Deliverable Report

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Deliverable Title	Improvement of larval weaning diets		
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Objective:

Despite the fact that meagre (*Argyrosomus regius*) larval development (Fernandez-Palacios et al., 2007; Hernandez-Cruz et al., 2007; Abreu et al., 2009; Cardiera et al., 2012) and larval rearing techniques (Estevez et al., 2007; Roo et al., 2010; Vallés & Estévez, 2013) have been extensively studied, weaning to dry diets remains to be an important bottleneck for this species as identified by DIVERSIFY partners. Unfortunately, there is a lack of information on nutritional requirements of meagre during weaning. Thus, the objective of Deliverable 8.1 was to better define the nutritional needs of meagre to improve the current larval weaning feeds for this species. In order to improve the weaning diets for meagre, several trials were conducted to determine the optimum levels of essential fatty acids and micronutrients that are known to be determinant of fish performance at larval stages in other species (Izquierdo & Koven, 2011; Hamre et al., 2013).

Description:

Two different trials were conducted in order to improve the fatty acid or micronutrients contents of meagre weaning diets.

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1.-Introduction

One of the most important nutritional factors for marine fish larvae is the dietary content of highly unsaturated fatty acids with 20 or more carbon atoms (HUFA) (Watanabe 1982). Namely, the n-3 series highly unsaturated fatty acid (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, in marine fish larvae 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 et al. 2008), due to the limited activity of Δ-5 and Δ-6 desaturases and elongases, and are considered essential (Sargent et al. 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 et al. 2001). The n-3 HUFA requirements have been extensively studied in marine fish larvae such as red seabream (*Pagrus major*) (Izquierdo et al. 1989), plaice (*Pleuronectes platessa*) (Rainuzzo et al. 1993), gilthead seabream (*Sparus aurata*) (Rodríguez et al. 1994; Izquierdo et al. 2000; Izquierdo 2005; Benítez-Santana et al. 2007), Japanese flounder (*Paralichtys olivaceus*) (Furuita et al. 1998), Atlantic halibut (*Hippoglossus hippoglossus*) (Shields et al. 1999). However, these fatty acids, particularly DHA, are very prone to oxidation and more exposed in formulated diets for marine fish larvae (Moren et al. 2011; Izquierdo et al. 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 et al. 2012). Thus, dietary inclusion of adequate levels of anti-oxidant nutrients is required to avoid in vivo lipid peroxidation of HUFA.

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 et al. 1970); Murai & Andrews 1974; González et al. 1995). Moreover, dietary vitamin E must be raised when dietary HUFA are high as it is found in carp (*Cyprinus carpio*) (Watanabe et al. 1981; Schwarz et al. 1988), tilapia (*Oreochromis niloticus*) (Satoh et al. 1987), Atlantic salmon (*Salmo salar* L.) (Hamre & Lie 1995), grouper (*Epinephelus malabaricus*) (Lin & Shiau 2005), gilthead seabream (Atalah et al. 2012); Izquierdo et al. 2013) or European seabass (*Dicentrarchus labrax* L.) (Betancor et al.2011). Thus, elevation of dietary PUFA causes a reduction in vitamin E contents in the liver of Atlantic salmon (Waagbø et al. 1993) or African catfish (*Clarias gariepinus*) (Lim et al. 2001). Recently, it has been shown that 3000 mg/kg dietary vitamin E seems to be adequate for good performance in seabass larvae (Atalah *et al.* 2012); in addition, at the same dietary vitamin E level an improvement in stress resistance has been recorded in marine fish larvae (Betancor *et al.* 2011). However, requirement measurements of vitamin E depend on interactions of this vitamin with other nutrients (Hamre 2011). The high vitamin E requirements of fish larvae have been associated with 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 et al. 1979). Ascorbic acid seems to play a significant role in α–tocopherol metabolism, reducing α-tocopheroxyl radicals and regenerating them to α-tocopherol (Niki et al. 1985). Consequently, optimum dietary vitamin E levels may also be determined by the levels of vitamin C (Hamre et al. 1997; Shiau & Hsu 2002; Sealey & Gatlin 2002; Chen et al. 2004; Atalah et al. 2010). For instance, elevation of dietary vitamin C from 1,800 to 3,600 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).

Vitamin A is involved in vision, growth, bone development, reproduction and normal maintenance of epithelial tissues. An increasing number of malformations were found in the caudal region and vertebrae of Japanese flounder and in vertebrae of turbot fed increasing dietary levels of vitamin A palmitate during metamorphosis. Villeneuve et al. (2005) fed European seabass larvae of 7 - 42 dph five isoproteic and isolipidic compound diets with graded levels of retinyl acetate (RA; RA0, RA10, RA50, RA250 and RA1000, containing 0, 10, 50, 250 and 1000 mg RA/kg DM, respectively). The analysed dietary levels were 12, 13, 31, 62 and 196 mg all-trans retinol/kg DM. Using malformation rate as indicator, the optimum level of retinol for European seabass was found to be around 31 $mg \cdot kg^{-1}$DM. Regarding vitamin D 19 IU D_3g^{-1} diet (0.5 mg Kg^{-1}) is required by European seabass larvae to obtain normal growth and development of the digestive system and the skeleton. Gradual increase in vitamin D_3 supplementation progressively increased bone mineralization in zebrafish (*Danio rerio*) larvae whereas excess of vitamin D_3 can induce vertebral deformities only in certain species. Finally, despite its important role in fish metabolism, there is a lack of studies in the requirements of vitamin K during larval development of fish. Besides, dietary levels, the type of vitamin K markedly affects larval development and the potential hypervitaminosis effect of vitamin K. For instance, in mummichog (*Fundulus heteroclitus*) larvae high levels of dietary menadione caused vertebral malformation, whereas the same levels of dietary phylloquinone (PK) were not harmful.

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

1.-Optimum essential fatty acids and related micronutrient levels in weaning diets for meagre

Materials and methods

To determine the effect of weaning diets on larval performance, 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 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 into 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 23.2±0.20 °C. All tanks (200 L fibreglass cylinder tanks with conical bottom and painted a light grey color) 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 efficient water renewal and a maintain high water quality, a daily check out was respected. Water was continuously aerated (125 ml min^{-1}). Average water dissolved O_2 reached 5.3±0.3 mg. Therefore, water quality in terms of temperature, dissolved O_2 and pH seemed 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 that complete weaning from 14 dah 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. 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 chloroform (i.e. 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. To determine larval performance and morphometry 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. To determine gut occupancy and digestive activity, 30 min after feeding, larvae were photographed under a binocular microscope and gut content was studied by image analysis. To determine the welfare status a stress resistance test was conducted at the end of the trial with 30 larvae that were handled out of the water in a scoop net for 30 sec. Final survival was calculated by individually counting all the larvae alive at the beginning and at the end of the experiment.

Table 1. Variable ingredients and proximate composition (g 100 g^{-1}dw) of early weaning diets containing several n-3 HUFA, vitamin E and vitamin C levels fed to meagre (*A. 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	0.00	0.00	0.00	10.00	10.00	10.00
Oleic acid[a]	10.00	10.00	10.00	0.00	0.00	0.00
Vitamin E*	150.00	300.00	300.00	150.00	300.00	300.00
Vitamin C*	180.00	180.00	360.00	180.00	180.00	360.00
Proximate composition						
Lipid	16.01	17.09	17.06	17.52	17.34	17.44
Protein	65.14	64.72	64.97	65.43	65.45	64.88
Moisture	10.32	10.59	9.38	9.67	9.39	9.35
Ash	5.47	5.55	5.70	5.88	5.73	5.81

To determine biochemical composition, 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. Moisture (A.O.A.C. 1995), crude protein (A.O.A.C. 1995) and crude lipid (Folch et al. 1957) contents of larvae and diets were 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 et al. (1990) and identified by comparison to previously characterized standards and gas-liquid chromatography-mass spectrometry.

For larval organ and skeleton development, hundred 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). For skeleton analysis, fixed larvae were stained with alizarin red and immediately photographed and examined for the occurrence of skeletal anomalies. Vertebrae were numerated from one to twenty four using Roman numerals in a cranial to caudal direction. The presence of supernumerary vertebral bodies, the presence of urinary calculus and anomalies were analysed separately. The effects of the different weaning diets on the axial skeleton mineralization were evaluated considering the total number of completely mineralized vertebral bodies within a larval size class.

