietary HUFA, such as in 3/150/180, showed enterocytes with large lipid vacuoles around the nucleus and in the basal part of the enterocyte (Fig. 2d,e), reflecting the higher lipid

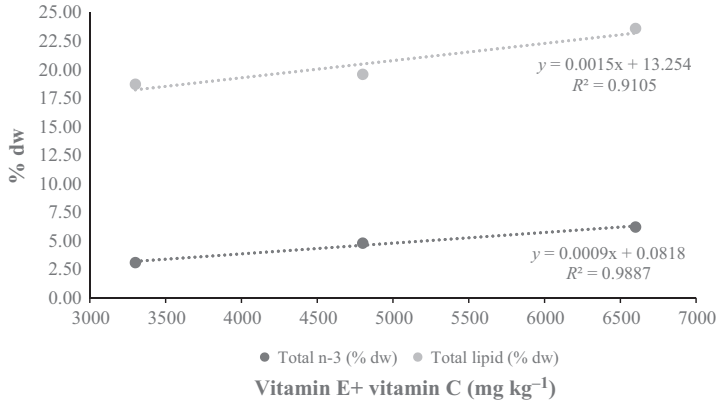

Figure 1 Effect of dietary vitamin E and C on total lipid and n-3 contents (dw) in meagre (*Argyrosomus regius*) larvae after 14 days of feeding 3% HUFA diets.

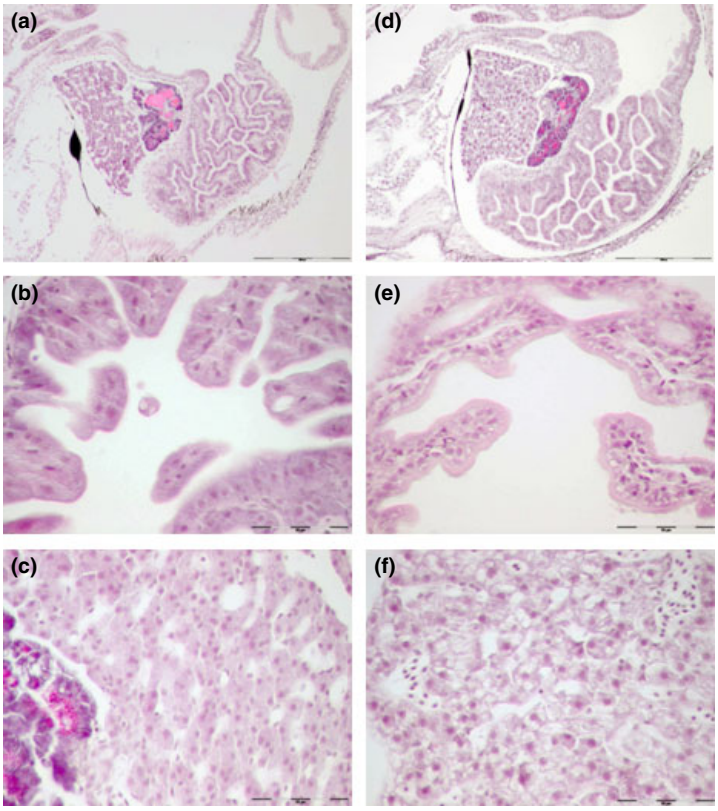

Figure 2 Sections of larvae intestine and liver of meagre *Argyrosomus regius* (28 dah) from different treatments. H&E stain. (a) larvae 0.4/150/180 (10X), (b) intestine larvae 0.4/150/180 (40X) scarce lipid vacuoles accumulation in the enterocytes; (c) liver larvae 0.4/150/180 (40X) condensed hepatocytes with centred nucleus and marked cytoplasm staining; (d) larvae 3/150/180 (10X), e: intestine larvae 3/150/180 (40X) large lipid vacuoles around the nucleus and in the basal part of the enterocytes; (f) liver larvae 3/150/180 (40X) hepatocytes with high lipid vacuoles deposition.

absorption activity. Similar features were observed in gut larvae fed diets 3/300/180 and 3/300/360. Regarding the liver, larvae fed low HUFA diets showed very condensed hepatocytes with centred nucleus and marked cytoplasm staining, observing a scarce deposition of lipid reserves (Fig. 2c). On the contrary, larvae fed higher HUFA levels showed hepatocytes with a higher accumulation of lipid vacuoles (Fig. 2f). No alteration in larval tissue was observed.

