Deliverable Report

Deliverable No:	D9.1	**Delivery Month:**		25
Deliverable Title	Optimum levels and ratios of essential fatty acids in relation to Tau and combined PUFA-carotenoids in greater amberjack enrichment products.			
WP No:	9	**WP Lead beneficiary:**		P2. FCPCT
WP Title:	Nutrition – greater amberjack			
Task No:	9.1	**Task Lead beneficiary:**		P2. FCPCT
Task Title:	Improve larval enrichment products to enhance production of larvae and juveniles			
Other beneficiaries:	P8. IEO	P15. ULL		
Status:	Delivered		**Expected month:**	24
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Other Scientists participating: Rodríguez, C. (ULL), Jérez, S. (IEO)

Objective: Optimum levels and ratios of essential fatty acids in relation to Tau and combined PUFA carotenoids in greater amberjack enrichment products: This deliverable will present a list of the optimum levels and ratios of essential fatty acids and carotenoids that should be included in enrichment products for rotifers to be fed to greater amberjack larvae. The deliverable will present the methodology employed and the main results that leaded to the recommended improvements including the consequences of the improvement in larval production. The deliverable will describe the effects of essential fatty acids and carotenoids and will include: a) the effects on larval performance b) welfare, c) fatty acid analysis, lipid classes, and carotenoid profiles of enrichment products, live preys and larvae.

Description: Three different trials were conducted to determine the optimum nutrient levels in enrichment products for live prey to be fed to greater amberjack larvae.

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1. -Introduction

A series of limitations constrain the production of greater amberjack (*Seriola dumerili*) juveniles in commercial hatcheries. Among those, the scarce knowledge on larval nutritional requirements results in inadequate larval feeds that lead to low larval survival and poor juvenile quality. Thus hatchery-produced juveniles tend to be weak, very sensitive to parasite infections, large mortalities by the incidence of the shock syndrome and high deformity rates, with overall larval survivals ranging between 2-9% (Kolkovski and Sakakura, 2004, Yamamoto et al., 2009).

Regarding nutritional requirements, the importance of essential fatty acids (EFA), for marine fish larvae performance has been widely discussed (Izquierdo & Koven, 2011). Specifically, the significance of highly unsaturated fatty acids (HUFA) as essential components of cellular membranes that modulate physiological processes, including membrane transport and receptors and enzymatic activities, has been reported. Among others, docosahexaenoic acid (DHA) plays an important role maintaining structural and functional integrity in fish cell membranes. Besides, it is very important for normal neural development and visual function, and more recently its role on bone and cartilage development and skeletal deformities prevention has been addressed (Bell et al., 1995; Roo et al., 2009; Izquierdo & Koven. 2011, Izquierdo et al., 2013, Hernández-Cruz et al., 2015). *Artemia* naupli and enriched *Artemia* are frequently used as food for young *Seriola* larvae from as early stages as 10 dph. However, the fatty acid composition of *Artemia*, even after enrichment greatly differs from wild marine zooplankton (McEvoy et al., 1995, Van der Meeren et al., 2008). Moreover, whereas on natural preys like copepods DHA is accumulated in phospholipids (PL), in *Artemia* DHA is preferentially incorporated into triglycerides (TG) or retroconverted to eicosapentaenoic acid (EPA), restraining the accumulation of DHA in *Artemia* tissues. Shock syndrome mortalities in fast growing species such as striped jack (*Caranx delicatisimus*) have been long associated to low DHA levels in *Artemia* (Arakawa et al., 1987). These facts suggest that DHA deficiencies or low content of DHA in live prey particularly during *Artemia* feeding stage could be closely related with sudden mortalities of Young fish from *Seriola* species and with the high susceptibility to stress caused by husbandry procedures such as tank transfer or grading (Marisol Izquierdo´s unpublished data). Besides, both low as well as high levels of dietary DHA fed to early developing larvae may cause mortalities and morphological alterations, particularly related with cartilage and bone development (Villeneuve et al., 2006; Ronnestad et al., 2013; Hernández-Cruz et al., 2015). Under this perspective, the objective of the present study was to determine the nutritional requirements and optimum levels of DHA at the time of *Artemia* stage for greater amberjack larvae, evaluating its effects on survival, growth, stress resistance/tolerance, and bone development. Results of this study are relevant to properly design well balance enrichment products and weaning diets for this species.

Lipid emulsions are arguably the most extended enrichment diets and a big variety of products are now commercially available. Composition of commercial lipid emulsions basically consists of fish oils, with other minor components including emulsifying agents, vitamins and fatty acid (FA) derivatives such as fatty acid ethyl esters added to compensate suboptimal natural profiles of fish oil (Monroig et al., 2007, Viciano et al., 2015). Several findings have shown that dietary phospholipids (PL) are a more efficient source of LC-PUFA (22:6n-3, DHA; 20:5n-3, EPA and 20:4n-6, AA) for larvae at rotifer and *Artemia* feeding stages. This probably reflects a limited ability of early larvae to carry through de novo synthesis of PL based on dietary TAG, and is in agreement with the finding that PL appears to be essential for fish larvae in their very early stages (Cahu et al., 2009, Coutteau et al., 1997, Tocher et al., 2008, Olsen et al., 2014, Li et al., 2014). However LC-PUFA rich marine PL have only occasionally been used to transfer this EFA into young larvae tissues (Olsen et al., 2014; Li et al., 2014). A good and more profitable alternative can be the formulation of a mixture of soybean lecithin with lipid sources rich in specific essential fatty acids (EFA). Although the efficiency of enrichment protocols with emulsified diets is normally higher than that of other enrichment products (Coutteau & Sorgeloos 1997; Sorgeloos et al., 2001), their use has been related with detrimental side effects. Among them, the autoxidation of HUFA (McEvoy et al., 1995; Sargent et al., 1997) and the consequent bioaccumulation of potentially toxic lipid peroxides into larvae fed emulsion-enriched *Artemia* (Monroig 2006) have been described. Among antioxidants, vitamin E and C but also carotenoids, including astaxanthin, with high oxygen quenching abilities can inhibit LC-PUFA peroxidation (Guerin et al., 2003, Atalah et al., 2011, Betancor et al., 2012, Hamre et al., 2013). Carotenoids are widely present in fish gonads

and eggs. They are precursors of Vit. A, being involved in reproduction and embryonic development, as well as in the prevention of oxidative stress processes (Guerin et al., 2003). According to Miki (1991), astaxanthin displays both a strong quenching effect against singlet oxygen and a strong scavenging effect against free radicals, and it can also inhibit lipid per-oxidation mediated by these active forms of oxygen. Carotenoids are actively mobilized into the gonads during sexual reproductive activity in aquatic animals. Although their role in the embryonic development is not very well established, there is evidence that the presence of carotenoids mitigates deleterious oxidative damage to the developing embryos and may be also present in the gonads to ensure larval visual function and adequate chromatophore responses. Specifically, carotenoids are found to be a determining factor for good egg quality in *Seriola* (Watanabe et al., 2003).

Under this perspective, the overall objective of the present task was to determine the nutritional requirements and optimum levels of DHA, EPA, and combined PUFA-carotenoids in greater amberjack enrichment products at the time of both rotifer and *Artemia* stages, evaluating their effects on survival, growth, welfare and stress resistance/tolerance, bone development and tissue composition. Results of this study will be used to properly design well-balanced enrichment products and weaning diets for this species.

For this purpose, three different feeding trials were conducted in order to determine the optimum levels of essential fatty acids and combined PUFA-carotenoids for greater amberjack larval performance by using different lipid and antioxidant sources. The two first trials, one for DHA and another one for EPA, were conducted at *Artemia* stage, within the frame of Sub-task 9.1.1 (FCPCT), whereas the third one was performed at rotifer feeding stage, within the frame of Sub-task 9.1.2 (IEO, ULL).

2.-Optimum docosahexaenoic acid (DHA) in enrichment products for live preys for greater amberjack

Methodology

Larval performance

Greater amberjack of 17 dph, at a total density of 1000 larvae per tank (mean total length 6.39± 0.44 mm; mean fresh weight 2.94 ± 0.57 mg), were randomly distributed in 15 experimental tanks of 200 l capacity. Water exchange was gradually increased from 0.80 l min^{-1} at 19 dph to 1.6 l min^{-1} at 25 dph and finally to 3.3 l min^{-1} from 30 dph to the end of the feeding trial. Average seawater temperature and dissolved oxygen during this period were 24.15 ± 0.35°C and 6.55 ± 0.41 ppm. From 17 to 22 dph, there was an overlap between rotifers (unenriched) and *Artemia* with a gradual reduction in the amounts of rotifers from 5000–0 individual's l^{-1} and a progressive increase of enriched *Artemia* from 125–500 individuals l^{-1}. From 23 to 35 dph, greater amberjack larvae were fed exclusively with enriched *Artemia* from one of the five dietary treatments.

In order to determine the optimum dietary DHA requirements for greater amberjack larvae during *Artemia* feeding stage, five experimental emulsions, which varied in the DHA content (0-50%) were formulated. Experimental emulsions were prepared, mixing increasing amounts of high DHA content commercial methyl ester oil (DHA-70, Maruha Nichiro Foods, Tokyo, Japan) containing 70% of Total Fatty Acid (TFA) as DHA, 12% as EPA and 2% as ARA ; Oleic Acid oil (Sigma-Aldrich; Madrid, Spain) including 77% of TFA as oleic acid and soya lecithin (SL, Korot SL, Alcoy, Spain) containing mainly 54% of TFA as linoleic acid (18:2n−6, LA) and trace amounts of EPA and DHA. In addition, to prevent the oxidation of high DHA levels, experimental emulsions were fortified with 3000 mg kg^{-1} vitamin E (DL-α-tocopherol acetate, Sigma-Aldrich, Madrid, Spain) and 2500 mg kg^{-1} vitamin C (L-ascorbic acid, Asc, Sigma-Aldrich, Madrid, Spain,) according to Atalah et al. (2011), Betancor et al. (2012); and Hamre et al. (2013); (see **Table 1**). Once prepared, emulsions were stored in a fridge at 4ºC until used. Three samples of each experimental emulsion collected along the experimental test were analyzed to determine fatty acid composition (**Table 2**). For

Artemia enrichment, 1.1 ml of each experimental emulsion was mixed with 100 ml of fresh water with a stirrer for 1 minute and added to a 10-l beaker filled with seawater and provided with aeration and oxygen supply. Enrichment time (18 hours) and density (150 individuals ml^{-1}) were equal for all experimental emulsions assayed. Temperature and salinity during enrichment were 28ºC and 37g l^{-1}. All larval sampling was carried out randomly from the experimental tanks. Total length was measured with a profile projector (Mitutoyo PJ-A3000, Kanagawa, Japan) and fresh body weight of 30 larvae/tank was determined initially (17 dph) and at 35dph.

Table 1. Emulsion ingredients, proximate and fatty acid composition of the resultant enriched *Artemia* containing increasing levels of DHA.

Experimental Emulsion	*DHA-0*	*DHA-1*	*DHA-2*	*DHA-3*	*DHA-4*
Ingredients (g kg^{-1} diet)					
DHA-7[a]	0	300	450	600	900
Oleic acid	900	600	450	300	0
Soy bean lecithin	100	100	100	100	100

Welfare

Larval survival was calculated by daily counting of dead larvae from 17 dph and by counting all the remaining alive larvae at the end of the experiment. Thirty larvae per tank at 35 dph were submitted to acute stress, handling them out of the water for 30 and 60 seconds and returning them to a bucket with aerated seawater. Survival rate was determined 24 hours later, counting all the surviving larvae (Izquierdo et al., 1989). To determine the skeletal anomalies incidence, 100 larvae were collected per tank at 35 dph. Fixed larvae were stained with alizarin red and immediately photographed to evaluate skeletal anomalies occurrence (Vandewalle et al., 1998). Different regions of the axial column were identified and divided according to Boglione et al. (2001). Observations were performed on the right side of the stained samples under a stereomicroscope. The numerical data set obtained was processed to calculate incidences for each descriptor (anomaly typology) and treatment.

Fatty acid analyses

The FA contents of the experimental emulsions (DHA 0-4) are listed in **Table 2, Fig. 1.** The lowest level of DHA in experimental emulsions was present in DHA-0 with 0.5% in TFA, while the DHA contents in DHA 1 to 4 increased from 10.5% to 52.5 % in TFA. The EPA and arachidonic acid (ARA) in the experimental emulsions increased in accordance with the DHA increase, from 0.1 – 10.5 % and from 0 – 2.70% in TFA, respectively. One final sample of larvae was collected from each experimental tank at 35 dph. Besides, 5 g of each experimental emulsion of recently hatched *Artemia* and enriched *Artemia* were stored. All samples were flushed with N_2 and kept frozen at -80 °C until analysis was carried out. Total lipids were extracted (Folch et al., 1957) and fatty acids prepared by trans-etherification (Christie, 1982). Separation and identification of the fatty acids was realized with gas chromatography (GC) (GC TERMO FINNIGAN FUCUS GC, Milan, Italia) under the conditions reported in Izquierdo et al. (1992). Dry matter, ash and protein content were calculated using the methods of analysis of the Association of Official Analytical Chemists (A.O.A.C, 2012).

Table 2. Selected fatty acid contents (percentage of total fatty acids, % TFA) of total lipids in the experimental emulsions.