Data were treated using the STATGRAPHICS (version 5.1 Plus for Windows; Graphic Software Systems, Inc. USA) and SPSS software (SPSS for Windows 11.5; SPSS, Chicago, IL, USA).T-student 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 for example), δ_j the fixed effect of the second factor (PUFA for example), $(\alpha\delta)_{ij}$ the interaction between fixed effects, and ε_{ijk} is the residual error.

Results

Gut occupancy, larval performance and morphometric parameters

The image analysis studies of the larval photographs of larvae fed the different diets denoted no significant differences in gut occupancy among fish fed the different diets. Daily weight gain in this study was ranging between 17.48±2.57% (treatment: 0.4/300/360) and 24.64±2.62% (treatment: 3/150/180); so being higher in larvae fed 3% (n-3 HUFA) (22.43±2.01%) compared to 0.4% (n-3 HUFA) larvae (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 lower in larvae fed diet 0.4/150/180 (**Table 2**), 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 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 ($y=9E-05x+0.18$ $R^2= 0.995$).

Similar trends were observed at the end of the feeding trial (28 dah). Thus, the two-way ANOVA analysis comparing the effect of dietary HUFA and vitamin E showed an improvement in growth, particularly body weight, when dietary HUFA levels were raised from 0.4 to 3%, whereas the effects of vitamin E or the interaction between both nutrients were not significant (Table 4). Similarly, the two-way ANOVA analysis comparing the effect of dietary HUFA and vitamin C showed the significant positive effect of dietary HUFA on fish weight, whereas the effects of vitamin C or the interaction between both nutrients were not significant (**Table 3**).

Larval organ and skeleton development

Histological study of larval foregut showed that larvae fed 0.4% HUFA presented condensed enterocytes with scarce accumulation of lipid vacuoles (**Fig. 1A 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. 1D and E**), reflecting the higher lipid 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. 1C**). On the contrary, larvae fed higher HUFA levels showed hepatocytes with a higher accumulation of lipid vacuoles (**Fig. 1F**). No other alterations were found in larval organ development. Moreover, no significant differences were found in skeleton development.

Survival and welfare status

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). In agreement, no significant differences were found in larval welfare status.

Table 2. Total length (mm), dry weight (mg), and survival of meagre 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**
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}
28 dah	5.15±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}
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}
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
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

Table 3. Results of two-way ANOVA analysis on total length (mm) and dry body weight (mg) of meagre larvae fed two dietary levels of HUFA, vitamin E and vitamin C.

		Total length			Dry weight		
		24 dah	28 dah	P	24 dah	28 dah	P
HUFA	0.4	4.85±0.05	5.18±0.05	< 0.05	0.20±0.01	0.22±0.01^{b}	< 0.05
	3	4.95±0.05	5.24±0.05		0.22±0.01	0.25±0.01^{a}	
Vitamin E	1500	4.85±0.06	5.22±0.06	< 0.05	0.20±0.01	0.25±0.11	< 0.05
	3000	4.96±0.04	5.20±0.04		0.21±0.01	0.23±0.01	
Interaction		NS	NS	< 0.05	NS	NS	< 0.05
HUFA	0.4	4.89±0.05	5.19±0.05	< 0.05	0.20±0.01	0.21±0.01^{b}	< 0.05
	3	4.98±0.05	5.25±0.05		0.22±0.01	0.25±0.01^{a}	
Vitamin C	1800	4.89±0.04	5.17±0.04	< 0.05	0.20±0.01	0.24±0.01	< 0.05
	3600	4.98±0.06	5.28±0.06		0.22±0.01	0.23±0.01	
Interaction		NS	NS	< 0.05	NS	NS	< 0.05

Table 4. 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 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	0.09	0.09	0.08	0.60	0.63	0.63
15:0	0.01	0.01	0.01	0.08	0.09	0.08
16:0	2.41	2.17	2.00	3.29	3.84	3.26
16:1n-7	0.03	0.03	0.03	0.69	0.64	0.76
16:1n-5	0.00	0.00	0.00	0.03	0.03	0.00
16:2n-4	0.00	0.00	0.00	0.05	0.05	0.06
16:3n-1	0.01	0.01	0.01	0.01	0.01	0.02
16:4n-3	0.00	0.00	0.00	0.06	0.05	0.06
18:0	0.28	0.58	0.52	0.80	0.94	0.79
18:1n-9	9.85	10.08	10.12	3.43	3.16	3.29
18:1n-7	0.09	0.15	0.15	0.43	0.42	0.47
18:1n-5	0.00	0.00	0.00	0.02	0.02	0.02
18:2n-9	0.00	0.00	0.00	0.01	0.01	0.01
18:2n-6	2.51	3.16	3.28	2.60	2.30	2.70
18:3n-6	0.00	0.00	0.00	0.05	0.05	0.05
18:3n-3	0.14	0.18	0.20	0.41	0.37	0.43
18:4n-3	0.00	0.00	0.00	0.15	0.14	0.16
20:0	0.04	0.03	0.03	0.05	0.06	0.05
20:1n-9	0.02	0.01	0.00	0.06	0.05	0.06
20:1n-7	0.12	0.13	0.13	0.48	0.67	0.53
20:1n-5	0.00	0.00	0.01	0.03	0.04	0.04
20:2n-9	0.00	0.00	0.00	0.01	0.01	0.01
20:2n-6	0.00	0.01	0.01	0.06	0.05	0.06
20:3n-6	0.01	0.00	0.00	0.02	0.02	0.02
20:4n-6	0.01	0.02	0.02	0.13	0.12	0.15
20:3n-3	0.03	0.01	0.01	0.04	0.03	0.04
20:4n-3	0.00	0.00	0.00	0.08	0.07	0.08
20:5n-3	0.09	0.10	0.11	0.95	0.86	0.99
22:1n-11	0.03	0.01	0.01	0.34	0.56	0.39
22:1n-9	0.03	0.01	0.02	0.07	0.09	0.07
22:4n-6	0.00	0.02	0.00	0.02	0.02	0.02
22:5n-6	nd	0.01	0.01	0.06	0.05	0.06
22:5n-3	0.00	0.01	0.00	0.18	0.16	0.19
22:6n-3	0.17	0.22	0.27	1.64	1.52	1.67
Saturated	2.82	2.89	2.65	4.87	5.60	4.86
Monoenoic	10.18	10.42	10.47	5.59	5.70	5.66
n-3	0.45	0.52	0.60	3.54	3.22	3.64
n-6	2.54	3.22	3.32	2.94	2.62	3.06
n-9	9.91	10.10	10.14	3.58	3.33	3.44
n-3HUFA	0.29	0.34	0.39	2.89	2.64	2.97
n-6HUFA	0.02	0.06	0.04	0.29	0.26	0.31
(n-3+n-6)HUFA	0.31	0.4	0.43	3.18	2.9	3.28
18:1n-9/n-3 HUFA	5.27	5.18	4.37	0.20	0.21	0.19
n-3/n-6	0.18	0.16	0.18	1.2	1.23	1.19
EPA/ARA	1.10	1.02	1.06	1.22	1.19	1.18
DHA/EPA	0.31	0.38	0.41	0.30	0.31	0.29