Discussion

Meagre larvae accepted very well the experimental diets and, despite the complete early weaning, larval growth rates were comparable to those obtained in previous studies. For instance, the best daily growth rates found in the present study (24% daily weight increase from initial weight) were in the range of those obtained by Hernández-Cruz *et al.* (2007) (23–36%). Despite survival was

relatively low due to the complete weaning in agreement with previous studies (Hernández-Cruz *et al.* 2007), the values were even higher than those obtained with similar weaning protocols (13% survival in Fernández-Palacios, Hernández-Cruz, Schuchardt, Izquierdo & Roo 2009 or 8% survival in Durán, Pastor, Grau, Massuti-Pascual, Valencia & Gil 2009).

The low growth obtained in larvae fed weaning diets with the low HUFA content (0.4% dw) together with the reduction in the HUFA contents in the larvae in comparison to the initial larvae, clearly indicated a deficiency in these fatty acids. Low growth has been found in larvae fed deficient levels of these fatty acids in several species such as turbot (*Scophtalmus maximus*) (Gatesoupe & LeMilinaire 1985) or gilthead seabream (Rodríguez *et al.* 1993, 1994; Salhi, Izquierdo, Hernández-Cruz, González & Fernández-Palacios 1994). In marine fish larvae, HUFA are an important source of metabolic energy, structural components in the phospholipids of cellular membranes and precursors of bioactive molecules, being required for larval growth and development (Izquierdo & Koven 2011). In this study, improved lipid absorption was observed at a histological level in gut as well as liver lipid deposition in larvae fed 3% n-3 HUFA, which was associated with a higher incorporation of essential fatty acids into larval lipids and larval growth, suggesting a high HUFA requirement in weaning diets for meagre. Optimum levels of n-3 HUFA for marine fish larvae vary between 0.05% and 3.9% in live food or formulated diets (Izquierdo & Koven 2011), requirements being higher than 3% in fast growing larvae of flounder (*Paralichthys olivaceus*) (Izquierdo, Arakawa, Takeuchi, Haroun & Watanabe 1992), in yellowtail (*Seriola quinqueradiata*) (Furuita, Takeuchi, Watanabe, Fujimoto, Sekiya & Imaizumi 1996), red seabream (Izquierdo *et al.* 1989) or common dentex (*Dentex dentex*) (Mourente, Tocher, Diaz-Salvago, Grau & Pastor 1999).

In this study, no gross signs of vitamin E or vitamin C deficiency, such as muscle dystrophy, liver damage or cranial deformities, could be found in the meagre larvae, denoting that neither of these vitamins were at critically low levels in the diets. Since liver constitutes the main lipid storage organ in fish and α-tocopherol is the principal fat-soluble antioxidant, vitamin E deficiency frequently damages this organ (Izquierdo & Betancor 2015). Besides, the abundance in the liver of haeme containing enzymes that favour oxidation, promotes the occurrence of pathological alterations in this organ (Hamre 2011), such as hepatocyte hypertrophy, inflammation, ceroidosis and necrosis (Thorarinsson, Landolt, Elliott, Pascho & Hardy 1994; Montero, Tort, Izquierdo, Socorro, Robaina, Vergara & Fernández-Palacios 1996). For instance, up to 37% gilthead seabream juveniles presented liver inflammation 15 weeks after feeding a non-α-tocopherol supplemented diet (Montero *et al.* 1996; Montero, Marrero, Izquierdo, Robaina, Vergara & Tort 1999).