	DHA-0	*DHA-1*	*DHA-2*	*DHA-3*	*DHA-4*
Fatty acid content (%TFA)					
Saturated	15.58	14.09	12.67	10.25	6.31
Monoenoics	75.32	63.46	56.27	43.34	17.08
n-3	1.29	14.59	23.22	38.33	68.39
n-6	7.78	7.71	7.61	7.75	7.62
n-9	72.72	61.41	52.01	38.48	10.72
Total n-3HUFA	0.64	13.91	22.49	37.56	67.53
14:0	0.02	0.04	0.06	0.07	0.12
16:0	11.73	9.79	8.20	5.87	1.42
16:1 n-7	0.76	0.77	0.75	0.70	0.62
18:0	3.22	3.52	3.61	3.49	3.80
18:1 n-9	72.71	60.94	51.34	37.55	9.12
18:1 n-7	1.52	0.01	1.61	1.66	1.47
18:2 n-6	7.76	6.22	5.24	4.01	0.97
18:3 n-3	0.64	0.56	0.51	0.47	0.32
20:1 n-9	*n.d*	0.13	0.20	0.28	0.49
20:4n-6 (ARA)	*n.d*	0.63	1.01	1.59	2.79
20:5n-3 (EPA)	0.10	2.27	3.72	5.97	10.57
22:6n-3 (DHA)	0.54	10.73	17.30	29.19	52.55
ARA/EPA	-	0.28	0.27	0.27	0.26
DHA/EPA	5.66	4.72	4.65	4.89	4.97
DHA/ARA	-	16.99	17.17	18.40	18.83
Oleic/DHA	134.66	5.68	2.97	1.29	0.17
Oleic/n-3HUFA	113.58	4.38	2.28	1.00	0.14
n-3/n-6	0.17	1.89	3.05	4.94	8.97

HUFA, highly unsaturated fatty acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; nd, not detected.

Statistical analysis

All the data were statistically treated using a SPSS Statistical Software System 15.0 (SPSS, www.spss.com). The significant level for all the analysis was set at 5% and results are given as mean values and standard deviation. All values presented as percentage were arcsine transformed. Also, all variables were checked for normality and homogeneity of variance, using the Kolmogorov–Smirnoff and the Levene tests, respectively. To compare means, the group data were statistically tested using one-way ANOVA. When variances were not homogenate, a non-parametric Kruskal–Wallis test was accomplished. To evaluate the differences in skeletal frequency of deformities log lineal statistical analysis were performed (Sokal & Rolf, 1995).

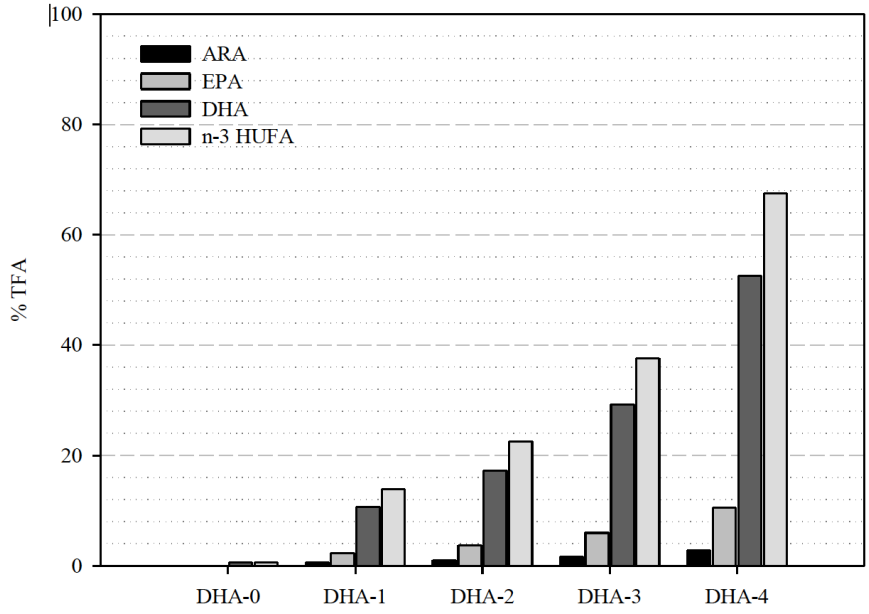


Figure 1. Fatty acids content (percentage of total fatty acids) in the experimental emulsions.

Results

Larval performance

Enrichment process with different experimental emulsions did not affect *Artemia* survival, which was about 96% on average. *Artemia* enriched with different experimental emulsions resulted in five isolipidic (*av.* 22.3%) enriched *Artemia* treatments, with no significant differences in the lipid contents among them (**Table 3**). The DHA content in *Artemia* (**Fig. 2**) was directly correlated to DHA content in the experimental emulsion ranging from 0.1 to 11%; (**Table 2**), other fatty acids such as EPA (range 0.8-6.4 % TFA) and oleic acid (range 15-33 % TFA) were also directly correlated with their emulsion content.

Larval growth was significantly affected by dietary DHA levels along the feeding trial. Mean values for all the dietary treatments were 11.98±0.51 mm in total length and 30.20±3.87 mg in fresh body weight at 35 dph. Fish final total length (TL) in group DHA-0 was similar to group DHA-1 and DHA-4, but significantly lower than those in groups DHA-2 and DHA-3 ($P \leq 0.05$), no differences were found among groups DHA-1, DHA-2, DHA-3 and DHA-4, respectively (**Fig. 3**). Fish fed DHA-2, and DHA-3 showed significantly higher final fresh weight (FW) ($P \leq 0.05$) but similar to DHA-4, whereas no difference was found between group DHA-0, DHA-1 and DHA-4 ($P > 0.05$) (**Fig. 3a and b**).

Table 3. Proximate (% dry matter) and fatty acids composition (%TFA) of *Artemia* nauplii after 18h enrichment with five experimental emulsions. (P ≤0.05)

	DHA-0	*DHA-1*	*DHA-2*	*DHA-3*	*DHA-4*
	Proximate analysis (% dry matter)				
Lipids	21.93±0.28	22.39±0.95	22.75±0.74	22.67±0.84	21.81±1.48
Moisture	90.19±0.20	90.18±0.16	90.12±0.48	90.04±0.18	89.06±0.39
Ash	10.58±0.31	10.75±0.07	10.27±0.06	10.45±0.76	10.53±0.63
	Fatty acid content (%TFA)				
Saturated	20.26^{a}	19.20^{a}	14.88^{b}	14.19^{b}	14.24^{b}
Monoenoics	43.65^{a}	41.60^{a}	36.38^{b}	31.10^{c}	25.57^{d}
n-3	26.16^{d}	29.09^{d}	38.32^{c}	44.17^{b}	49.33^{a}
n-6	7.82	7.86	8.21	8.24	8.33
n-9	34.46^{a}	31.99^{a}	27.79^{b}	22.87^{c}	17.02^{d}
Total n-3HUFA	2.74^{e}	5.60^{d}	12.09^{c}	16.95^{b}	20.59^{a}
14:0	0.49^{a}	0.45^{ab}	0.35^{bc}	0.33^{c}	0.34^{c}
16:0	10.87^{a}	10.15^{ab}	7.95^{bc}	7.62^{c}	7.55^{c}
16:1 n-7	1.70^{a}	1.73^{ab}	1.50^{bc}	1.45^{c}	1.52^{bc}
18:0	7.84^{a}	7.52^{a}	5.65^{b}	5.34^{b}	5.42^{b}
18:1 n-9	33.46^{a}	30.88^{b}	26.68^{c}	21.72^{d}	15.73^{e}
18:1 n-7	6.17^{a}	6.32^{a}	5.67^{b}	5.37^{c}	5.52^{bc}
18:2 n-6	6.53^{a}	6.10^{b}	5.83^{b}	5.39^{c}	5.16^{c}
18:3 n-3	20.62^{b}	20.92^{b}	22.84^{ab}	23.57^{ab}	24.92^{a}
20:1 n-9	0.03^{e}	0.06^{d}	0.08^{c}	0.09^{b}	0.11^{a}
20:4n-6 (ARA)	0.33^{d}	0.60^{c}	0.90^{b}	1.10^{a}	1.23^{a}
20:5n-3 (EPA)	0.87^{d}	2.19^{c}	4.33^{b}	5.52^{ab}	6.41^{a}
22:6n-3 (DHA)	0.13^{e}	1.43^{d}	5.15^{c}	8.47^{b}	10.92^{a}
ARA/EPA	0.40^{a}	0.29^{b}	0.21^{c}	0.20^{c}	0.19^{c}
DHA/EPA	0.15^{d}	0.67^{c}	1.19^{b}	1.54^{a}	1.71^{a}
DHA/ARA	0.39^{d}	2.36^{c}	5.75^{b}	7.75^{a}	8.86^{a}
Oleic/DHA	279.96^{a}	23.23^{b}	5.33^{c}	2.57^{d}	1.45^{e}
Oleic/n-3HUFA	12.87^{a}	5.86^{b}	2.22^{c}	1.28^{d}	0.77^{e}
n-3/n-6	3.33^{c}	3.69^{c}	4.67^{b}	5.36^{b}	5.92^{a}

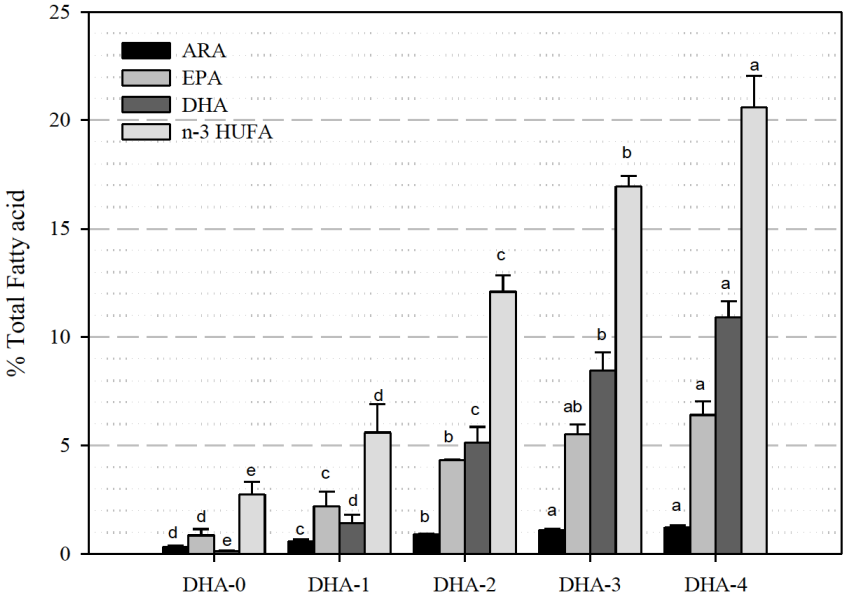


Figure 2. Fatty acids content (percentage of total fatty acids) in the enriched *Artemia* experimental emulsions.

The relationship between final total length and *Artemia* DHA content was described by the second-order polynomial regression: $y = 11.244 + 0.428x - 0.034\ x^2$ *(y= Total length; x= dietary DHA (% TFA);* $R^2=0.993$; $P \leq 0.05$). Also, final fresh weight was described by the equation: $y = 24.495 + 2.900x - 0.2141\ x^2$ *(y= Fresh weight; x= dietary DHA (% TFA);* $R^2=0.956$; $P \leq 0.05$). Under the experimental conditions applied, both growth models suggest that maximum growth was achieved in the range of dietary DHA concentrations tested, between 5-10 %TFA with a maximum around 6.5-7% DHA content in *Artemia*.

Welfare

Larval survival was significantly ($P \leq 0.05$) affected by dietary DHA at 35 dph, the lowest survival was recorded in those larvae receiving the lowest DHA in the *Artemia* (DHA-0) (**Fig. 4a**). On the contrary, larval resistance to stress test, determined as the survival rate 24 hours after handling, was not significantly affected by dietary DHA, although the poorest survival was recorded in those larvae fed the lowest DHA in the diet (DHA-0) (**Fig. 4b**).

The incidence of total acute skeletal deformities measured as sum of lordosis, kyphosis, scoliosis, vertebral anomalies and cranial anomalies were relatively low for all the dietary DHA levels assayed. This parameter was not significantly affected by the dietary DHA with an average value for all the dietary treatments of 5.01±1.09% (**Fig. 5a**). Regardless of the DHA content, most of the acute skeletal deformities were major alterations in the pre-haemal region, related mainly with lordosis apparition, while in the haemal region, the most important affection was recorded as vertebral malformation, particularly identified as deformation, ossification ridges, marked reduction in length or elongation of the vertebral bodies or intervertebral bony plates (**Fig. 5b**). The relationship between total acute deformities, sum of column deformities and *Artemia* DHA content was described by the second-order polynomial regression: $y = y_0 + ax + bx^2$ *(y= Total acute deformities or sum of column deformities; x= dietary DHA (% TFA);* with a determination coefficient (R^2), ranging from 0.65-0.75 and $P > 0.05$ (**Fig. 5a and b**).