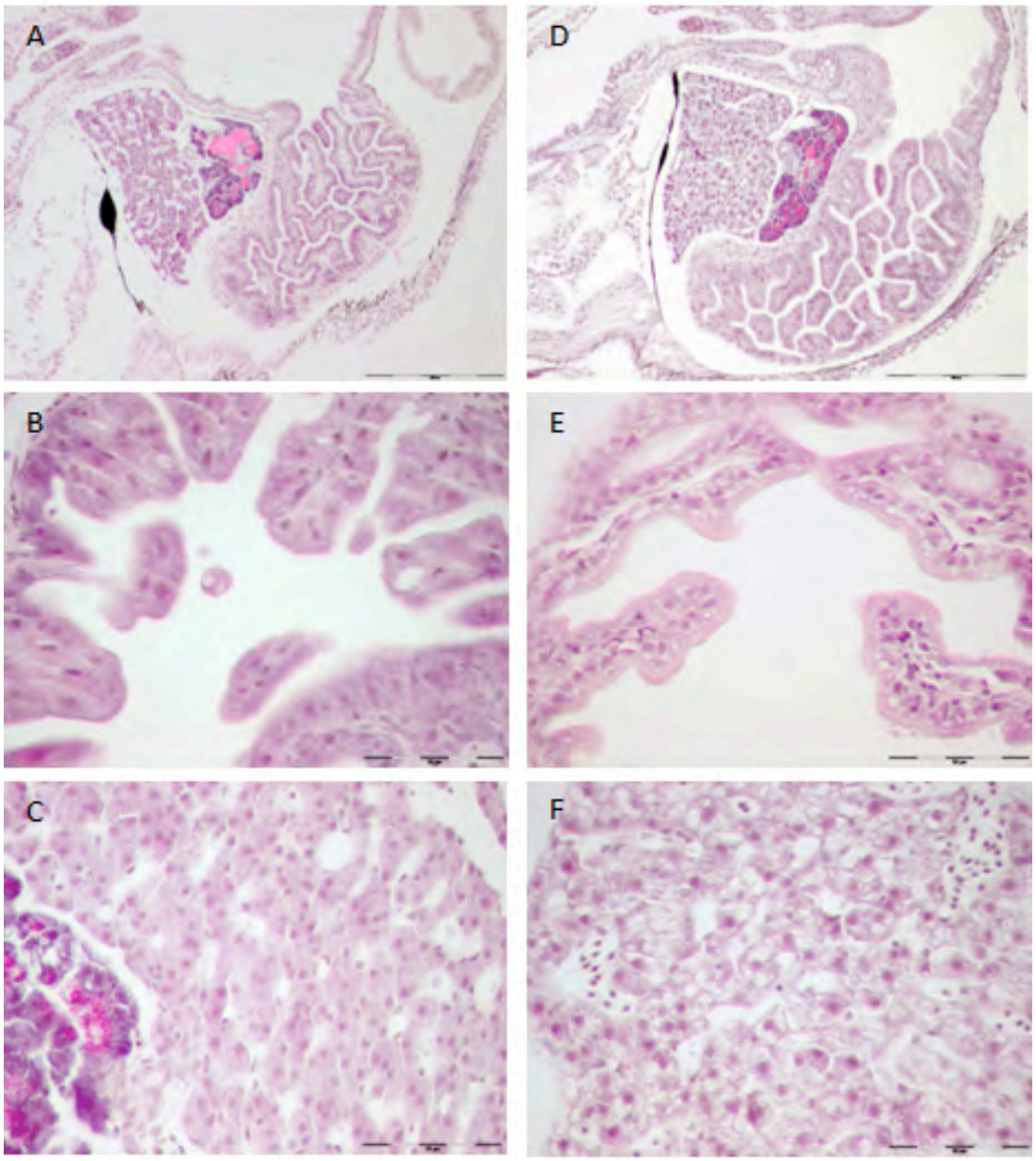

Figure 1. Sections of larvae intestine and liver of meagre (28 dah) from different treatments. H&E.

Biochemical composition

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 4**). Accordingly, a high ratio OA/n-3 HUFA was obtained in these diets (**Table 4**). 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 4**).

Despite dietary lipids levels were similar among diets, elevation of dietary HUFA tended to increase larval total lipid contents (**Table 5**). Moreover, lipid contents in larvae fed 3% HUFA were increased by dietary vitamin E+ vitamin C levels, and a significant positive correlation was found between the two parameters ($y=0.0151x+13.26$, $R^2=0.91$) (**Fig. 2**).

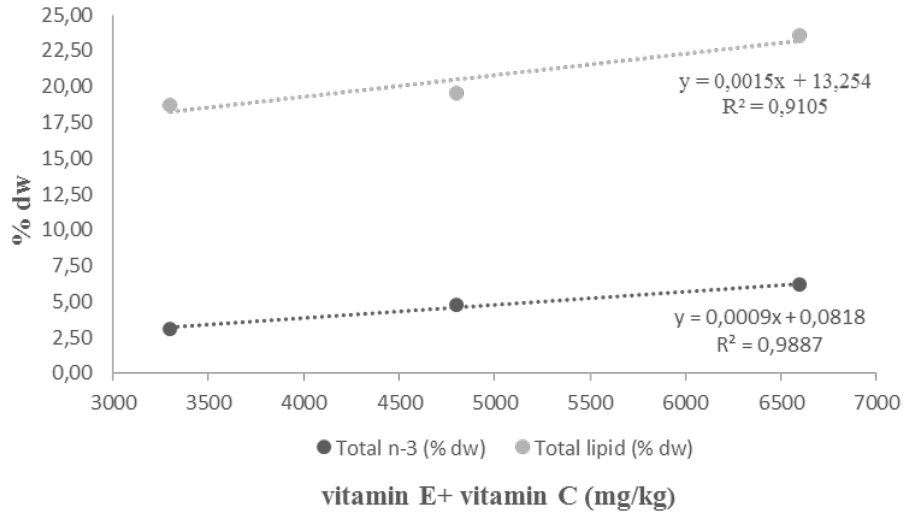

Figure 2. Effect of dietary vitamin E and C on lipid and n-3 contents (dw) in meagre (*A. regius*) larvae after 14 days of feeding 3% HUFA diets.

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 5**). T-student analysis showed that larval contents of n-3 HUFA were significantly ($P<0.05$) higher in larvae fed high n-3 HUFA than low 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, the n-3, n-3 HUFA, n-6 contents and n-3/n-6 ratios (**Table 5**), regardless that similar levels were found in the respective diets (**Table 2**). Particularly, increase in dietary vitamin E+ vitamin C levels led to a significant linear increase in the DHA ($y=0.008x-0.45$, $R^2=0.97$) and n-3 fatty acid ($y=0.009x+0.084$, $R^2=0.99$, 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 an antioxidant protection by these vitamins (**Table 5**).

Table 5. Total lipid content (mg/g dw) and fatty acid composition (%dw) of whole body meagre larvae, after 14 days of feeding several n-3 HUFA, vitamin E and C dietary contents.

	14 dah	28 dah					
		0.4/150/180	0.4/300/180	0.4/300/360	3/150/180	3/300/180	3/300/360
Lipids	19.49±2.81	17.54±2.12[b]	17.63±3.79[b]	21.11±1.12[ab]	18.72±1.71[b]	19.59±0.13[ab]	23.61±0.86[a]
14:0	0.17	0.08	0.07	0.21	0.14	0.12	0.14
15:0	0.10	0.05	0.05	0.09	0.08	0.07	0.08
16:0	4.86	3.82	3.88	5.24	4.50	4.21	4.67
16:1n-7	1.10	0.14	0.13	0.24	0.24	0.29	0.34
16:1n-5	0.12	0.04	0.04	0.05	0.05	0.05	0.06
16:2n-4	0.20	0.10	0.09	0.12	0.17	0.17	0.20
16:3n-1	0.50	0.32	0.34	0.29	0.35	0.34	0.43
16:4n-3	0.09	0.14	0.14	0.10	0.07	0.09	0.11
18:0	1.81	2.43	2.36	3.16	2.95	2.46	2.81
18:1n-9	3.85	4.87	5.00	5.54	3.01	2.58	3.12
18:1n-7	0.72	0.38	0.37	0.47	0.54	0.49	0.59
18:1n-5	0.04	0.02	0.01	0.01	0.03	0.02	0.04
18:2n-9	0.17	0.03	0.03	0.02	0.01	0.02	0.03
18:2n-6	1.46	2.45	2.55	2.65	1.70	1.83	2.31
18:3n-6	0.06	0.05	0.05	0.06	0.07	0.06	0.07
18:3n-3	0.19	0.10	0.07	0.24	0.42	0.12	0.17
18:4n-3	0.02	0.02	0.01	0.06	0.02	0.01	0.02
20:0	0.12	0.14	0.13	0.21	0.14	0.12	0.14
20:1n-9	0.04	0.01	0.01	0.01	0.02	0.02	0.03
20:1n-7	0.36	0.40	0.41	0.42	0.34	0.33	0.42
20:1n-5	0.08	0.03	0.03	0.03	0.04	0.04	0.05
20:2n-9	0.05	0.00	0.01	0.01	0.01	0.02	0.02
20:2n-6	0.13	0.11	0.11	0.17	0.13	0.12	0.16
20:3n-6	0.08	0.04	0.04	0.03	0.04	0.05	0.06
20:4n-6	0.51	0.28	0.29	0.21	0.41	0.55	0.70
20:3n-3	0.04	0.01	0.01	0.02	0.03	0.03	0.04
20:4n-3	0.05	0.03	0.01	0.05	0.05	0.04	0.06
20:5n-3	0.27	0.14	0.14	0.21	0.36	0.65	0.85
22:1n-11	0.03	0.01	0.01	0.02	0.09	0.07	0.10
22:1n-9	0.09	0.24	0.22	0.25	0.29	0.28	0.24
22:4n-6	0.04	0.02	0.02	0.02	0.03	0.04	0.06
22:5n-6	0.09	0.04	0.04	0.02	0.09	0.14	0.18
22:5n-3	0.13	0.06	0.06	0.06	0.17	0.31	0.43
22:6n-3	1.44	0.67	0.66	0.64	1.94	3.51	4.50
Saturated	7.09	6.53	6.51	8.93	7.84	6.99	7.87
Monoenoic	6.49	6.16	6.24	7.09	4.67	4.19	5.00
n-3	2.26	1.19	1.11	1.38	3.08	4.79	6.20
n-6	2.37	2.99	3.09	3.17	2.47	2.79	3.54
n-9	4.22	5.16	5.27	5.83	3.35	2.92	3.45
n-3HUFA	1.93	0.92	0.89	0.97	2.55	4.55	5.88
n-6HUFA	0.85	0.49	0.5	0.45	0.7	0.9	1.16
ı-6+n-3)HUFA	2.78	1.41	1.39	1.42	3.25	5.45	7.04
:1n-9/n-3HUFA	0.39	0.93	1.00	1.21	0.22	0.11	0.13
n-3/n-6	0.19	0.07	0.06	0.09	0.23	0.34	0.41
EPA/ARA	0.11	0.09	0.09	0.21	0.17	0.23	0.29
DHA/EPA	1.02	0.83	0.84	0.65	1.00	1.06	1.25