Nevertheless, increase in dietary vitamin E and vitamin C levels, in larvae fed 3% HUFA, raised the DHA, n-3 fatty acids and total lipid contents in the larvae and promoted larval growth. Being vitamin E the major hydrophobic antioxidant, the increase in dietary HUFA would accelerate the autocatalytic peroxidation of vitamin E, increasing the requirement for this vitamin (Watanabe 1982; Sargent, McEvoy & Bell 1997; Izquierdo *et al.* 2001). For instance, elevation of dietary HUFA contents in weaning diets for larval gilthead seabream required the increase in dietary vitamin E to promote incorporation of HUFA in fish membranes and promote larval growth (Atalah *et al.* 2012). Moreover, vitamin C not only protects tissues from oxidative stress by neutralizing the reactive oxygen species (ROS), but also would play an important role indirectly protecting HUFA from oxidation, since it is essential to regenerate α-tocopheroxyl radicals to α-tocopherol. Thus, result of an increase in vitamin E and vitamin C in meagre larvae fed 3% HUFA agree well with previous studies that demonstrated that these vitamins protected HUFAs from oxidation, increased their incorporation into larval tissues and promoted larval growth (Betancor *et al.* 2011, 2012; Hamre 2011; Atalah *et al.* 2012; Izquierdo *et al.* 2013). Despite information in the synergistic effect of vitamin E and vitamin C in larvae is very scarce. For instance, an antioxidant synergism was demonstrated in seabass larvae fed high 5% DHA (Betancor *et al.* 2012) as well as in juveniles of several species such as rainbow trout (*Oncorhynchus mykiss*) (Frischknecht, Wahli & Meier 1994), Atlantic salmon (Hamre *et al.* 1997), yellow perch (*Perca flavescens*) (Lee & Dabrowski 2003), golden shiner (*Notemigonus crysoleucas*) (Chen *et al.* 2004), channel catfish (*Ictalurus punctatus*) (Yildirim-Aksoy, Lim, Li & Klesius 2008), hybrid striped bass (*Morone chrysops X M. saxatilis*) (Sealey & Gatlin

2002) or red seabream (Gao, Koshio, Ishikawa, Yokoyama, Nguyen & Mamauag 2013). On the contrary, no synergistic effect of vitamin E and vitamin C could be found on growth performance and disease resistance in fingerling Nile tilapia (*Oreochromis niloticus* L.) (Kim, Wang, Choi, Park, Koo & Bai 2003).

Few studies have been aimed to determine the vitamin E and vitamin C requirements in marine fish larvae (Atalah *et al.* 2010). The requirements are suspected to be high, at the g/kg level, according to Moren *et al.* (2011), in view of the high risk of oxidative stress faced by the intensively reared larvae. Indeed, both vitamin C and vitamin E markedly improve stress resistance in marine fish (Ortuño, Esteban & Meseguer 2003). In agreement, the results of the present study suggest high vitamin E and vitamin C requirements in meagre larvae (higher than 1500 and 1800 mg kg^{-1} for vitamin E and vitamin C respectively), close to those suggested for gilthead seabream (Atalah *et al.* 2012) or European seabass (Betancor *et al.* 2011, 2012). Similarly, increase in vitamin E contents in rotifers or weaning diets improved growth of cod larvae (*Gadus morhua*) (Zheng, Takeuchi, Kobayashi, Hirokawa & Watanabe 1997), gilthead seabream (Saleh 2013) or European seabass (Betancor *et al.* 2011), without affecting survival. Growth in terms of weight gain is also affected by the dietary vitamin E levels in juveniles of a closely related species, the red drum (*Sciaenops ocellatus*) (Peng, Wang & Gatlin 2008). Nevertheless, in juveniles dietary vitamin E seems to affect growth only in fish under situations of high oxidative risk (Izquierdo & Betancor 2015). Increase in dietary vitamin C also improves growth in juveniles of several teleost species (Ai, Mai, Zhang, Xu, Duan, Tan & Liufu 2004; Roosta, Hajimoradloo, Ghorbani & Hoseinifar 2014).

In summary, the results of this study have shown that 0.4% dietary HUFA is not enough to cover the essential fatty acid requirements of larval meagre and, since their elevation up to 3% markedly improved lipid absorption, essential fatty acids levels and growth, a high HUFA requirement in weaning diets is foreseen for this species. Besides, the results also pointed out the importance of dietary vitamins E and C to protect these essential fatty acids from oxidation, increase their contents in larval tissues and promote growth, suggesting as well high vitamin E and vitamin C requirements in meagre larvae (higher than 1500 and 1800 mg kg^{-1} for vitamin E and vitamin C respectively). Further studies are being conducted to determine the HUFA requirements of meagre larvae in diets fortified with vitamin E and vitamin C.

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