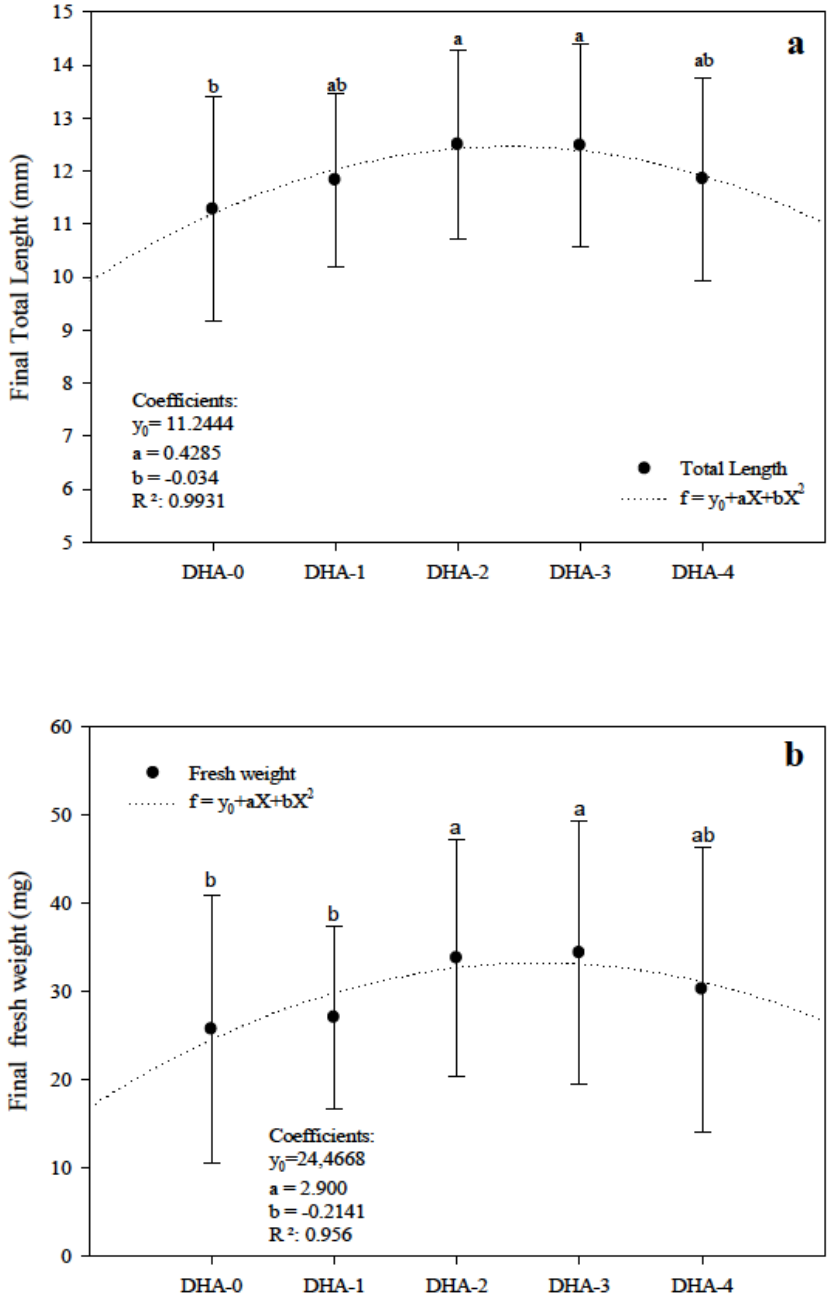


Figure 3. Relationship between (a) total length (mm) and (b) fresh weight (mg) and dietary *Artemia* DHA (22:6n-3) content in greater amberjack larvae 35 days posthatch (mean ± S.D., n=3). Data are fitted to a quadratic regression analysis ($f=y_0+ax+bx^2$).

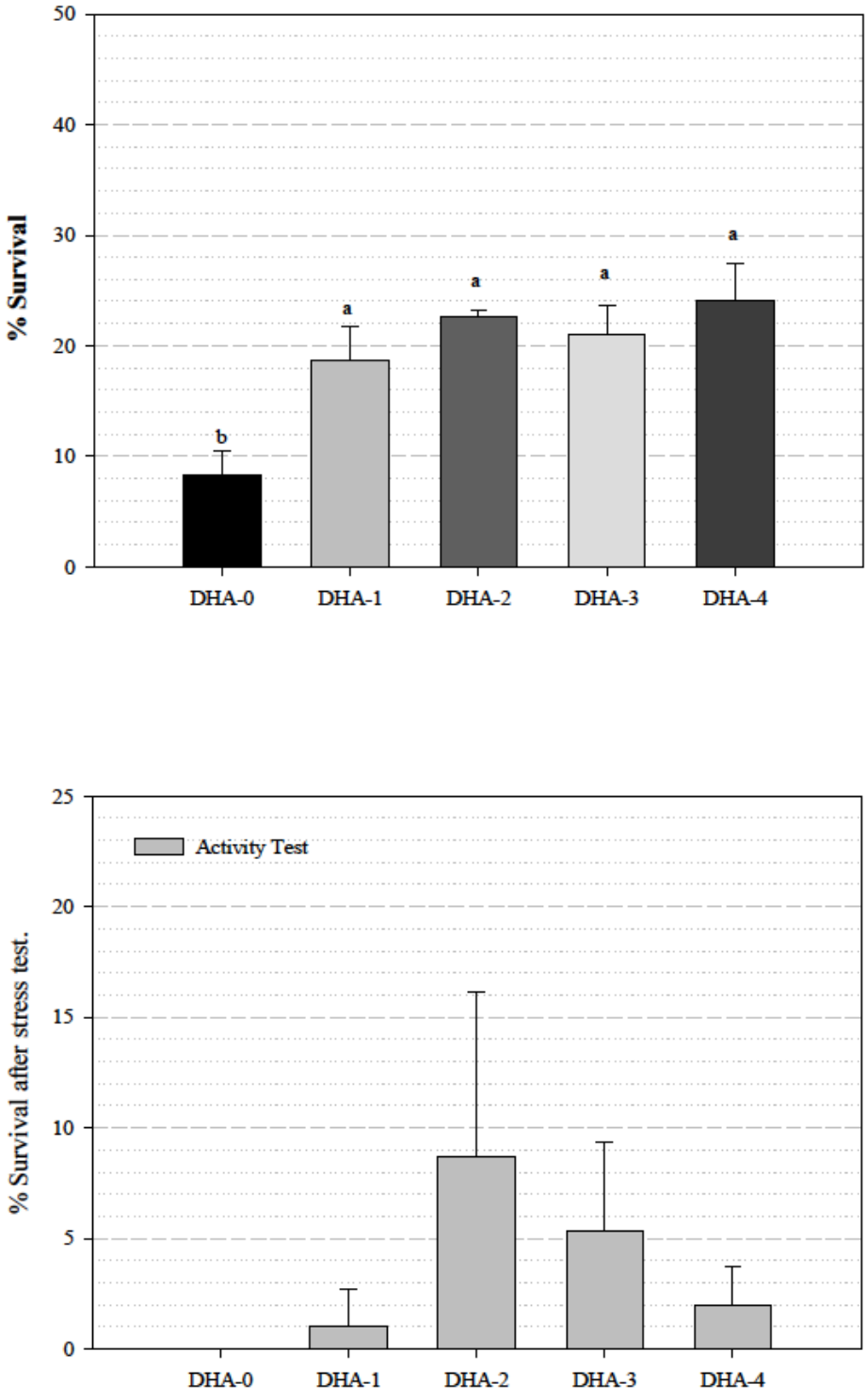
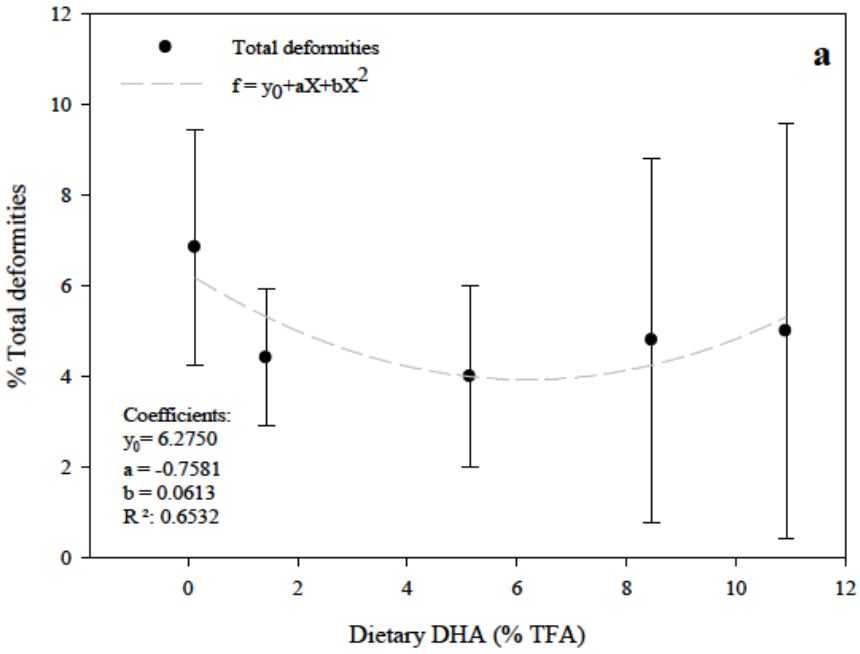
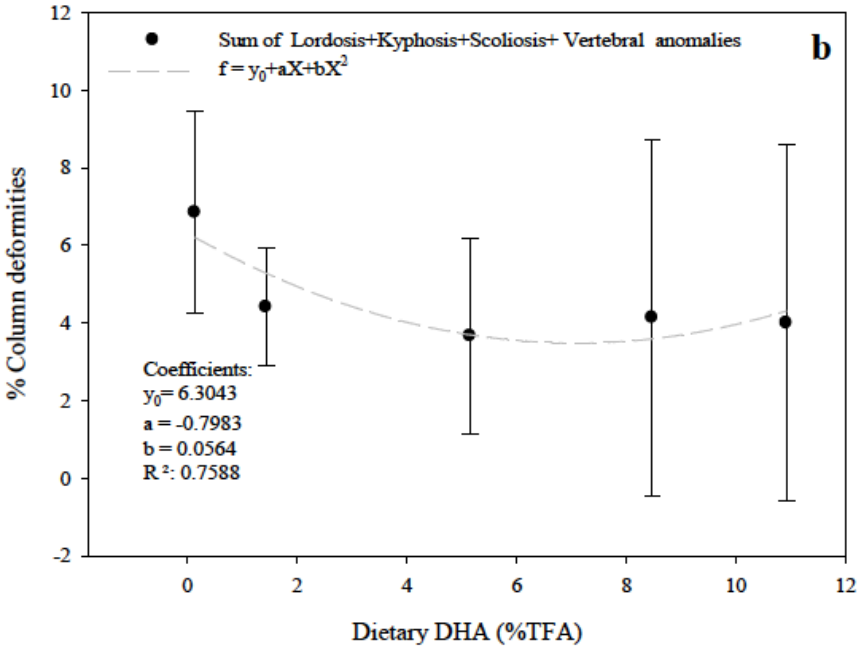


Figure 4. (a) Final survival and (b) survival after stress test (air exposure 30 seconds) in 35 dph greater amberjack larvae, fed different dietary *Artemia* DHA (22:6n-3) content. Values are mean + S.D., n=3.


Total deformities
f = y0+aX+bX2
a
% Total deformities
Coefficients:
y0= 6.2750
a = -0.7581
b = 0.0613
R ²: 0.6532
Dietary DHA (% TFA)


Sum of Lordosis+Kyphosis+Scoliosis+ Vertebral anomalies
f = y0+aX+bX2
b
% Column deformities
Coefficients:
y0= 6.3043
a = -0.7983
b = 0.0564
R ²: 0.7588
Dietary DHA (%TFA)

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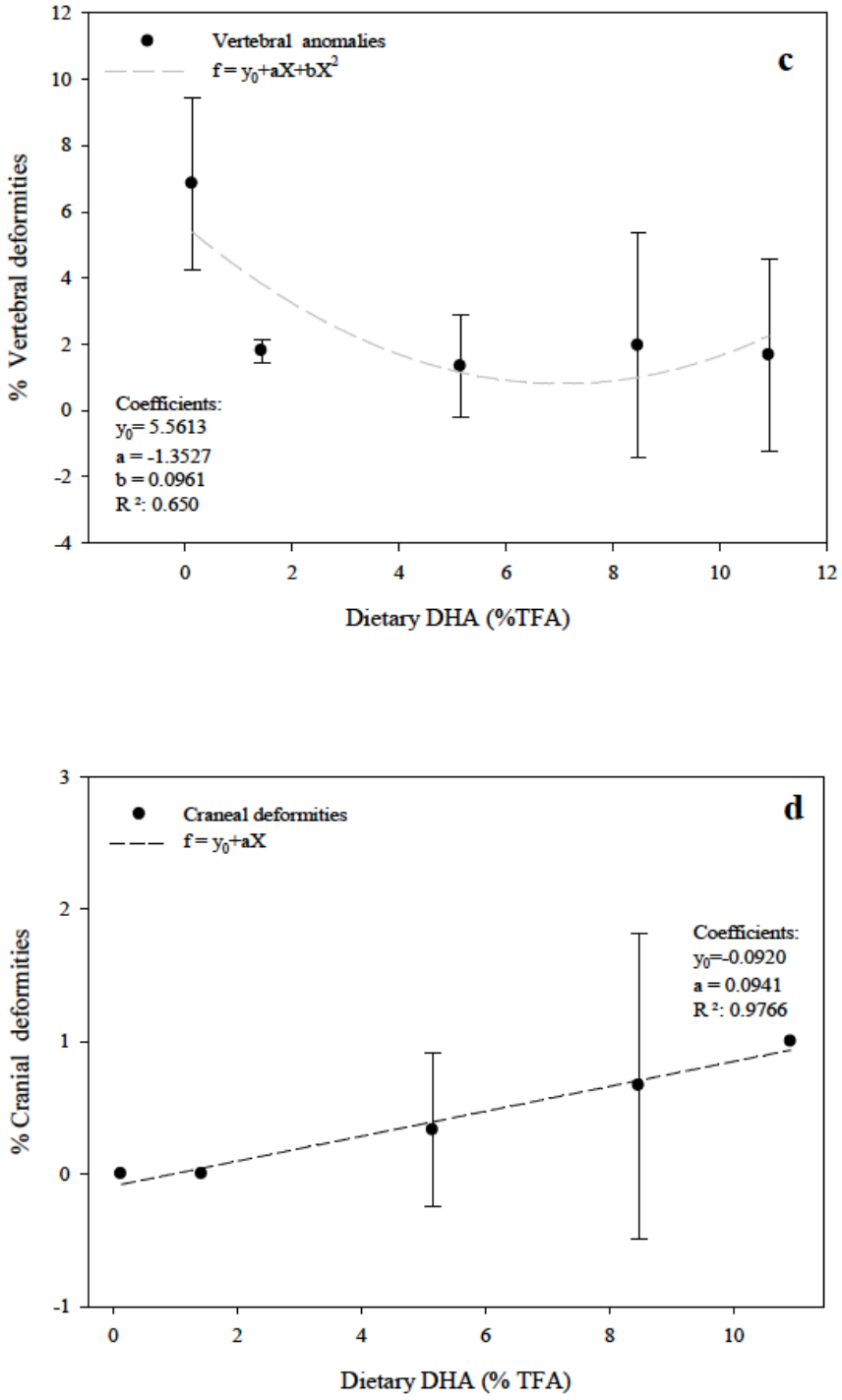

Figure 5. Relationship between (a) total acute deformities (%), (b) sum of column deformities, (c) vertebral malformation (%), (d) cranial malformation (%) to dietary *Artemia* DHA (22:6n-3) content (%TFA) in larval greater amberjack at 35 days post-hatch (mean + S.D., n=3). Data are fitted to a quadratic regression analysis ($f=y_0+ax+bx^2$) for a, b and c and to a linear regression analysis ($f=y_0+ax$) for d.