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 and co-workers (2007) (23-36%). Despite that survival was relatively low due to the complete early 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 et al., 2009b or 8% survival in Durán et al., 2009).

The low growth obtained in larvae fed weaning diets with the low HUFA content (0.4% dw) as well as 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 & Le Milinaire 1985) or gilthead seabream (Rodríguez et al. 1993, 1994; Salhi et al. 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 the present study, improvement was observed in lipid absorption in gut and lipid deposition in liver 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 et al. 1992), in yellowtail (*Seriola quinqueradiata*) (Furuita et al. 1996), red seabream (Izquierdo et al. 1989) or common dentex (*Dentex dentex*) (Mourente et al. 1999).

In the present 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 heme containing enzymes that favour oxidation, promotes the occurrence of pathological alterations in this organ (Hamre 2011), such as hepatocyte hypertrophy, inflammation, ceroidosis and necrosis (Montero et al. 1996; Thorarinsson et al. 1994). 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 et al. 1999).

Nevertheless, an 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. For vitamin E the major hydrophobic antioxidant, the increase in dietary HUFA would accelerate the autocatalytic peroxidation and increase the requirement for this vitamin (Watanabe 1982; Sargent et al. 1997; Izquierdo et al. 2001). For instance, elevation of dietary HUFA contents in weaning diets for larval gilthead seabream required an 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); vitamin C would play an important role indirectly protecting HUFA from oxidation, since it is essential to regenerate α-tocopheroxyl radicals to *α*-tocopherol. Thus, the 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, an antioxidant synergism was demonstrated in seabass larvae fed high 5% DHA (Betancor *et al.* 2012); the synergistic effect between these vitamins has been also described 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 et al. 2008), hybrid striped bass (*Morone chrysops X M. saxatilis*) (Sealey & Gatlin 2002) or red seabream (Gao et al. 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 et al. 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 (g/kg) are suspected to be high, at the g/kg level, , in view of the high risk of oxidative stress faced by the intensively reared larvae according to Moren et al. (2011). Indeed, both vitamin C and vitamin E markedly improve stress resistance in marine fish (Ortuño et al. 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, an increase in vitamin E contents in rotifers or weaning diets improved growth of cod larvae (*Gadus morhua*) (Zheng et al. 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 et al. 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 et al. 2004; Roosta et al. 2014).

Conclusion

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 vitamin E and vitamin 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).

2.-Importance of dietary vitamins A, K and D in weaning diets for meagre

Materials and methods

To determine the effect of weaning diets on larval performance and morphometry, meagre larvae were obtained from an induced spawning from broodstock that was kept in 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 five microdiets in triplicates. Larvae were previously fed enriched rotifers (DHA Protein Selco; INVE, Dendermonde, Belgium) until they reached 20 days after hatching (dah). Meagre larvae (initial total length 7.2±0.7 mm; dry body weight 0.5±0.1mg) were randomly distributed in 15 experimental tanks at a density of 2100 larvae per tank and were fed one of five experimental diets tested in triplicates for 14 days, at an average water temperature of 24.5±0.5 °C. All tanks were 200 L fibreglass cylinder tanks with conical bottom and painted a light grey color 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 carried out. Water was continuously aerated (125 ml min^{-1}). Average water dissolved O_2 reach 5.6±0.1 mg and water quality in terms of temperature, dissolved O_2 and pH were appropriate for this species. The 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 2 and 4 g per tank during the first and second week of feeding respectively.

Five isonitrogenous and isolipidic experimental microdiets (pellet size <250 µm & 250-500 µm) were formulated using squid powder non defatted as source of protein and lipid, and were completed with Krill-PL as source of marine phospholipids, and level of vitamin E (1.500 mg kg^{-1}), vitamin C (3.600 mg kg^{-1}), gelatin (3.0), mineral premix (4.5 g/100 g), vitamins premix (6.0 g/100g) without menadione, ergocalciferol and retinol acetate (**Table 6**). Additional, menadione as source of vitamin K was added to the vitamin mix (175 mg kg^{-1}) in all diets except to C-Vit K (diet without vitamin K supplementation diet), ergocalciferol as source of vitamin D was added to the vitamin mix (37 mg kg^{-1}) in all diets except to C-Vit D (diet without vitamin D supplementation) and retinol acetate as source of vitamin A was added to the vitamin mix (3 mg kg^{-1}) in all diets except to C-Vit A (diet without vitamin A supplementation). Taurine (2.000 mg kg^{-1}) was added only to C+Taurine diet (diet with taurine addition diet). The diet with vitamin K, D and A supplementation and without taurine addition was considered as a control diet (C) (**Table 6**).

To determine gut occupancy, after feeding, larvae were observed under the binocular microscope to determine feed acceptance. Growth was determined by measuring dry body weight (Faustino and Power, 1998) and total length (Profile Projector; Nikon V-12A, Tokyo, Japan) of 30 fish per tank at the beginning and at the end of the trial. For welfare status, a stress resistance test was conducted at the end of the trial with 30 larvae that were handled out of the water in a scoop net for 30 sec. Final survival was calculated by individually counting all the larvae alive at the beginning and at the end of the experiment. Additional, 30 larvae per tank were collected for histological study and 100 larvae per tank for osteological study and kept at 10% formalin buffered to pH 7 with 0.1M phosphate buffer for 1 and 2 days. 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.

Moisture (A.O.A.C. 1995), crude protein (A.O.A.C. 1995) and crude lipid (Folch et al., 1957) contents of larvae and diets were 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 et al. (1990) and identified by comparison to previously characterized standards and gas-liquid chromatography-mass spectrometry.

For the larval organ development studies, fixed larvae were dehydrated through graded alcohols, then by use of xylene and finally embedded in paraffin wax. Four paraffin blocks containing five larvae per tank (12 blocks per diet) were sectioned at 5 µm, and sections were stained with haematoxylin and eosin (H&E) and Zhiel-Neelsen for histopathological evaluation (Martoja & Martoja-Pearson 1970). For skeleton development studies, fixed larvae were stained with alizarin red and immediately photographed and examined for the occurrence of skeletal anomalies (Vandewalle et al. 1998). The effects of experimental diets on axial skeleton mineralisation were evaluated quantifying the surface corresponding to bone (red) using a computerized image analysis package (Imagen-Pro Plus, Media Cybernetics, Inc, Silver Springs, MD, USA) that allows the selection and quantification of pixels color red. The value of red pixels was associated to the degree of bone mineralization with respect to total surface larvae.

All values presented as percentage (skeletal anomalies, mineralization of the column, total survival and incidencia of granuloma) were arc cosine transformed (Ennos, 2007). Statistical differences were checked with one-way ANOVA for multiple comparisons of means using SPSS software (SPSS for Windows 11.5; SPSS, Chicago, IL, USA). In the case of statistical differences among the treatment groups, a Tukey test was applied in order to evaluate inter-group differences. If variances were not homogeneous, a parametric test was applied (homogeneity of the variance with Levene's test, data normality with Kolmogorov-Smirov test) (Zar, 2009). The significance level was fixed at 95%.

Gut occupancy, larval performance and morphometric parameters

The image analysis studies of the larval photographs of larvae fed the different diets denoted no significant differences in gut occupancy among fish fed the different diets. After only 7 days of feeding (26 dah), growth in terms of total length and dry body weight was only significantly ($P<0.05$) higher in larvae fed diet C-Vit D (8.9±1.0) (mean±SD) (**Table 7**). However, at the end of the feeding trial (33 dah), the larvae feeding

diets without supplementation of vitamin K (C-Vit K), vitamin D (C-Vit D) and vitamin A (C-Vit A) increased significantly ($P<0.05$) growth in terms of total length (12.8±1.6; 12.6±1.3; 12.2±1.7, respectively). This same trend was found in body weight (3.2±0.2; 3.3±0.2 for C-Vit K and C-Vit D, respectively) except for larvae fed C-Vit A (2.5±0.3) that did not show significant differences with larvae fed Control (**Table 8**).

Table 6. Ingredients and proximate composition of early weaning diets fed to meagre larvae from 20 to 33 dah. (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation).

	Diets				
	C	**C+Taurine**	**C-Vit K**	**C-Vit D**	**C-Vit A**
Ingredients					
Taurine[i]	0.0	200.0	0.0	0.0	0.0
Vit K[j]	17.3	17.3	0.0	17.3	17.3
Vit D[k]	3.7	3.7	3.7	0.0	3.7
Vit A[l]	0.3	0.3	0.3	0.3	0.0
Proximate composition (%)					
Crude lipids	16.4	16.2	16.5	17.1	17.9
Crude protein	76.0	75.9	76.4	76.4	76.1
Moisture	13.7	13.6	13.6	13.8	13.8
Ash	6.5	6.5	6.5	6.6	6.5
Taurine[1]	4.0	5.8	4.0	4.0	4.0
Vitamin K[2]	2.4	2.4	0.0	2.6	2.2
Vitamin D[3]	28.9	29.0	30.4	2.3	27.4
Vitamin A[4]	4.2	4.3	4.2	4.3	4.1

Table 7. Total length (mm), dry weight (mg) and survival of meagre larvae fed early weaning diets from 20 to 33 dah (initial total length 7.2±0.7 mm and dry body weight 0.5±0.1mg). (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation).

		Diets				
		C	**C+Taurine**	**C-Vit K**	**C-Vit D**	**C-Vit A**
Total length	26 dah	8.3±1.0 [a]	8.5±0.9 [a]	8.5±1.0 [a]	8.9±1.0 [b]	8.6±1.0 [a]
	33 dah	11.5±1.7 [a]	11.7±1.3 [a]	12.8±1.6 [b*]	12.6±1.3 [b]	12.2±1.7 [b]
Body weight	26 dah	0.7±0.1 [a]	0.8±0.1 [a]	0.7±0.1 [a]	0.9±0.2 [b]	0.8±0.2 [a]
	33 dah	2.4±0.6 [a]	2.3±0.4 [a]	3.2±0.2 [b*]	3.3±0.2 [b]	2.5±0.3 [a]
Survival (%)		16.7±6.5	12.9±1.2	7.1*	17.7±12.3	19.0±0.5

Larval organ and skeleton development

By histopathological examination, granulomas were detected in the liver of some larvae of the different experimental diets. Granulomas displayed different stages of growth and morphology: initial granulomas or "Stage I", granuloma of 0.5 mm composed of a central cluster of voluminous macrophages (**Fig. 3a**). Late granulomas or "Stage II", granuloma of 1.8 mm composed of an eosinophilic necrotic central area surrounded by several concentric layers of macrophages that presented vacuolized cytoplasm, observing more flattened in the outer layers. Hepatic cells around of the granulomas were compressed (**Fig. 3b**).

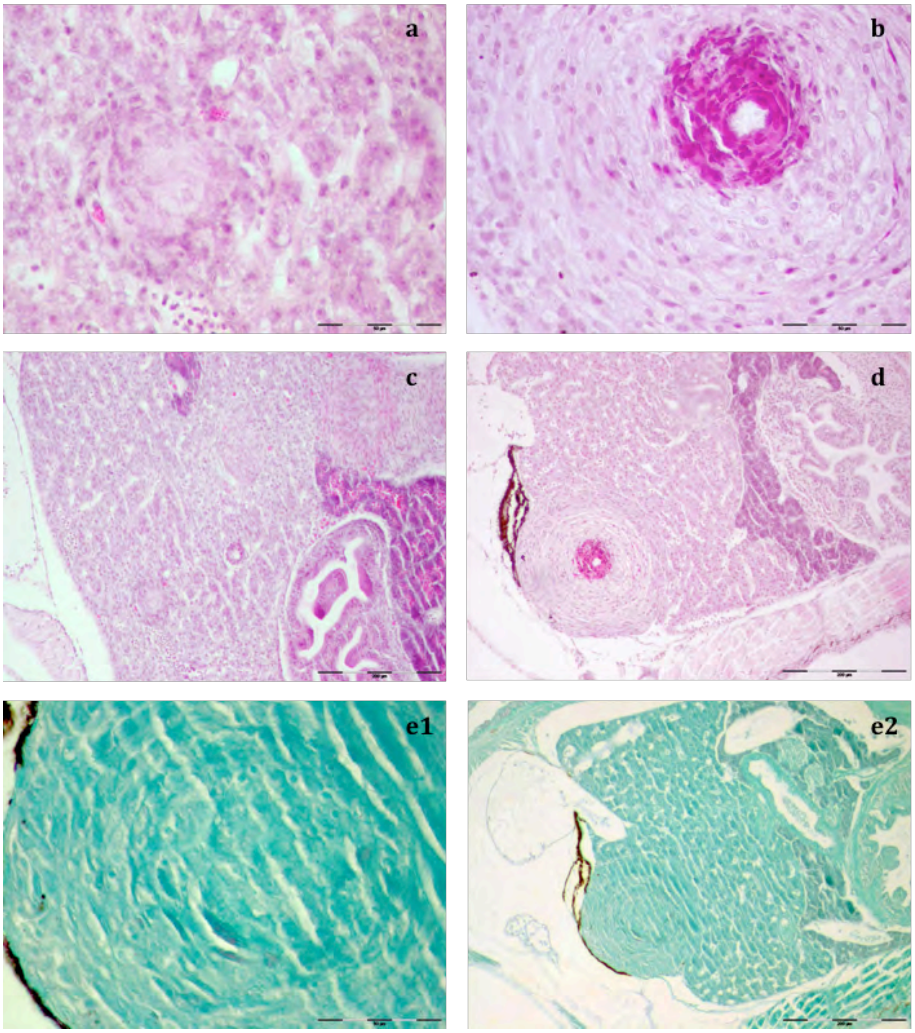

Figure 3. **a**. Initial or "Stage I" granuloma in the liver (40x). **b**. "Stage II" granuloma in the liver (40x). **c**. "Stage I" granuloma in the liver of larvae fed without vitamin K supplementation diet (C-Vit K) (10x). **d**. "Stage II" granuloma in the liver of larvae fed without vitamin K supplementation diet (C-Vit K) (10x). **e1.** (40x) and **e2.** (10x) staining with Ziehl-Neelsen technique, granuloma in the liver of larvae fed without vitamin K supplementation diet (C-Vit K).

At 33 dah, high incidence of Stage I and II granulomas was found in larvae fed without supplementation of vitamin K (C-Vit K) (12.5%) (**Fig. 3c and d**), followed by larvae fed without supplementation of vitamin A (C-Vit A) and without supplementation of vitamin D (8.3% and 3.3%, respectively) (**Table 8**). Larvae from control and taurine diets did not show granulomas.

Larvae with granulomes were stained with Ziehl-Neelsen technique for Mycobacteria detection being negative for all cases (absence of alcohol-acid resistant bacillus, **Fig. 1e1 and e2**).

Table 8. Percentage of total larvae affected with granuloma and percentage of larvae affected with granuloma in state I and state II at 33 dah. (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation)

	33 dah		
Diets	Total	Stage I	Stage II
C	0.0±0.0	0.0±0.0	0.0±0.0
C+Taurine	0.0±0.0	0.0±0.0	0.0±0.0
C-Vit K	12.5±10.6	5.0±7.1	7.5±3.5
C-Vit D	3.3±2.9	1.7±2.9	1.7±2.9
C-Vit A	8.3±10.4	6.7±7.6	1.7±2.9

Osteological study

No significant differences on frequency of severe anomalies of the total anomalies were found among the groups of larvae, accounted for 40% for larvae fed Control, C+Taurine and C-Vit A diets, 45% for larvae fed C-Vit K diet and 38% for larvae fed C-Vit D diet. The index of severe anomalies among groups varied from 1.5% for larvae fed C+Taurine diet, 1.4% for larvae fed C-Vit D diet, 1.3% for larvae fed C-Vit K and C-Vit A diets to 1.2% for Control larvae, but without significant differences among groups.