Similarly, the relationship between vertebral malformation and *Artemia* DHA content was described by the same model stated above (**Fig. 5c**), suggesting a higher level of vertebral malformations when low dietary DHA was used. On the contrary, a significant relationship between cranial deformities and *Artemia* DHA content was described by the linear regression: *y = 0.092 +0.0941x (y= %Cranial malformation; x= dietary DHA (% TFA);* R^2*=0.9766;* $P \leq 0.05$). These results suggest that higher levels of DHA tended to intensify the occurrence of this type of deformity during the live stage of development evaluated (Fig. 5d). Under the experimental conditions assayed and the regression models applied, the best quality in terms of lower skeletal deformities incidence were achieved between 4-6%TFA with a maximum around 5% DHA content in *Artemia*, which matched with the data obtained to reach best growth.

Fatty acid analysis

Results of total lipids and fatty acids analysis of greater amberjack larvae at 35 dph are shown in **Table 4.** Total lipids, ash content and moisture were equal for the larvae fed the different DHA *Artemia* treatments. Regarding FA composition, saturated fatty acids were similar for all groups. Monounsaturated fatty acids were positively correlated to 18:1 n−9, in the diet, since this FA represented 70% of total monoenes on average. Total n−3 fatty acids, significantly increased with the increase in DHA in the *Artemia*. Similarly, the DHA level in larvae fed Diet 0–4 was significantly increased by the increase in dietary DHA. ARA and EPA contents in larval tissues were also affected by dietary treatments, with the lowest ($P \leq 0.05$) values in DHA-0 fish, which was significantly lower than those in fish fed DHA-1, DHA-2, DHA-3 and DHA-4 *Artemia*.

Table 4. Proximate (lipid, moisture and ash content, % dry matter) and fatty acids composition (%TFA) of greater amberjack larvae 35 dah fed enriched *Artemia* with different experimental emulsions. Proximate and FA analysis data represent means ± SD (n=3). Different superscripts within each row indicate a significant difference between DHA emulsions (ANOVA (P ≤0.05); Tukey's HSD).

	DHA-0	*DHA-1*	*DHA-2*	*DHA-3*	*DHA-4*
Proximate analysis (% dry matter)					
Lipids	13.12±0.27	14.32±2.05	14.76±1.61	14.82±1.66	15.33±1.41
Moisture	85.58±3.66	86.95±2.60	88.98±0.50	88.11±2.71	88.94±0.89
Ash	16.07±0.97	16.80±0.65	17.54±1.95	17.39±0.38	18.00±0.54
Fatty acid content (%TFA)					
Saturated	25.39±1.27^{a}	26.92±0.96	26.45±0.40	27.02±0.94	27.04±0.70
Monoenoics	33.36±1.54^{a}	29.67±0.58^{ab}	27.72±0.45^{bc}	25.64±0.64^{c}	20.99±2.58^{d}
n-3	26.04±0.37^{e}	27.87±0.34^{d}	32.18±0.29^{c}	33.69±0.52^{b}	38.15±0.86^{a}
n-6	11.65±0.39^{a}	11.97±0.53^{a}	10.53±0.10^{b}	10.42±0.47^{b}	9.89±0.11^{b}
n-9	25.12±1.22^{a}	22.66±0.88^{b}	20.00±0.36^{c}	17.89±0.55^{c}	15.33±1.17^{d}
Total n-3HUFA	10.99±1.02^{d}	15.92±0.60^{c}	21.19±0.49^{b}	22.57±0.28^{b}	26.32±0.86^{a}
14:0	0.40±0.03^{a}	0.36±0.04^{ab}	0.32±0.02^{b}	0.33±0.02^{ab}	0.35±0.03^{ab}
16:0	13.45±0.82	13.74±0.78	13.78±0.23	14.21±0.54	14.13±0.45
16:1 n-7	1.17±0.09^{a}	0.83±0.05^{b}	0.84±0.03^{b}	0.84±0.07^{b}	0.95±0.06^{b}
18:0	10.25±0.55^{b}	11.50±0.34^{a}	11.17±0.24^{ab}	11.32±0.45^{a}	11.44±0.34^{a}
18:1 n-9	23.65±1.53^{a}	20.89±0.76^{b}	19.01±0.34^{bc}	16.88±0.54^{c}	13.90±0.59^{d}
18:1 n-7	6.27±0.05	5.70±0.34	5.76±0.05	5.79±0.02	3.82±3.29
18:2 n-6	7.22±0.06^{a}	6.28±0.22^{b}	5.14±0.07^{c}	4.66±0.08^{d}	4.48±0.23^{d}
18:3 n-3	13.09±1.34^{a}	10.40±0.30^{b}	9.47±0.27^{b}	9.57±0.67^{b}	10.63±0.73^{b}
20:1 n-9	0.23±0.35^{ab}	0.68±0.06^{a}	0.04±0.00^{b}	0.05±0.00^{b}	0.27±0.39^{ab}
20:4n-6 (ARA)	2.66±0.42^{b}	3.53±0.21^{a}	3.32±0.09^{a}	3.40±0.16^{a}	3.36±0.08^{a}
20:5n-3 (EPA)	3.78±0.30^{b}	4.72±0.38^{a}	5.16±0.11^{a}	4.99±0.17^{a}	5.30±0.25^{a}
22:6n-3 (DHA)	3.54±0.53^{e}	7.58±0.03^{d}	12.60±0.42^{c}	14.53±0.16^{b}	17.81±0.50^{a}
ARA/EPA	0.70±0.06	0.75±0.10	0.64±0.01	0.68±0.04	0.63±0.02
DHA/EPA	0.93±0.07^{e}	1.61±0.13^{d}	2.44±0.11^{c}	2.91±0.10^{b}	3.36±0.07^{a}
DHA/ARA	1.33±0.02^{e}	2.15±0.13^{d}	3.80±0.11^{c}	4.28±0.16^{b}	5.30±0.13^{a}
Oleic/DHA	6.84±1.58^{a}	2.76±0.11^{b}	1.51±0.08^{bc}	1.16±0.05^{bc}	0.78±0.01^{c}
Oleic/n-3HUFA	2.17±0.36^{a}	1.31±0.10^{b}	0.90±0.04^{bc}	0.75±0.03^{c}	0.53±0.01^{c}
n-3/n-6	2.24±0.10^{c}	2.33±0.13^{c}	3.06±0.01^{b}	3.24±0.17^{b}	3.86±0.05^{a}

HUFA, highly unsaturated fatty acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

3.-Optimum eicosapentaenoic acid (EPA) in enrichment products for live preys for *Seriola dumerilii*

Methodology

Larval performance

At 17 dph, a total of 1000 larvae per tank (mean total length 6.39± 0.44 mm; mean fresh weight 2.94 ± 0.57 mg) were randomly distributed in 15 experimental tanks of 200 l capacity. Water exchange was gradually increased from 0.80 l min^{-1} at 19 dph to 1.6 l min^{-1} at 25 dph and finally to 3.3 l min^{-1} from 30 dph to the end of the feeding trial. Average seawater temperature and dissolved oxygen during this period were 24.15 ± 0.35°C and 6.55 ± 0.41 ppm. From 17 to 22 dph, there was an overlap between rotifers (unenriched) and *Artemia* with a gradual reduction in the amounts of rotifers from 5000–0 individual's l^{-1} and a progressive increase on enriched *Artemia* from 125–500 individuals l^{-1}. From 23 to 35 dph, greater amberjack larvae were fed exclusively with enriched *Artemia* from one of the five dietary treatments.

In order to determine the optimum dietary EPA requirement for greater amberjack larvae during *Artemia* feeding stage, five experimental emulsions, which varied in the EPA content (0-60%) were formulated. Experimental emulsions were prepared, mixing increasing amounts of high EPA content commercial triglycerides oil (Incromega EPA 500 TG, Croda, Barcelona, Spain) containing 63% of Total Fatty Acid (TFA) as EPA, 8% as DHA and 3% as ARA ; Oleic Acid oil (Sigma-Aldrich; Madrid, Spain) including 77% of TFA as oleic acid and soya lecithin (SL, Korot SL, Alcoy, Spain) containing mainly 54% of TFA as linoleic acid (18:2n−6, LA) and trace amounts of EPA and DHA. In addition, to prevent the oxidation of high DHA levels, experimental emulsions were fortified with 3000 mg kg^{-1} vitamin E (DL-α-tocopherol acetate, Sigma-Aldrich, Madrid, Spain) and 2500 mg kg^{-1} vitamin C (L-ascorbic acid, Asc, Sigma-Aldrich, Madrid, Spain,) according to Atalah et al. (2011), Betancor et al. (2012a) and Hamre et al. (2013) (see **Table 5).** Once prepared, emulsions were stored in a fridge at 4º C until used. Three samples of each experimental emulsion collected along the experimental test were analyzed to determine fatty acid composition (**Table 6**). For *Artemia* enrichment, 1.1 ml of each experimental emulsion was mixed with 100 ml of fresh water with a stirrer for 1 minute and added to a 10-l beaker filled with seawater and provided with aeration and oxygen supply. Enrichment time (18 hours) and density (150 indv ml^{-1}) were equal for all the experimental emulsions assayed. Temperature and salinity during enrichment were 28ºC and 37g l^{-1}.

All larval sampling was carried out randomly from the experimental tanks. Total length was measured with a profile projector (Mitutoyo PJ-A3000, Kanagawa, Japan) and fresh body weight of 30 larvae/tank was determined initially (17 dph) and at 35dph.

Table 5. Emulsion ingredients, proximate and fatty acid composition of the resultant enriched *Artemia* containing increasing levels of EPA

Experimental Emulsion	*EPA-0*	*EPA-1*	*EPA-2*	*EPA-3*	*EPA-4*
			Ingredients (g kg^{-1} diet)		
EPA 500TG[a]	0	300	450	600	900
Oleic acid	900	600	450	300	0
Soy bean lecithin	100	100	100	100	100

Table 6. Selected fatty acid contents (percentage of total fatty acids, % TFA) of total lipids in the experimental emulsions.

	EPA-0	*EPA-1*	*EPA-2*	*EPA-3*	*EPA-4*
			Fatty acid content (%TFA)		
Saturated	15.21	12.66	11.46	9.27	2.61
Monoenoics	74.63	58.63	50.00	36.95	7.60
n-3	2.16	20.48	30.11	45.09	79.52
n-6	7.93	7.43	7.16	6.95	7.65
n-9	72.17	56.07	47.47	34.57	5.56
Total n-3HUFA	1.37	17.35	25.60	38.74	70.62
14:00	0.02	0.12	0.20	0.27	0.35
16:00	11.45	9.21	7.99	6.01	0.95
16:1 n-7	0.76	0.81	0.88	0.89	0.87
18:00	3.14	2.54	2.25	1.72	0.41
18:1 n-9	72.13	55.84	47.13	34.06	3.94
18:1 n-7	1.40	1.46	1.33	1.16	0.94
18:2 n-6	7.80	6.14	5.16	4.05	1.96
18:3 n-3	0.67	0.65	0.64	0.66	1.18
20:1 n-9	0.02	0.01	0.01	0.02	0.41
20:4n-6 (ARA)	0.06	0.82	1.25	1.83	3.53
20:5n-3 (EPA)	0.84	14.28	21.18	31.97	60.16
22:6n-3 (DHA)	0.30	2.36	3.34	5.11	6.79
ARA/EPA	0.07	0.06	0.06	0.06	0.06
DHA/EPA	0.36	0.17	0.16	0.16	0.11
DHA/ARA	5.41	2.89	2.68	2.80	1.92
Oleic/DHA	240.20	23.67	14.13	6.66	0.58
Oleic/n-3HUFA	52.71	3.22	1.84	0.88	0.06
n-3/n-6	0.27	2.76	4.21	6.49	10.40

Welfare

Larval survival was calculated by daily counting of dead larvae from 17 dph and by counting all the remaining alive larvae at the end of the experiment. Thirty larvae per tank at 35 dph were submitted to acute stress, handling them out of the water for 30 and 60 seconds and returning them to a bucket with aerated seawater. Survival rate was determined 24 hours later, counting all the surviving larvae (Izquierdo et al., 1989). To determine the skeletal anomalies incidence, 100 larvae were collected per tank at 35 dph. Fixed larvae were stained with alizarin red and immediately photographed to evaluate skeletal anomalies occurrence (Vandewalle et al., 1998). Different regions of the axial column were identified and divided according to Boglione et al. (2001). Observations were performed on the right side of the stained samples under a stereomicroscope. The numerical data set obtained was processed to calculate incidences for each descriptor (anomaly typology) and treatment.

Fatty acid analysis

One final sample of larvae was collected from each experimental tank at 35 dph. Besides, 5 g of each experimental emulsion of recently hatched *Artemia* and enriched *Artemia* were stored. All samples were flushed with N_2 and kept frozen at -80 °C until analysis was carried out. Total lipids were extracted (Folch et al., 1957) and fatty acids prepared by trans-etherification (Christie, 1989). Separation and identification of the fatty acids was realized with gas chromatography (GC) (GC TERMO FINNIGAN FUCUS GC, Milan, Italia) under the conditions reported in Izquierdo et al (1992). Dry matter, ash and protein content were calculated using the methods of analysis of the Association of Official Analytical Chemists (A.O.A.C, 2012).

Statistical analysis

All the data were statistically treated using a SPSS Statistical Software System 15.0 (SPSS, www.spss.com). The significant level for all the analysis was set at 5% and results are given as mean values and standard deviation. All values presented as percentage were arcsine transformed. Also, all variables were checked for normality and homogeneity of variance, using the Kolmogorov–Smirnoff and the Levene tests, respectively. To compare means, the group data were statistically tested using one-way ANOVA. When variances were not homogenate, a non-parametric Kruskal–Wallis test was accomplished.

Results

Larval performance

Artemia enriched with different experimental emulsions (**Fig. 6**) resulted in five enriched *Artemia* treatments (**Table 7, Fig. 7**). The EPA content in *Artemia* was directly correlated to EPA content in the experimental emulsion ranging from 1.08 to 22.9 %; (**Table 7**), other fatty acids such as DHA (range 0.14-3.01 % TFA), ARA (range 0.39-1.72 % TFA) and total n-3 HUFA (range 3.24-29.07 % TFA) were also directly correlated with their emulsion content.

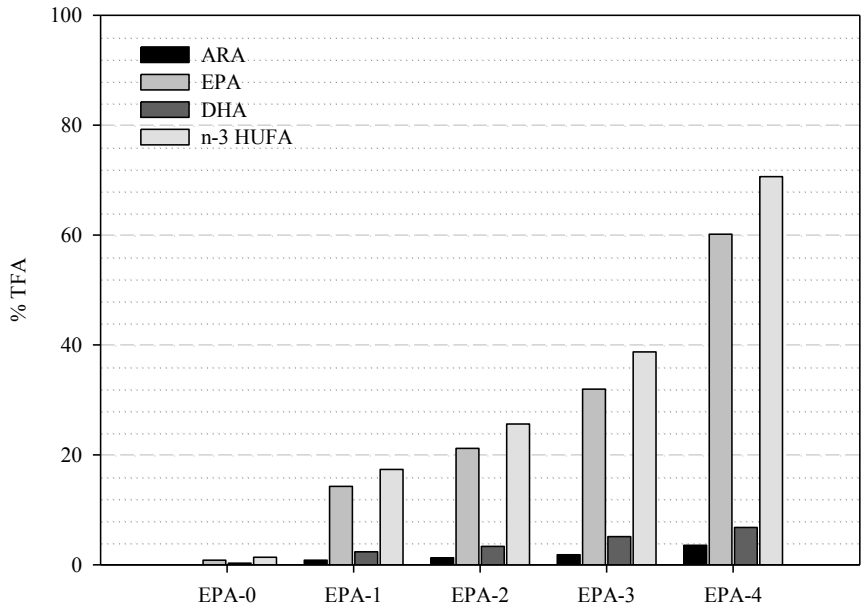

Figure 6. Fatty acids content (percentage of total fatty acids) in the experimental emulsions.

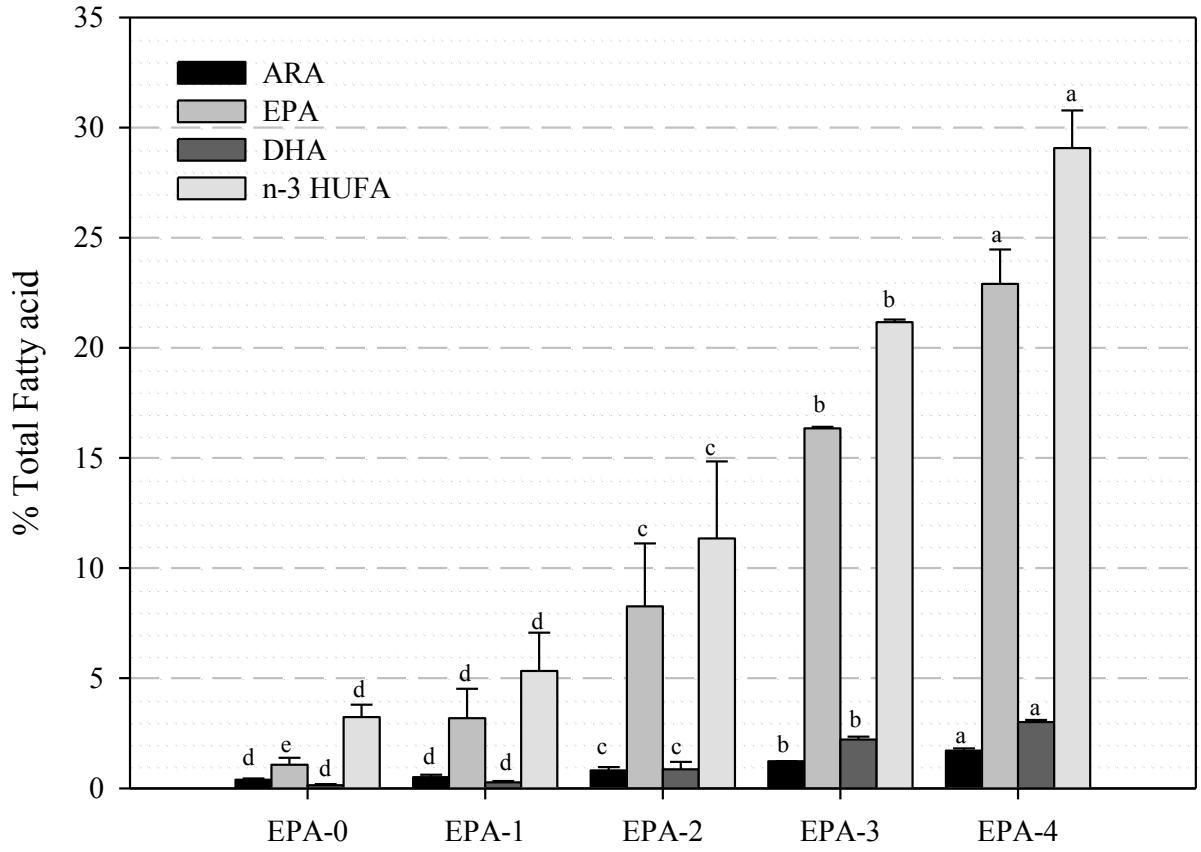

Figure 7. Increase in fatty acids composition (EPA, DHA, ARA and total n-3 HUFA; in %TFA) of *A. salina* nauplii after 18h enrichment with five experimental emulsions. Data represent means and error bars are standard deviation (n=3). Treatments means for each FA, with different superscripts indicate significant differences (ANOVA (P ≤0.05); Tukey's HSD).

Larval growth was significantly affected by the dietary EPA levels along the feeding trial (**Fig. 8**). Mean values for all the dietary treatments were 11.72±0.47 mm in total length and 27.13±3.21 mg in fresh body weight at 35 dph. Fish final total length (TL) in group EPA-0 was similar to group EPA-1 and EPA-4, but significantly lower than those in groups EPA-2 and EPA-3 (P ≤ 0.05), no differences were found among groups EPA-1, EPA-2, EPA-3 and EPA-4, respectively. Fish fed EPA-3 showed significantly higher final fresh weight (FW) (P≤ 0.05) than EPA-0 and EPA-1 but similar to EPA-2 and EPA-4 (P> 0.05), (**Fig. 8a and b**).

The relationship between final total length and *Artemia* EPA content was described by the second-order polynomial regression: $y = 10.893 + 0.214x - 0.080\ x^2$ *(y= Total length; x= dietary EPA (% TFA);* R^2*=0.965;* P ≤ 0.05). Also, final fresh weight was described by the equation: $y = 21.503 + 1.460x - 0.0544\ x^2$ *(y= Fresh weight; x= dietary EPA (% TFA);* R^2*=0.989;* P ≤ 0.05). Under the experimental conditions applied, both growth models suggest that maximum growth was achieved in the range of dietary EPA concentrations tested, between 8-16 %TFA with a maximum around 13-14% DHA content in *Artemia,* when low dietary DHA were provided (0.8-2.2% TFA).

Table 7. Proximate (% dry matter) and fatty acids composition (%TFA) of *A. salina* nauplii after 18h enrichment with five experimental emulsions. Proximate analysis data represent means ± SD, (n=3), FA content data are presented as mean. Different superscripts within each row indicate a significant difference between DHA emulsions (ANOVA (P ≤0.05); Tukey's HSD).

	EPA-0	*EPA-1*	*EPA-2*	*EPA-3*	*EPA-4*
			Proximate analysis (% dry matter)		
Lipids	19.72±1.04	19.45±1.97	24.29±3.16	25.36±1.06	26.31±0.68
Moisture	90.33±0.21	90.39±0.21	90.05±0.35	90.00±0.30	89.05±0.12
Ash	10.79±0.61	10.16±0.43	10.09±0.69	8.61±1.49	9.76±048
			Fatty acid content (%TFA)		
Saturated	19.07^{a}	19.93^{a}	16.18^{ab}	12.56^{bc}	10.21^{c}
Monoenoics	41.31^{a}	40.38^{a}	37.26^{a}	30.28^{b}	22.44^{c}
n-3	29.51^{b}	29.64^{b}	36.16^{b}	46.74^{a}	55.40^{a}
n-6	7.76^{b}	7.56^{b}	8.00^{b}	7.92^{b}	8.87^{a}
n-9	31.80^{a}	30.08^{a}	28.51^{a}	23.14^{b}	15.06^{c}
Total n-3HUFA	3.24^{d}	5.33^{d}	11.34^{c}	21.16^{b}	29.07^{a}
14:00	0.46^{ab}	0.57^{a}	0.40^{b}	0.32^{b}	0.34^{b}
16:00	10.66^{a}	11.11^{a}	8.83^{ab}	6.84^{b}	6.90^{b}
16:1 n-7	1.75^{ab}	1.93^{a}	1.63^{ab}	1.40^{b}	1.50^{b}
18:00	6.86^{a}	7.15^{a}	6.00^{a}	4.66^{ab}	2.23^{b}
18:1 n-9	30.70^{a}	29.00^{a}	27.44^{a}	22.07^{b}	13.77^{c}
18:1 n-7	6.39^{ab}	6.83^{a}	5.92^{b}	4.83^{c}	4.97^{c}
18:2 n-6	6.30^{a}	6.06^{a}	6.10^{a}	5.53^{b}	5.70^{b}
18:3 n-3	23.03	21.61	21.66	21.56	21.80
20:1 n-9	0.03^{a}	0.03^{a}	0.03^{a}	0.03^{a}	0.09^{b}
20:4n-6 (ARA)	0.39^{d}	0.51^{d}	0.81^{c}	1.23^{b}	1.72^{a}
20:5n-3 (EPA)	1.08^{e}	3.18^{d}	8.26^{c}	16.34^{b}	22.91^{a}
22:6n-3 (DHA)	0.14^{d}	0.27^{d}	0.87^{c}	2.22^{b}	3.01^{a}
ARA/EPA	0.38^{a}	0.17^{b}	0.11^{bc}	0.07^{c}	0.07^{c}
DHA/EPA	0.13^{ab}	0.09^{c}	0.10^{bc}	0.14^{a}	0.13^{a}
DHA/ARA	0.35^{c}	0.53^{c}	1.02^{b}	1.81^{a}	1.76^{a}
Oleic/DHA	253.82^{a}	115.64^{b}	38.41^{b}	9.98^{b}	4.58^{b}
Oleic/n-3HUFA	9.87^{a}	6.22^{ab}	2.74^{bc}	1.04^{c}	0.47^{c}
n-3/n-6	3.79^{b}	3.90^{b}	4.52^{b}	5.90^{a}	6.24^{a}

Welfare

Larval survival was significantly (P≤ 0.05) affected by dietary EPA at 35 dph, the lowest survival was recorded in those larvae receiving the lowest EPA in the *Artemia* (EPA-0) (**Fig. 9a**). Hence, larval resistance to stress test, determined as the survival rate 24 hours after handling, was significantly affected by dietary EPA, the lower survival was recorded in those larvae fed the lowest EPA in the diet (EPA-0) (**Fig. 9b**).