Severe anomalies of hemal vertebra were quite common among groups (32.7%), followed by pre-hemal (13.7%) and caudal vertebra (9.6%) (**Fig. 4**). Anal fin (12.5%) showed higher anomalies than caudal fin (0.1%). Malformations involving spines and rays were very common in all groups of larvae, being up to 46.8% for spines of arch and 26.9% for branquiostegal rays (**Fig. 4**).

No differences among larvae fed the different experiment diets were found regarding the mineralisation of the bones at 33 dph (**Table 9**), although larvae without supplementation of vitamins K, D and A tended to have higher mineralization than the control larvae.

Survival and welfare status

Larval survival at the end of the experiment was not significantly different among the different groups of larvae, being in average 16.6±5.1% (**Table 7**), except for the group of larvae fed C-Vit K that presented low survival (7.1%) with 100% of mortality in two tanks. No significant differences were found in larval welfare status.

Biochemical composition

The fatty acid composition of the diets was not different among diets, being high in saturated acids (SAFA) (5.8%DW), specially lauric (14:0) (1.4%DW) and palmitic acid (16:0) (3.9%DW) acids (**Table 10**) and high in monosaturated acids (MUFA) (4.1%DW), particularly oleic acid (18:1n-9) (1.5%DW). The levels of n-3 PUFA (5.9%DW) were highest than n-6 PUFA (0.4%DW) for all diets, and subsequently the n-3/n-6 PUFA ratio was high (13.4%DW). Standing out the fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexanoic acid (DHA, 22:6n-3) as the n-3 fatty acids found in highest levels (2.6 and 2.6 %dw, respectively) for all diets (**Table 10**).

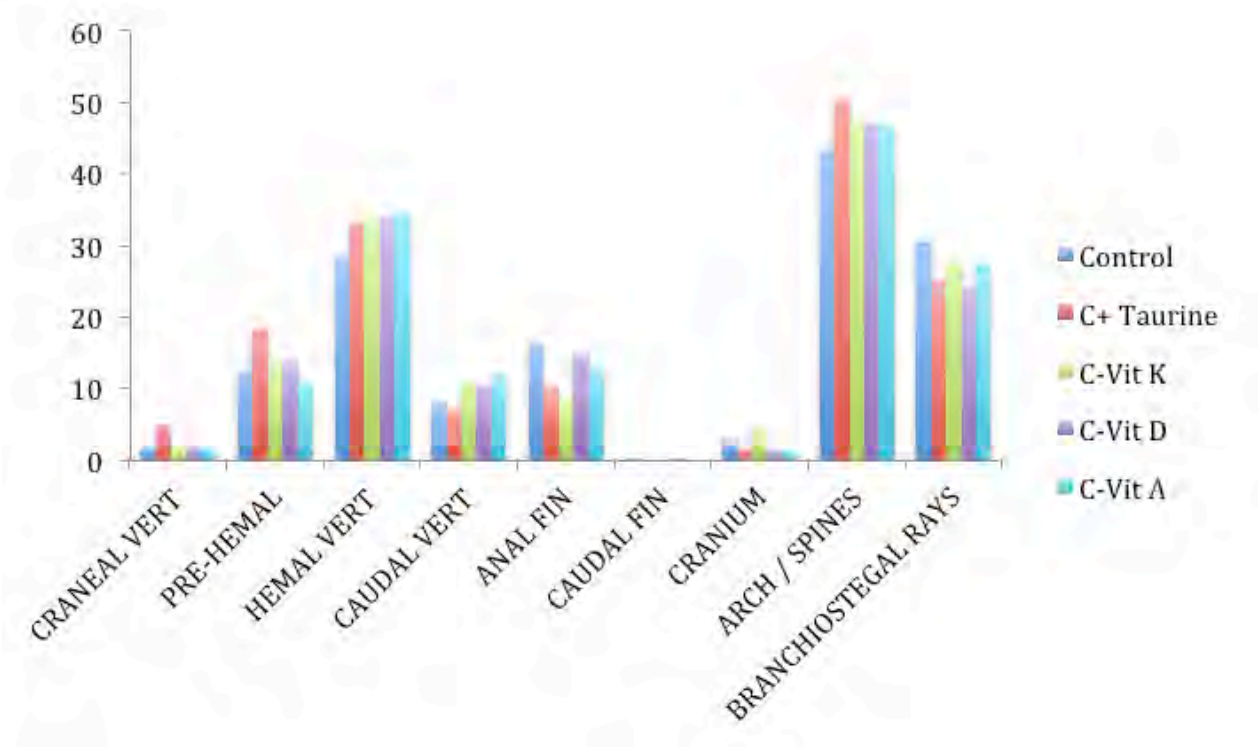

Figure 4. Frequencies (%) of larvae with different anomalies in each experimental diet (33 dah). (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation). Data are referred to the total of individuals of each group.

Table 9. Percentage of mineralization (%) of larvae from different diets at 33dah. (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation)

Diets	Mineralization
C	32.6±0.9
C+Taurine	32.9±2.2
C-Vit K	35.4*
C-Vit D	35.7±1.4
C-Vit A	36.5±2.4

Table 10. Main fatty acid composition (% dw) of the early weaning diets used to fed larval meagre (*Argyrosomus regius*) from 20 to 33 dah. (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control without vitamin D supplementation).

	Diets				
	C	**C+Taurine**	**C-Vit K**	**C-Vit D**	**C-Vit A**
14:0	1.3	1.4	1.4	1.4	1.5
16:0	3.7	3.9	3.8	3.8	4.1
18:0	0.5	0.5	0.5	0.5	0.5
Total SAFA[a]	5.7	5.4	5.7	5.9	6.2
16:1n-7	0.8	0.9	0.9	0.9	0.9
18:1n-9	1.5	1.5	1.5	1.5	1.6
20:1n-7	0.5	0.5	0.5	0.5	0.5
22:1n-9	0.1	0.2	0.1	0.1	0.2
Total MUFA[b]	3.9	4.0	4.1	4.2	4.5
18:2n-6	0.2	0.2	0.2	0.2	0.2
20:4n-6	0.1	0.1	0.1	0.1	0.1
Total n-6 PUFA[c]	0.4	0.4	0.4	0.5	0.5
18:3n-3	0.2	0.1	0.2	0.2	0.2
18:4n-3	0.4	0.4	0.4	0.5	0.5
20:3n-3	0.1	0.1	0.1	0.1	0.1
20:4n-3	0.1	0.1	0.1	0.1	0.1
20:5n-3	2.6	2.4	2.6	2.7	2.9
22:5n-3	0.1	0.1	0.1	0.1	0.1
22:6n-3	2.5	2.3	2.5	2.6	2.7
Total n-3 PUFA[d]	6.0	5.3	5.9	6.2	6.4
Total n-3 LC PUFA[e]	5.3	4.8	5.3	5.6	5.8
Total PUFA	6.3	5.8	6.3	6.6	6.9
n-3/n-6 PUFA	15.4	11.9	13.2	13.3	13.0

Despite dietary lipids levels were similar among diets, larvae fed without supplementation of vitamin K (C-Vit K) and vitamin D (C-Vit D) tended to increase larval total lipid contents (**Table 11**), and a significant positive correlation between larvae lipids and body weight at 33 dah ($y=0.3814x-2.4621$, $R^2=0.83$) was found.

The total saturated fatty acids, specially stearic acid (18:0), total monounsaturated acids, specially oleic acid (18:1n-9) were higher in the initial larvae than those of the experimental groups (**Table 11**). Regarding the diets, total amount of SAFA, specially 16:0 was higher in the larvae fed without supplementation of vitamin K (C-Vit K) and vitamin D (C-Vit D) (3.2%DW and 3.0%DW respectively) than those found in larvae fed C+Taurine and C-Vit A (2.5%DW), although without significant differences with the control larvae. Similarly, total amount of MUFA, especially 18:1n-9 were highest in the larvae fed without supplementation of vitamin K (C-Vit K) and vitamin D (C-Vit D) than those of C+Taurine and C-Vit A.

Total n-6 PUFA contents, especially linoleic acid (18:2n-6), were highest in the initial larvae and consequently higher than those of the experimental groups (**Table 11**). Levels were but similar among larvae fed the different diets at 33dah. On the contrary, the total n-3 PUFA content was lowest in initial larvae (2.5%DW) mainly by reduction of levels of eicosapentanoic acid (20:5n-3) (0.4%DW) and docosahexanoic acid (22:6n-3) (1.6%DW), and subsequently the n-3/n-6 PUFA ratio was lower (1.0%DW) than those of the experiment groups (**Table 4**). Among larvae fed experimental diets, those feeding the C-Vit K and C-Vit D

diets increased significantly the level 20:5n-3 (1.9% DW and 1.8% DW, respectively), and additionally these feeding groups tended to increase total n-3 PUFA, n-3 LC PUFA and n-3/n-6 PUFA ratio (**Table 4**), although for this ratio without significant differences. Nevertheless, an increase of larvae 22:6n-3 content (≈3.5%DW) compared with the initial larval level (1.6% DW) was found regardless of the different diets.

Table 11. Total lipid content and fatty acid composition (% dw) of whole body meagre larve (33 dah) feeding on diets (C control diet; C+Taurine control with taurine supplementation; C-Vit K control without vitamin K supplementation; C-Vit D control without vitamin D supplementation; C-Vit A control withouth vitamin D supplementation).

		Diets				
	Initial larvae	**C**	**C+Taurine**	**C-Vit K**	**C-Vit D**	**C-Vit A**
Lipids	14.8±2.8	13.3±2.1	12.6±0.8	15.2±0.7	14.4±0.8	12.7±1.1
14:0	0.1±0.0	0.3±0.0^{abc}	0.1±0.1^{c}	0.4±0.0^{a}	0.3±0.0^{ab}	0.2±0.0^{bc}
16:0	2.7±0.1	2.8±0.1^{ab}	2.5±0.2^{a}	3.2±0.2^{b}	3.0±0.0^{b}	2.5±0.1^{a}
18:0	1.8±0.2	1.0±0.1	1.0±0.1	1.0±0.1	0.9±0.0	0.9±0.1
20:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
Total SAFA[a]	4.9±0.3	4.2±0.2^{ab}	3.7±0.4^{a}	4.7±0.3^{ab}	4.3±0.1^{b}	3.7±0.3^{a}
16:1n-7	0.6±0.0	0.4±0.0^{ab}	0.3±0.1^{a}	0.5±0.0^{b}	0.5±0.0^{ab}	0.3±0.1^{ab}
18:1n-9	2.2±0.1	1.3±0.0^{ab}	1.2±0.0^{a}	1.5±0.1^{c}	1.4±0.0^{bc}	1.2±0.0^{a}
20:1n-7	0.3±0.0	0.3±0.0^{ab}	0.2±0.0^{a}	0.3±0.0^{b}	0.3±0.0^{b}	0.2±0.0^{a}
22:1n-9	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
Total MUFA[b]	4.2±0.1	2.8±0.1^{ab}	2.5±0.1^{a}	3.2±0.1^{c}	3.0±0.1^{bc}	2.5±0.2^{a}
18:2n-6	1.7±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
20:4n-6	0.4±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
Total n-6 PUFA[c]	2.5±0.0	0.5±0.1	0.6±0.0	0.6±0.0	0.5±0.0	0.5±0.0
18:3n-3	0.3±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
18:4n-3	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.1±0.0
20:4n-3	0.1±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:5n-3	0.4±0.0	1.5±0.1^{a}	1.4±0.1^{a}	1.9±0.1^{b}	1.8±0.1^{b}	1.5±0.2^{ab}
22:5n-3	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
22:6n-3	1.6±0.1	3.3±0.0	3.4±0.2	3.6±0.4	3.6±0.1	3.4±0.2
Total n-3 PUFA[d]	2.5±0.2	5.3±0.1^{ab}	5.2±0.2^{a}	6.1±0.2^{b}	6.0±0.1^{ab}	5.4±0.3^{ab}
Total n-3 LC PUFA[e]	2.1±0.0	5.1±0.1^{a}	5.1±0.3^{a}	5.8±0.5^{b}	5.8±0.2^{b}	5.2±0.4^{ab}
Total PUFA	5.1±0.2	5.8±0.1^{a}	5.8±0.2^{a}	6.6±0.2^{ab}	6.5±0.1^{b}	5.9±0.3^{a}
n-3/n-6 PUFA	1.0±0.1	9.7±0.1	9.0±0.2	10.5±0.2	11.1±0.1	10.3±0.3

Discussion

There are very few studies and very little information about requirements and effects of certain liposoluble vitamins on fish larvae, such as vitamin A (Takeuchi *et al.*, 1995, Tauri *et al*., 2006), vitamin D (Haga *et al.*, 2004, Darias *et al.*, 2010) and vitamin K (Udagawa *et al*., 2001), as well as taurine (Pinto, 2010 and 2013), and even lower on meagre.

When the survival results were analyzed, there was a low larvae survival with the treatment without vitamin K supplement, due to a massive and sudden mortality of all larvae in two of the three tanks of this treatment. This could indicate that the vitamin K basal levels on meagre larvae were not enough to cover their requirements. Furthermore, the appearance of cannibalism was seen for this treatment, could reflect a nutritional deficiency. The transition of meagre larvae from live prey to dry feeds is the most critical phase in the early developmental stages of this fish larvae (Hamlin et al., 2000; Garcia-Ortega et al., 2003), and high mortalities are observed due to a decreased feed intake and low assimilation of the dry feeds (Watanabe and Kiron, 1994).

In the present study, an effect of the supplement or the absence of liposoluble vitamins (A, D y K) on the total length and dried weight of meagre larvae was observed. At 26 dph, larvae fed with microdiet with non addition of vitamin D grew more than on the other treatments in terms of size and weight. This could indicate that vitamin D supplement on the rest of the treatments could lead to a hypervitaminosis D that affects negatively to the growth. In support of this, Darias *et al*. (2010) also reported the high sensitivity of European seabass larvae (45 dph) to vitamin D hypervitaminosis.

At 33 dph, larvae size was higher in treatments with absence of vitamin A and D, compared to larvae fed the control treatment. Regarding larvae dry weight, it was significantly higher for treatments with no vitamin D supplement than on larvae given the control treatment. As mentioned, these results may show an intense hypervitaminosis D as we have mentioned before and a light hypervitaminosis A for larvae fed the control and taurine excess treatments. Some studies have shown that larval fish diets with deficient or excess vitamin A had a compromised development, showing reduced growth and survival rates, delayed digestive system maturation and high incidence of skeletal deformities (Cahu et al, 2003). Best growth and survival of European sea bass larvae have been found 30 mg all-*trans* retinol/kg diet dry matter (Villeneuve et al., 2005), whereas higher or lower levels of vitamin A induced poorer growth and survival.

Roy and Lall (2007) showed that haddock, *Melanogrammus aeglefinus* growth was not affected at 8 weeks although at 20 weeks, the average body weights of the two groups became slightly different and those receiving vitamin K diets grew more rapidly. Others studies with vitamin K deficient feed caused no detectable deficiency symptoms in rainbow trout (Kitamura et al. 1967), channel catfish, *Ictalurus punctatus* (Murai & Andrews 1977) and in brook trout, *Salvelinus fontinalis* (Poston, 1964), but reduced growth and increased mortality in amago salmon, *Oncorhynchus rhodurus* (Taveekijakarn et al. 1996), and increased mortality in mummichog, *Fundulus heteroclitus* (Udagawa and Hirose 1998).

After analyzing the results on larvae fed with taurin excess microdiets, they were not significantly different than larvae fed with control microdiet, regarding growth and survival. Pinto et al. 2010 also did not find significant differences between treatments with taurine and control without taurine for growth and survival of Senegalese sole, *Solea senegalensis* larvae until the end of the pelagic phase (25 dph). However, by the end of the trial (32 dph), Senegalese sole larvae from the taurine treatment presented a significantly higher growth than larvae from the control treatment, the same was observed by Chen et al. (2004, 2005) in red sea bream (*Pagrus major)* larvae and Japanese flounder, *Paralichthys olivaceus* larvae. In another experiment, (Pinto *et al* 2013), no significant differences were found between treatments for growth or survival of gilthead sea bream larvae during the experimental period. Positive correlations between dietary taurine levels and larval growth have been demonstrated for turbot, *Scophthalmus maximus* (Conceição *et al*., 1997), red sea bream *Pagrus major* (Chen *et al*., 2004), Japanese flounder, *Paralichthys olivaceus* (Chen *et al*., 2005), Pacific cod, *Gadus macrocephalus* (Matsunari *et al*., 2005) and cobia, *Rachycentron canadum* (Salze *et al*., 2011). However, the underlying mechanisms leading to growth enhancement have not been clarified, since taurine, lacking a carboxyl group, cannot be incorporated into tissue proteins. Nevertheless, the few studies available with fish larvae show that dietary taurine supplementation affect larval quality, through an enhancement on the ontogenetical development of the sensory systems, as observed in cobia (Salze et al., 2011), or through an enhancement in metamorphosis completion in flatfish species, such as Japanese flounder (Chen et al., 2005).