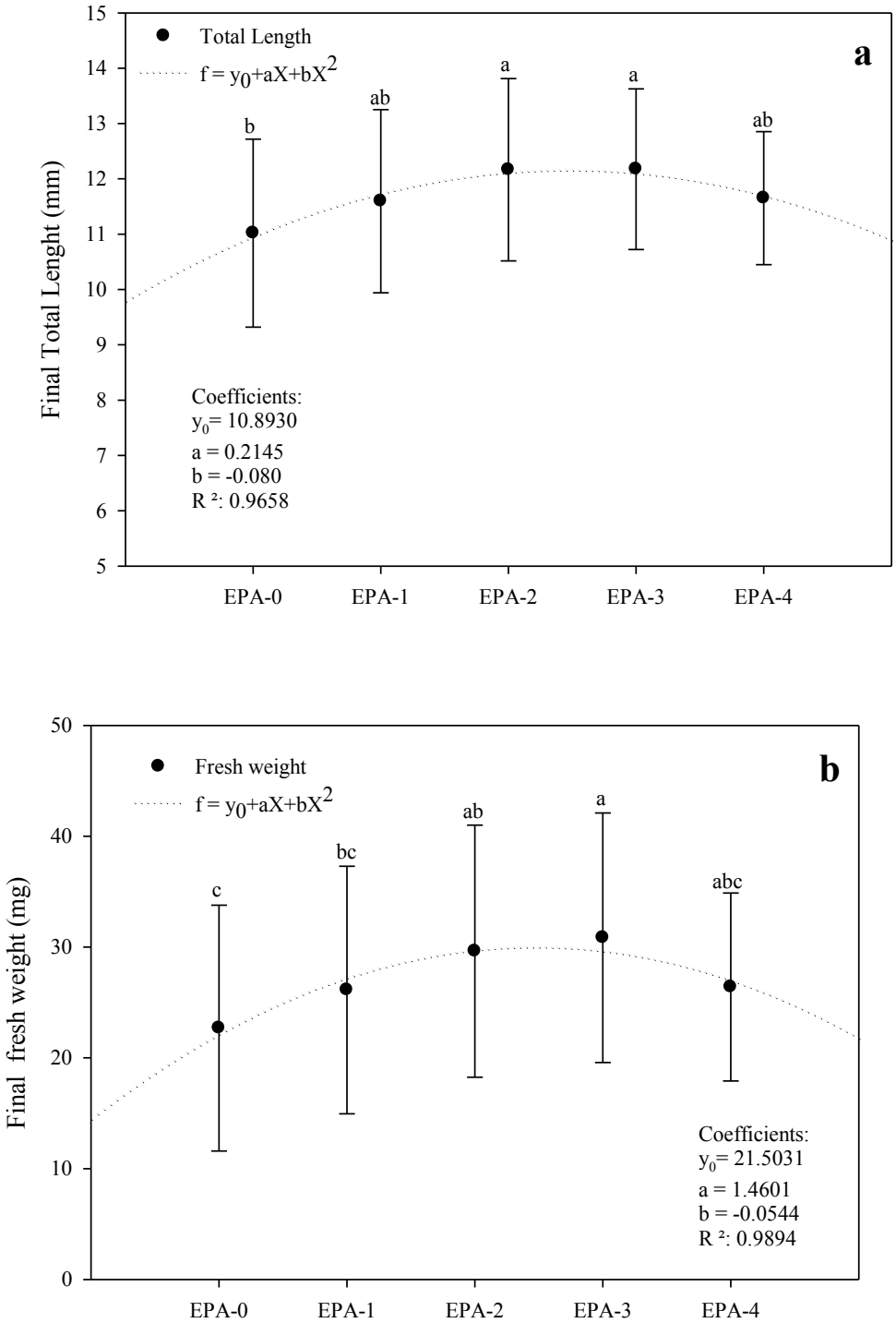

Figure 8. Relationship between (a) total length (mm) and (b) fresh weight (mg) to dietary *Artemia* EPA (20:5n-3) content in larval greater amberjack at 35 days posthatch (mean + S.D., n=3). Data are fitted to a quadratic regression analysis ($f=y_0+ax+bx^2$).

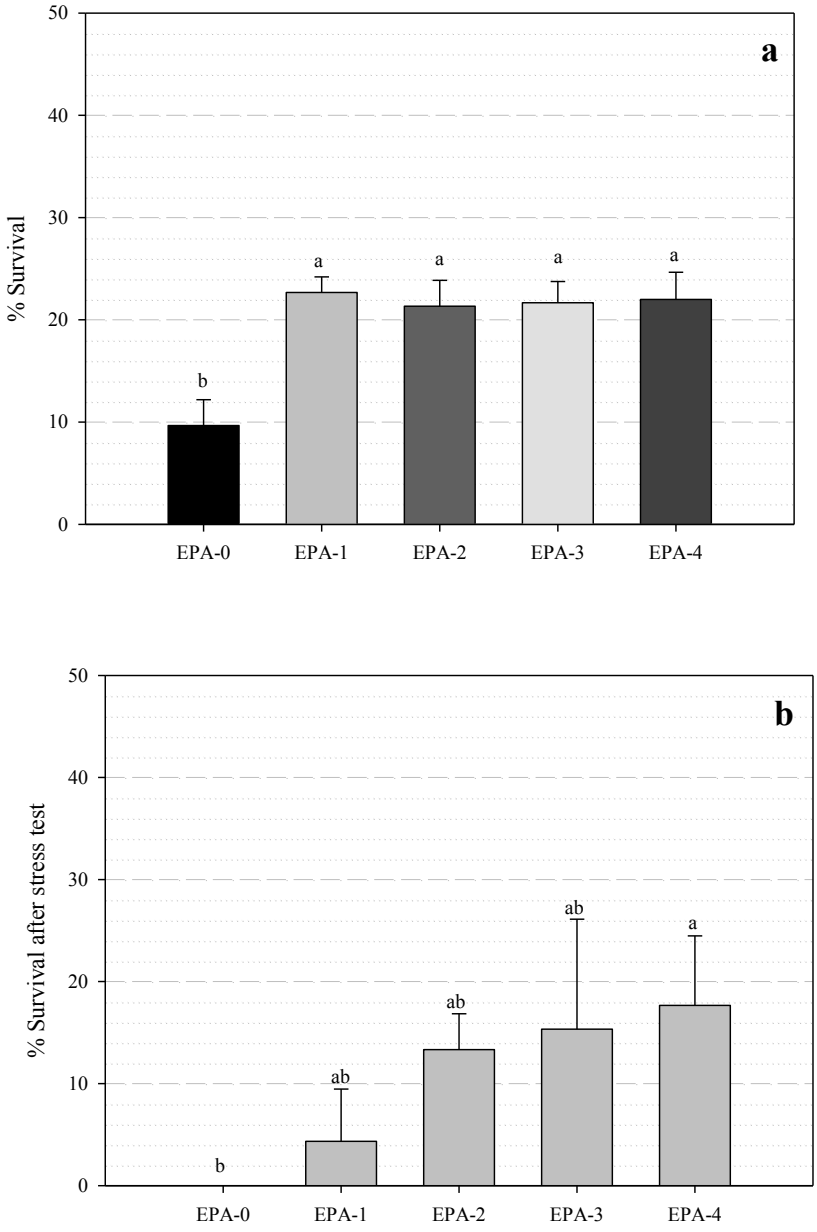

Figure 9. (a) Final survival and (b) survival after stress test in 35 dph greater amberjack larvae, fed different dietary *Artemia* EPA (20:5n-3) content. Values are mean + S.D., n=3.

The incidence of total acute skeletal deformities was not significantly affected by the dietary EPA, with an average value for all the dietary treatments of 22.63±6.25% (**Fig. 10a**). Regardless of the EPA content, most of the acute skeletal deformities were major alterations in the haemal region, related with column deformities (**Fig. 10b)**, the most important affection was recorded as scoliosis**.** The relationship between total acute deformities, and *Artemia* EPA content was described by the lineal regression: $y = y_0 + ax$ *(y= Total acute deformities; x= dietary EPA (% TFA);* with a determination coefficient (R^2) of 0.85 and P> 0.05 (Fig. 5a). Also, the relationship between the sum of column deformities and *Artemia* EPA content was described by

the same model stated above represented as: *y = 29.109 -0.645x (y= % column deformities; x= dietary EPA (% TFA);* R^2*=0.860;* P ≤ 0.05) (**Fig 10b**), suggesting a higher level of vertebral malformations when low dietary EPA was used. These results suggest that higher levels of EPA tended to intensify the apparition of this type of deformity during the live stage of development evaluated (**Fig. 10b**).

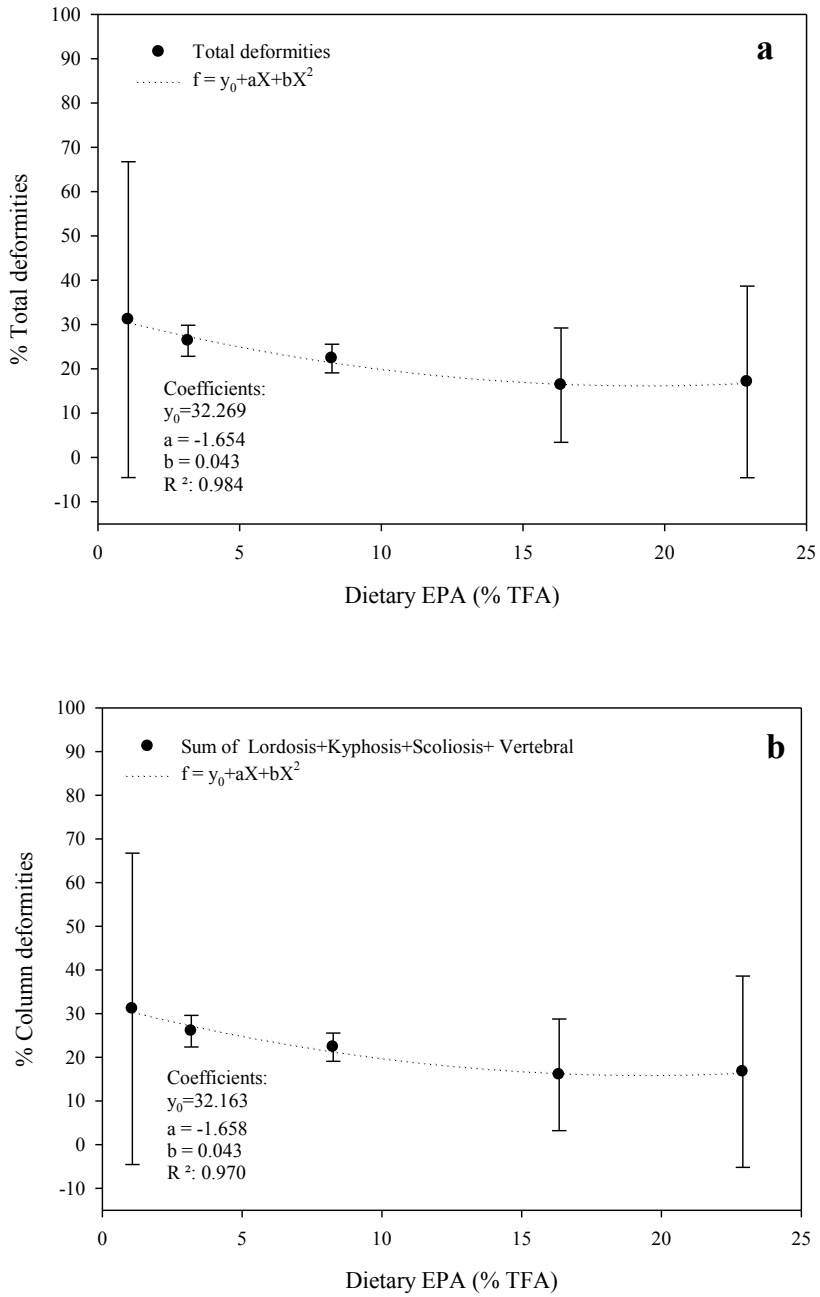

Figure 10. Relationship between (a) total deformities (%), (b) sum of column deformities to dietary *Artemia* EPA (20:5n-3) content (%TFA) in larval greater amberjack at 35 days post-hatch (mean + S.D., n=3). Data are fitted to a quadratic regression analysis ($f=y_0+ax+bx^2$) for a, b and c data, while a linear regression analysis ($f=y_0+ax$) in d.

Fatty acid composition

Results of total lipids and fatty acids analysis of greater amberjack larvae at 35 dph are shown in **Table 8.** Total lipids, ash content and moisture were equal for the larvae fed the different EPA *Artemia* treatments. Regarding FA composition, total n-3 and n-3 HUFA were significantly increased with the increase in dietary EPA. The same way, EPA level in larvae fed Diet 0–4 was significantly increased with the increase in the dietary EPA content. DHA contents in larval tissues were also affected by dietary treatments, with the lowest values in group EPA-0, which was significantly lower than those in groups EPA-4 ($P \leq 0.05$). On the other hand, ARA content was not significantly affected by dietary EPA.

Table 8. Proximate (lipid, moisture and ash content, % dry matter) and fatty acids composition (%TFA) of greater amberjack larvae 35 dah fed enriched *Artemia* with different experimental emulsions. Proximate and FA analysis data represent means ± SD, (n=3). Different superscripts within each row indicate a significant difference between DHA emulsions (ANOVA ($P \leq 0.05$); Tukey's HSD).

	EPA-0	*EPA-1*	*EPA-2*	*EPA-3*	*EPA-4*
			Proximate analysis (% dry matter)		
Lipids	17.27±3.85	14.71±1.70	16.08±6.94	15.00±2.75	20.10±1.39
Moisture	84.58±2.35	86.87±1.10	87.24±3.66	85.59±0.70	87.71±1.12
Ash	15.99±0.02	16.43±1.41	16.03±1.49	15.95±2.40	14.19±0.93
			Fatty acid content (%TFA)		
Saturated	27.00±1.30^{ab}	28.93±1.71^{a}	27.61±0.59^{ab}	24.11±0.82^{c}	24.93±0.27^{bc}
Monoenoics	29.07±3.23^{a}	28.75±0.81^{a}	28.61±0.27^{a}	27.98±0.37^{a}	23.00±0.24^{b}
n-3	28.78±5.25^{b}	27.83±0.56^{b}	30.31±0.21^{b}	33.37±2.16^{ab}	38.68±0.42^{a}
n-6	11.62±1.08	11.02±0.43	10.28±0.18	10.01±0.79	9.97±0.23
n-9	21.60±3.07^{b}	21.58±0.60^{b}	21.45±0.22^{b}	20.01±1.56^{ab}	15.84±0.25^{b}
Total n-3HUFA	15.59±6.24^{b}	17.31±0.59^{b}	20.09±0.23^{ab}	21.84±0.91^{ab}	26.31±0.26^{a}
14:00	0.51±0.16	0.40±0.15	0.31±0.02	0.36±0.09	0.35±0.03
16:00	14.22±0.32^{ab}	14.60±0.36^{a}	14.36±0.20^{ab}	12.50±0.83^{c}	13.24±0.23^{cb}
16:1 n-7	0.57±0.04	0.54±0.01	0.54±0.00	0.76±0.36	0.57±0.01
18:00	11.37±1.08^{ab}	13.13±1.81^{a}	12.13±0.44^{ab}	10.16±0.55^{b}	10.59±0.11^{ab}
18:1 n-9	18.83±3.29^{a}	19.16±0.41^{a}	19.28±0.18^{a}	17.23±2.56^{ab}	13.50±0.22^{b}
18:1 n-7	6.11±0.32	5.84±0.14	5.85±0.06	5.76±0.37	5.80±0.02
18:2 n-6	6.55±0.71^{a}	5.82±0.23^{ab}	5.35±0.14^{b}	4.79±0.43^{b}	5.05±0.32^{b}
18:3 n-3	11.25±1.27	8.99±0.45	8.76±0.29	9.56±1.69	10.51±0.32
20:1 n-9	0.40±0.02	0.41±0.04	0.38±0.01	0.45±0.22	0.33±0.00
20:4n-6 (ARA)	3.09±0.26	3.45±0.11	3.37±0.09	3.11±0.15	3.30±0.07
20:5n-3 (EPA)	6.65±4.48^{b}	8.50±0.29^{ab}	10.54±0.11^{ab}	11.47±1.25^{ab}	13.55±0.25^{a}
22:6n-3 (DHA)	4.81±1.64^{b}	4.51±0.28^{b}	5.11±0.22^{b}	5.73±0.27^{b}	8.02±0.19^{a}
ARA/EPA	0.59±0.29^{a}	0.41±0.01^{a}	0.32±0.01^{a}	0.27±0.04^{a}	0.24±0.01^{a}
DHA/EPA	0.82±0.22^{a}	0.53±0.02^{a}	0.49±0.02^{a}	0.50±0.06^{a}	0.59±0.02^{a}
DHA/ARA	1.56±0.53^{a}	1.31±0.09^{a}	1.52±0.06^{a}	1.85±0.13^{a}	2.43±0.06^{a}
Oleic/DHA	4.34±1.86^{a}	4.26±0.18^{a}	3.77±0.17^{a}	3.01±0.42^{a}	1.68±0.05^{a}
Oleic/n-3HUFA	1.37±0.63^{a}	1.11±0.01^{a}	0.96±0.02^{a}	0.79±0.09^{a}	0.51±0.01^{a}
n-3/n-6	2.52±0.72^{a}	2.53±0.05^{a}	2.95±0.03^{a}	3.36±0.46^{a}	3.88±0.12^{a}