Regarding the results of the proximal composition of meagre larvae, a positive relation between larvae lipid percentage on each treatment and larvae size at 33 dph was observed. This shows that there was a good nutrient utilization to promote larval growth. However, minor dietary lipid changes do not seem to affect larval growth. For instance, larvae growth was affected when the difference on the lipid percentage among microdiets was over 7% on gilthead bream larvae (Salhi, 1997). On this study, the difference on lipid percentage on the microdiets did not exceed the 2%. In general, larvae fatty acid composition was constant among all treatments.

When analyzing histology, presence of granulomas was found in the liver of meagre larvae. The origin of granuloma occurrence on fish larvae is not well understood. In fact, this is the first time this disease is detected on larvae culture. In larger fish, it is well known that Mycobacteria and *Nocardia spp* are the most common cause of granulomas, particularly of those occurring in the spleen (Ferguson, 1989). Nevertheless,

other authors have related the appearance of granulomas to non infectious reasons. On this way, Herman, (1996) described granulomatous lesions in culture of juveniles of Salmonidae, Sparidae and Turbot occurring in the absence of an infectious agent. Granulomas lesions in brook trout (*Salvelinus fontinalis*) were related to the use of cottonseed meal in the diet (Dunbar and Herman, 1971) and to the use of formulate feeds or fish meal or frozen fish stored for prolonged periods in gilthead seabream (*Sparus aurata*, Paperna, 1987). Similar pathology condition have been reported from cultured fish such as turbot (*Scophthalmus maximus*) in France. Messager *et al* (1986) experimentally induced typical granulomatosis hypertyrosinemia in turbot fed diets deficient in ascorbic acid. Coustans *et al* (1990) confirmed the effect of ascorbic acid deficiency and further showed that the condition is exacerbated by hypovitaminosis of B group vitamins. Tyrosine catabolism is impaired leading to deposition of crystalline tyrosine and subsequent granulomatosis. While collage metabolism is impacted, skeletal deformities do not appear to be associated with this disease. Reduced growth, general melanism and mortality may be the first indication of the problem. In meagre, there is only recent evidence of microscopic liver granulome in juveniles fed different vitamin E levels (Rodriguez *et al*., in preparation).

On the present study, we can confirm that the existence of granulomas on meagre larvae was not due to Mycobacterium, associating its origin to nutritional composition of experimental microdiets. This way, the presence of granulomas at 33 dph affected on a high percentage larvae fed with microdiets with non vitamin K (12,5 %), D (3,3 %) and A (8,3 %) supplement. Vitamin K is well known for its effect on vascular biology. Thus, vitamin K deficiency is characterized by anaemia, increased blood clotting time, histopathological changes in liver and gills and affects the synthesis of plasma (Krossøy, 2011). In relation to vitamin A, it has essential roll on normal maintenance of epithelial tissue in all vertebrates (Ross et al., 2000). The damages on the vascular tissue or/and epithelial cells could be the reasons for the granuloma development. Further studies are required to study the granulomatosis in meagre culture.

Several physical anomalies have been described in fish larvae, in a study, Boglione et al. (2001) showed that only 4% of wild caught animals showed body deformations, in contrast to the very high values observed in hatchery-reared larvae (Divanach et al., 1996). Malformations are often associated with growth depression and high mortality rate. On this study, despite there were no significantly differences found among larvae at different treatments, there was a higher percentage of severe deformities of larvae fed with taurine excess microdiets than on larvae fed the other diets. For this reason, we suggest that a taurine excess can have a negative effect on corvine larvae development. Regarding larvae fed with non vitamin A addition microdiets, percentage of deformities was similar to the control treatment. Several studies have shown that larval fish fed diets with deficient or excess vitamin A had a compromised development and high incidence of skeletal deformities (Cahu et al, 2003). Other authors have observed an increasing number of malformations in the caudal region and vertebrae of Japanese flounder (Dedi et al. 1997) and in the vertebrae of turbot (Estévez and Kanazawa 1995) fed increasing dietary levels of vitamin A palmitate during metamorphosis, Villeneuve et al. (2005) fed European seabass larvae of 7–42 dph five isoproteic and isolipidic compound diets with graded levels of retinyl acetate, using malformation rate as an indicator, the optimum level of retinol was found to be around 31 mg kg^{-1}. Hypervitaminosis A is known to cause skeletal deformities and other malformations (Dedi et al. 1997; Fernández et al. 2008; Fernández and Gisbert 2010), although this effect is not observed on the experience made. Larvae fed with non vitamin D addition microdiet also showed a high percentage of deformities, although this was not significantly different as compared with the control treatment. Although there is scarce information concerning vitamin D requirements during the larval stage, a recent study performed in European sea bass showed that larvae performed well with 0,67 mg kg^{-1} of vitamin D supplemented diet and exhibited lower deformities score (20% deformities) while lower and upper levels of such vitamins induced skeletal malformations. It was also noticed that a deficiency of these vitamins is more harmful for developing European sea bass than an excess (Darias et al., 2010, 2011). When we take a look at severe deformities percentage on larvae fed with non vitamin K microdiet, we find that they were similar to larvae fed the control microdiet. On the contrary, studies on mummichog (*Fundulus heteroclitus*) larvae have shown that diets without vitamin K supplementation caused a higher incidence of deformities in the vertebrae and caudal skeleton (Udagawa 2001). Moreover, a deficiency vitamin K caused the formation of thin and weak bone, and induces bone structure abnormalities such as vertebral fusion and row irregularity, both in early development and during later growth in mummichog (Udagawa 2001, 2004).

Regarding mineralization, in the present study higher percentage of mineralization on larvae fed with non vitamin A supplement microdiet was found. This can lead to conclude that an hypervitaminosis A could modify bone mineralization in the rest of treatments, since these besides retinol acetate addition to the microdiets, they have vitamin A from krill oil used in the microdiet formulation. For larvae fed with non vitamin D microdiets and control larvae, no significant differences were found. Although, addition of vitamin D to the embryo medium increased bone mineralization in developing yolk-sac larvae of zebrafish, *Danio rerio* in a dose-dependent manner (Fleming *et al*. 2005). Regarding vitamin K, Roy and Lall (2007) showed that vitamin K deficiency decreased bone mineralization and increased the occurrence of bone deformities in haddock, *Melanogrammus aeglefinus L.*. However, in our study larvae fed non vitamin K addition treatment had the second higher percentage of mineralization. More studies are required to understand better these development processes in meagre larvae.

Conclusion

The present study has demonstrated the importance of supplementation of meagre weaning diets with 2.4 mg/kg vit K, since the absence of this vitamin markedly reduced larval survival. However, meagre seemed to be very sensitive to hypervitaminosis D and, only midly to hypervitaminosis A, since supplementation with these vitamins leaded to a growth reduction. On the contrary, taurine supplementation did not have any effect in meagre larvae performance.

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Deviations

This Deliverable was prepared according to the DOW description of "WP 8, Task 8.1 Improvement of larval weaning feeds", whose objective was to determine "the optimum levels of essential fatty acids and related micronutrients in weaning diets for meagre", including "the effect of nutrients on culture performance, morphometric parameters, gut occupancy, larval organ and skeleton development, and biochemical composition". As stated in the DOW, "This Task will result in deliverable D8.1 Improvement of larval weaning diets". However in the same DOW (page 68 of 187), the description of D8.1 Improvement of larval weaning diets is somehow wrong, since it describes "A report with recommendation of the optimum levels of Lys in on-growing diets for meagre". On-growing diets do not correspond to weaning diets, the latter being the target of WP8, Task 8.1 and D8.1. On-growing diets correspond to diets for juveniles but not for larvae. Therefore, the reference for Lys requirements for juveniles in the description of the DOW for this deliverable was a typographical error, and we apologize for this. Indeed, the study of Lys requirements for juvenile meagre was proposed by P1.HCMR in the initial proposal submission, but it was removed from the DOW following the instructions of the EU Scientific Officer the during the negotiation process.

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