4.- Combined effect of PUFA-rich lipids and carotenoids in enrichment products for live prey (rotifers) for greater amberjack

Methodology

Preliminary rotifer enrichment assays

A first experiment was carried out using rotifers at an initial density of 300 rot ml^{-1} in 10-l triplicate tanks. The rotifers were enriched with three experimental emulsions added at 8% concentration to the enrichment tanks. Taking into account that viable greater amberjack wild eggs have around 17% of total lipid (TL) in DM: 30% TAG and 20% PL, with 26% of DHA and 5% of EPA in both TL and PL, and 3 and 4% of AA in TL and PL, respectively (Rodríguez-Barreto et al., 2014), the emulsions were designed with different lipid sources varying in their chemical structure and combined to supply high LC-PUFA levels and DHA/EPA/ARA ratios resembling those of wild amberjack eggs total and polar lipids. E1 was based on a polar rich (PL-rich) emulsion containing a marine natural lecithin LC60 (PhosphoTech Laboratories, France) with up to 60 % phospholipids (40% PC + 20 PE) rich in DHA, and a DHA/EPA ratio of 2.5/1. E3 was based on a mixture of different TAG sources (Incromega DHA500 TAG and cod liver oil) although a slight supplementation with soybean lecithin was performed to help emulsification and absorption of lipids. Finally, E2 emulsion was formulated on a blend of these three lipid sources. The three experimental emulsions were additionally supplemented with free arachidonic acid (AA) (Sigma Aldrich, Madrid, Spain) and emulsified with 0.5 g of egg yolk. A commercial booster rich in TAG was also used as a control (C). The commercial protocol consisted of a rotifer conditioning with Espresso product, followed by DHA-PROTEIN SELCO enrichment (INVE-AQUACULTURE, Belgium). Ingredients and fatty acid composition of the experimental emulsions is shown in **Table 9.** For rotifers enrichment, 800 mg of each experimental emulsion was mixed with fresh water with a stirrer and added to 10-l tanks filled with seawater and provided with aeration and oxygen supply. Enrichment time and initial density (300 indv ml^{-1}) were equal for all the treatments assayed. Temperature, salinity and oxygen saturation during enrichment were 21.3ºC, 37g l^{-1} and 96 %, respectively. Triplicate samples of rotifers were taken at 3, 6, 10 and 24 h of enrichment for evaluation of survival (**Table 10**) and lipid and fatty acid analysis (**Table 11**).

Regardless of dietary treatment, longer enrichment periods (10 and 24h) tended to decrease oxygen saturation of media (**Table 10**) and LC-PUFA in rotifers PL decreased with time. In addition, treatment E2 gave the worst results in terms of rotifer population condition (lower survival and ovigerous females) (**Table 10**). As shown in **Table 11** and according to greater amberjack eggs composition, the best combination in terms of rotifer PL absolute contents and proportions of DHA, EPA and ARA was achieved with the marine lecithin used in treatment E1 for 3h.

In a second preliminary experiment, the lipid emulsion achieving the best combination in terms of rotifer PL/TAG as well as a the higher LC-PUFA in rotifer polar lipids without negatively affecting rotifer population, was selected (E1), mixed with three different proportions of carotenoids (50, 100 or 150 ppm Naturose ~2% astaxanthin), and added to the enrichment tanks at 6% concentration, under the same rearing conditions and sampling times, for evaluation of survival and rotifer total lipid **(Fig. 11)** and carotenoid content assessment **(Fig. 12).**

No significant variation in rotifers population condition was registered when E1 was combined with increasing proportions of carotenoids. Regardless of the treatment, maximum absorption of carotenoids was reached after 3h **(Fig. 12).**

From these two preliminary experiments and according to the carotenoid and lipid and fatty acid composition of wild greater amberjack female gonads and eggs, it was concluded that rotifers enriched for short periods (3-6h) with 6% of the marine lecithin with a slight supplementation of AA (E1) in combination with a range of carotenoids well below 50 ppm, might improve species larval performance at early life stages.

Table 9. Ingredients and main fatty acid composition (% of total fatty acids) of the experimental lipid emulsions.

	E1	E2	E3
Ingredients (mg L^{-1})			
Marine lecithin	78	23.6	0
Incromega DHA 500	0	39.3	47.2
Cod liver oil	0	15.7	31.5
Arachidonic acid	2	1.4	1.3
Fatty acids (% TFA)			
Total SFA	33.42	17.62	16.8
14:0	1.29	1.47	1.31
16:0	26.57	11.29	10.6
18:0	4.60	4.02	4.39
Total MUFA	13.15	23.66	27.4
16:1	1.40	2.39	2.86
18:1	8.89	12.40	16.1
20:1	2.86	4.19	3.82
22:1	nd	3.52	3.52
Total n-6 PUFA	11.36	8.98	17.4
18:2	3.57	3.08	13.2
20:4	7.45	3.86	2.54
Total n-3 PUFA	39.52	49.72	38.2
18:3	0.19	0.52	1.92
18:4	0.24	0.95	1.11
20:4	7.45	3.86	2.54
20:5	10.96	9.21	6.74
22:5	0.00	1.59	1.29
22:6	28.12	36.92	26.7
n-3/n-6	3.48	5.54	2.19
Total n-3 HUFA	39.09	48.25	35.2

nd, not detected. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids (≤C20 ≤3 double bounds).

Larval rearing

Newly hatched larvae of greater amberjack, at a total density of 5000 larvae per tank (mean total length 3.14± 0.08 mm), were randomly distributed in 12 experimental tanks of 100 l capacity. Water exchange and continuous light conditions were 0.30 l min^{-1} and 700 lux (surface of the water) during the feeding trial. Average seawater temperature and dissolved oxygen during this period were 22.4 ± 0.4°C and 7.56 ± 0.12 mg l^{-1} (98.8±1.8 % oxygen saturation). From 3 to 11 dph, rotifers in the tanks were adjusted to 5 individuals ml^{-1} and increased to 10 individuals ml^{-1} until the end of the trial. Rotifers were enriched with one of four treatments (one commercial and three experimental) and added to the larval rearing tanks twice a day.

The effect of new combinations of PUFA-rich lipids and carotenoids formulated from results attained in the preliminary enrichment assays, were assessed on greater amberjack larval performance, welfare and body

composition. To this purpose the rotifer enrichment commercial protocol (C) was compared with three experimental emulsions (E1; E1,10 and E3,10) added at a 6% concentration for 3h to the rotifer enrichment tanks, under the same rearing conditions. E1 and E3 consisted of the same emulsions described above (see **Table 9**), with E1,10 and E3,10 consisting of these two lipid emulsions combined with 10 ppm (mg l^{-1}) of Naturose.

All larval sampling at each age (1, 6, 10 and 14 dph) was carried out randomly from the experimental tanks. Total length was measured with a digital imaging system (Nikon Digital Sight) and the percentage of larvae with swim bladder inflated was determined. The eye diameter was measured and their proportion with respect to the total length calculated. At the end of the trial (14 dph) larvae of each tank were counted and the percentage of survival calculated. Daily prey intake was also estimated by the differences between added and remaining rotifers in larval rearing tanks.

Welfare

Samples of 15 larvae at 14 dph were collected in triplicate from each treatment, homogenized in distilled water (1 ml) and centrifuged (8,000 g, 15 min, 4°C) to collect the supernatant. Protein concentration was then analyzed according to Bradford (1976) using bovine serum albumin (BSA) as standard to report the activities per mg of protein. Cortisol level was determined by radioimmunoassay using ELISA kits (Arbor Assay, Michigan, USA). Commercially available laboratory assay kits were used to determine whole body levels of glucose, lactate, sodium and potassium determined in duplicates using standard spectrophotometric assays (Spinreact).

Table 10. Data (mean ± SD) of survival (%), ovigerous female (%), Temperature (ºC) and oxygen saturation (%) from the different experimental treatments (E1, E2 and E3) vs. control treatment (C) used to live prey (rotifers) enrichment, for 24 hours.

Treat.	Time (h)	Survival (%)		Ovigerous (%)		Temp. (ºC)	Oxygen (%)	
C	0	100.0 ± 0.0	a	14.2 ± 0.0		22.2 ± 0.1	95.1 ± 0.7	a
	3	72.0 ± 8.0	bc 2	10.9 ± 2.4	12	22.6 ± 0.0	81.5 ± 0.7	ab 2
	6	74.4 ± 3.2	b 1	9.8 ± 2.3	1	22.8 ± 0.0	81.5 ± 2.1	ab 2
	10	60.1 ± 2.9	c 2	8.5 ± 1.9	1	22.9 ± 0.1	71.0 ± 8.7	b 2
	24	66.8 ± 5.3	bc 1	9.8 ± 3.6	12	22.9 ± 0.2	73.2 ± 13.1	b 2
E1	0	100.0 ± 0.0	a	14.2 ± 0.0		22.1 ± 0.0	95.3 ± 1.0	a
	3	79.3 ± 3.0	b 12	10.3 ± 2.1	12	22.8 ± 0.0	84.9 ± 3.6	b 2
	6	76.6 ± 6.4	b 1	10.2 ± 2.5	1	22.9 ± 0.1	82.7 ± 2.9	b 2
	10	68.7 ± 3.4	b 12	6.4 ± 1.9	1	23.0 ± 0.1	80.5 ± 3.1	b 2
	24	78.4 ± 14.4	b 1	9.0 ± 4.2	12	23.0 ± 0.1	94.2 ± 2.1	a 1
E2	0	100.0 ± 0.0	a	14.2 ± 0.0		22.1 ± 0.0	95.0 ± 3.7	a
	3	70.8 ± 1.5	b 2	9.7 ± 2.4	a	22.7 ± 0.1	83.8 ± 0.7	b 2
	6	18.6 ± 4.9	c 2	1.7 ± 1.5	b 12	22.9 ± 0.1	83.0 ± 1.9	b 2
	10	12.7 ± 5.2	c 3	0.0 ± 0.0	b 2	23.0 ± 0.1	76.4 ± 0.8	c 2
	24	18.4 ± 1.4	c 2	8.5 ± 1.3	a 2	22.9 ± 0.0	94.6 ± 1.5	a 1
E3	0	100.0 ± 0.0	a	14.2 ± 0.0	12	22.1 ± 0.1	95.2 ± 1.1	a
	3	80.1 ± 4.7	ab 12	15.6 ± 5.2	a 1	22.7 ± 0.1	84.5 ± 1.7	b 2
	6	72.6 ± 5.2	ab 1	8.5 ± 1.5	ab 12	22.9 ± 0.1	81.3 ± 0.9	b 2
	10	70.6 ± 15.7	ab 12	5.5 ± 4.1	b 12	22.9 ± 0.0	78.1 ± 3.4	b 2
	24	50.7 ± 18.9	b 1	3.4 ± 1.3	b 2	22.8 ± 0.1	93.7 ± 4.5	a 1

Different letters indicate significant differences ($P<0.05$) among enrichment periods (0, 3, 6, 10 and 24 hours) within a treatment. Different numbers indicate significant differences ($P<0.05$) between treatments for a particular enrichment period.

Table 11. TL (% dry matter, DM), TAG and TPL (% TL) and main FA composition (% of total FA) of TPL of rotifers enriched for 3, 6 and 10 h with the control and experimental lipid emulsions.

	Control			E1			E2			E3		
	3h	6h	10h	3h	6h	10h	3h	6h	10h	3h	6h	10h
TL	12.8±1.1^{a,B}	16.6±1.7^{b,B}	17.6±1.2^{b,A}	19.9±1.4^{b,A}	16.0±1.6^{a,B}	15.6±0.3^{a,A}	20.6±1.5 ^{A}	22.9±0.8 ^{A}	26.0±3.5 ^{B}	12.7±1.6^{a,B}	13.9±2.1^{ab,B}	16.5±1.0^{b,A}
TAG	33.5±0.7^{a,B}	39.8±1.8^{b,A}	46.1±1.9^{c,B}	24.5±0.9^{a,C}	33.5±0.3^{b,B}	36.1±1.3^{c,A}	43.4±0.8 ^{A}	42.2±2.4 ^{A}	42.5±0.4 ^{B}	35.9±2.5^{a,B}	41.6±2.4^{ab,A}	44.9±3.2^{b,B}
TPL	26.1±1.9 ^{A}	26.0±2.2 ^{A}	23.3±1.3 ^{B}	31.5±2.9 ^{A}	31.1±0.8 ^{A}	29.4±1.5 ^{C}	13.6±0.5 ^{C}	17.5±2.7 ^{C}	13.1±2.1 ^{A}	24.9±0.2 ^{B}	24.1±1.9 ^{B}	22.8±1.8 ^{B}
TPL-Fatty acids												
∑ SFA	14.3±0.9^{a,A}	16.2±0.3^{b,A}	17.0±0.5^{b,B}	22.5±0.3^{a,C}	19.7±1.2^{b,B}	19.8±0.2^{b,C}	18.5±0.9 ^{B}	18.4±1.8 ^{AB}	17.6±0.5 ^{B}	14.5±0.3 ^{A}	14.7±1.7 ^{A}	14.9±0.7 ^{A}
∑ MUFA	61.6±0.3^{c,D}	44.1±0.5^{b,B}	38.3±0.8^{a}	37.6±1.2^{a,A}	39.1± 0.4^{b,A}	36.9±0.4^{a}	42.1±0.8^{b,B}	38.7±0.8 ^{a,A}	35.8±2.9^{a}	49.8±0.6^{c,C}	46.3±0.3^{b,C}	41.6±2.3^{a}
∑ n-6 PUFA	8.9±0.1^{a,A}	15.7±0.3^{b,B}	16.4±0.3^{b,C}	9.7±0.0^{a,B}	11.3±0.4^{b,A}	11.0±0.3^{b,B}	10.7±0.4^{b,C}	11.3±0.2 ^{b,A}	9.1±0.9^{a,A}	15.6±0.6 ^{D}	16.1±0.3 ^{B}	14.8±1.1 ^{C}
20:4	0.8±0.1^{a,B}	1.6±0.0^{b,C}	1.7±0.1^{b,A}	4.0±0.0 ^{A}	4.6±0.5 ^{A}	4.8±0.1 ^{C}	3.7±0.3^{ab,A}	4.4±0.3 ^{b,A}	3.5±0.3^{a,B}	3.7±0.2 ^{A}	3.6±0.0 ^{B}	3.6±0.1 ^{B}
∑ n-3 HUFA	4.3±0.3^{a,C}	16.0±0.7^{b,B}	16.8±0.9^{b,A}	23.2±0.5 ^{A}	23.7±2.4 ^{A}	22.2±0.5 ^{B}	21.0±1.7^{a,A}	25.6±2.2 ^{b,A}	26.9±0.6^{b,C}	11.2±0.4^{a,B}	14.9±0.5^{b,B}	19.0±2.9^{b,AB}
20:5	1.1±0.1^{a,D}	3.3±0.2^{b,B}	3.3±0.2^{b,A}	6.4±0.2 ^{A}	6.5±0.7 ^{A}	6.3±0.3 ^{C}	5.0±0.5^{a,B}	6.4±0.6 ^{b,A}	5.5±0.4^{ab,BC}	3.1±0.3 ^{C}	4.1±0.6 ^{B}	4.4±0.8 ^{AB}
22:6	2.4±0.2^{a,C}	11.4±0.4^{b,B}	11.5±0.6^{b,A}	16.3±0.5^{b,A}	16.5±2.8^{ab,A}	14.4±0.2^{a,A}	15.3±1.1 ^{A}	18.1±2.8 ^{A}	19.7±0.6 ^{B}	7.4±0.1^{a,B}	9.5±0.1^{ab,B}	12.2±2.1^{b,A}

Values are expressed as mean ± SD (n=3). C, commercial booster; E1, 100% marine lecithin; E2, 30% marine lecithin + 70% blend of oils rich in TAG; E3, 100% blend of oils rich in TAG. Lowercase letters within a row denote significant differences among enrichment periods for a particular dietary treatment (P <0.05); capital letters denote differences among treatments for a particular enrichment period. TL, total lipid content; TAG, triacylglycerides; TPL, total polar lipids; FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids (≥C20 ≥3 double bounds).

Table 12. Whole body glucose (mg mg protein $^{-1}$), lactate (mg mg protein $^{-1}$), Na^+ (mg mg protein $^{-1}$) and K^+ (mg mg protein $^{-1}$) in 14 dph greater amberjack larvae fed with rotifers enriched with commercial (C) and experimental (E1; E1,10 and E3,10) emulsions.

	C		E1		E1,10		E3,10	
Glucose	0.14	± 0.05	0.10	± 0.04	0.09	± 0.05	0.15	± 0.06
Lactate	1.76	± 0.06 a	0.68	± 0.13 b	0.65	± 0.35 b	0.75	± 0.14 b
Na^+	250.22	± 26.05	160.12	± 124.18	104.46	± 6.09	261.78	± 74.17
K^+	17.22	± 3.77	11.36	± 4.51	8.83	± 1.41	7.69	± 2.23

Values are mean ± SD (n=3). Different letters indicate a significant difference between treatments (P< 0.05).

Lipid, fatty acid and carotenoid analysis

One final sample of larvae was collected from each experimental tank at 14 dph. Besides, each experimental emulsion, and triplicate samples of enriched rotifers were stored. All samples were flushed with N_2 and kept frozen at -80 °C until analysis was carried out. Total lipids were extracted (Folch et al., 1957) and fatty acids from total and polar lipids were obtained by acid - catalysed transmethylation of 1 mg of lipid extracts according to Christie (1989). The fatty acid methyl esters (FAME) were purified by thin-layer

chromatography (TLC) (Christie, 2003), separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Scientific) equipped with an on-column injector, a flame ionisation detector and a fused silica capillary column, Supelcowax TM 10 (Sigma-Aldrich, Madrid, Spain). Identification of individual FAME was confirmed by GC–MS chromatography (DSQ II, Thermo Scientific).

Carotenoids were obtained according to the method of Barua et al. (1993), using ethyl acetate/ethanol (10 mL, 1:1 v/v), ethyl acetate (5 ml) and hexane (10 ml) as extracting solvents. Afterwards, carotenoid contents were quantified by spectrophotometry at 470 nm.

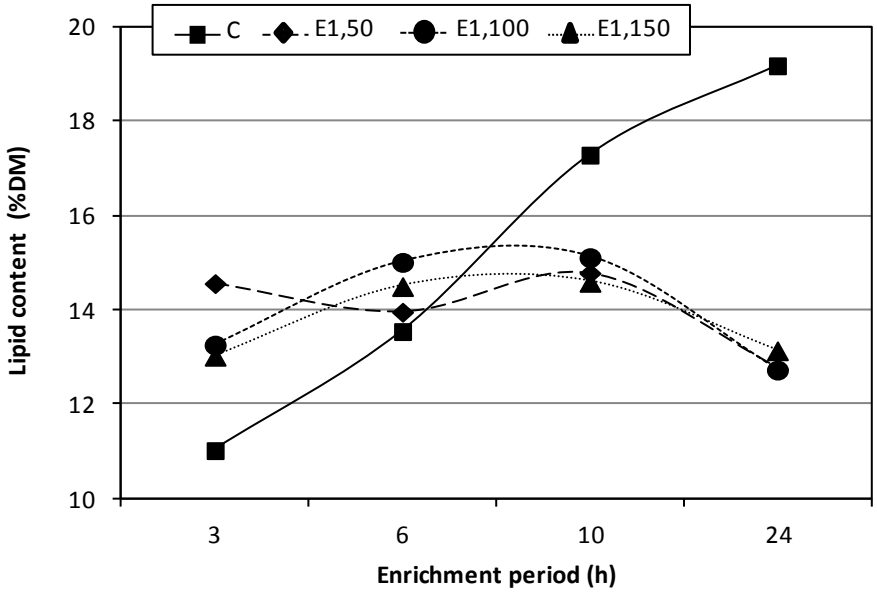

Figure 11. Time course of lipid content (% DM) in rotifers enriched with lipid emulsion C or E1 supplemented with increasing levels of astaxanthin (50, 100 and 150 ppm).

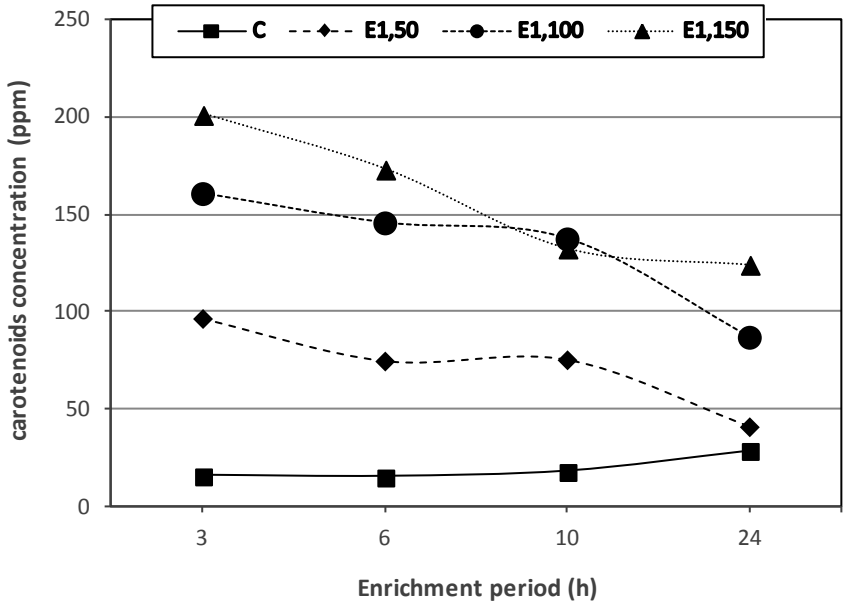

Figure 12. Time course of carotenoids content (ppm) in rotifers enriched with lipid emulsion C or E1 supplemented with increasing levels of astaxanthin (50, 100 and 150 ppm).

Statistical analysis

All the data were statistically treated using a SPSS Statistical Software System 15.0 (SPSS, www.spss.com). The significant level for all the analysis was set at 5% and results are given as mean values and standard deviation. All values presented as percentage were arcsine transformed. Also, all variables were checked for normality and homogeneity of variance, using the Kolmogorov–Smirnoff and the Levene tests, respectively. To compare means, the group data were statistically tested using one-way ANOVA. When variances were not homogenate, a non-parametric Kruskal–Wallis test was accomplished.

Results

Rotifer and larval performance

Enrichment process with different experimental emulsions did not affect rotifers survival, which was about 94% on average.

Larval growth was significantly affected by dietary treatments. Mean values for all the dietary treatments were 5.47±0.43 mm in total length at 14 dph. Total length of larvae was similar between the four enrichment treatments at 6 and 10 dph. However, the different treatments assayed showed significant differences at 14 dph. Fish total length (TL) in Control group was significantly lower at 14 dph while larvae from treatment E3,10 showed the significantly highest size but similar to treatment E1,10. Although E1 larvae were smaller than E3,10 larvae, no differences were found between groups E1,10 and E1 at the same age ($P > 0.05$) **(Fig. 13).**

Larval survival was significantly ($P \leq 0.05$) affected by treatments assayed at 14 dph. The lowest survival was recorded in those larvae receiving the treatment E3,10 (**Fig. 14**) and it was significantly different to treatment E1,10, whereas no differences were found between treatments E1 and Control.

The eye diameter to total length ratio (%) for all treatments was 9.07±0.23 % at 14 dph. The treatment E1,10 showed the highest ratio and it was significantly different ($P \leq 0.05$) to treatment Control, whereas treatments E1 and E3,10 were similar between them (**Fig. 15**). The percentage of larvae with the swim bladder inflated was similar for all treatments (84 ± 16 % of larvae) at 14 dph.

Welfare

Elevated and significantly higher ($P < 0.05$) whole body cortisol levels were observed in larvae fed treatment E1 at 14 dph (**Fig. 16**). On the contrary the cortisol level of larvae from treatment E1,10 was the lowest one. No significant differences ($P < 0.05$) in whole body glucose levels were observed among the different treatments (**Table 12**) although a similar trend as with the cortisol was observed for E1,10 treatment. Whole body lactate showed higher values in Control larvae treatment when compared to the other groups, whereas whole body Na^+ and K^+ levels in larvae fed with different treatments were not significantly different.

Lipid, carotenoid and fatty acid contents

In spite of the trend of total carotenoids to increase in rotifers with dietary supplementation, the differences were not significant among experimental treatments. In addition only Control rotifers lipid contents displayed a significantly higher lipid content with respect to the experimental diets (**Figs 17** and **18).** A good correlation among total carotenoid level in rotifers can be also observed in **Fig. 17**, with Control and E1 larvae displaying the lowest contents and a higher value being present in both E1,10 and E3,10. The very low standard deviation of carotenoid data from E3,10, made this treatment significantly different from that of Control and E1 larvae. The high lipid contents of the commercial emulsion is not only evident in rotifers total lipids but in corresponding larvae. Although a good correlation was found among the lower lipid levels

of the experimental larvae and the corresponding values in rotifers, an apparent trend for a higher incorporation of lipids in E1,10 and E3,10 larvae can be observed (**Fig. 18**).

Lipid classes profiles were confirmed in rotifers sampled in present experiment (data not shown), so that higher contents of TG in Control and E3 rotifers, as well as a higher total polar lipid content of rotifers enriched with treatment E1, resembled values shown in **Table 11**. Due to sudden mortalities displayed by two of the Control larval replicates, only a very small sample was available for lipid analysis and its lipid profile is consistent with rotifer composition, since a higher content of TG and lower levels of TPL were present. On the contrary, independently of rotifers lipid classes profiles, experimental larvae composition was quite similar among treatments with a significant amount of TPL compared to TG. According to the larval TL fatty acid composition, there were higher contents of DHA in both E1 and E1,10 treatments (24.8 ±1.3 and 22.0 ± 2.5%, respectively), whereas values of DHA for E3,10 was 17.8 ± 1.4 %. The same trend was even more evident for the polar lipids.

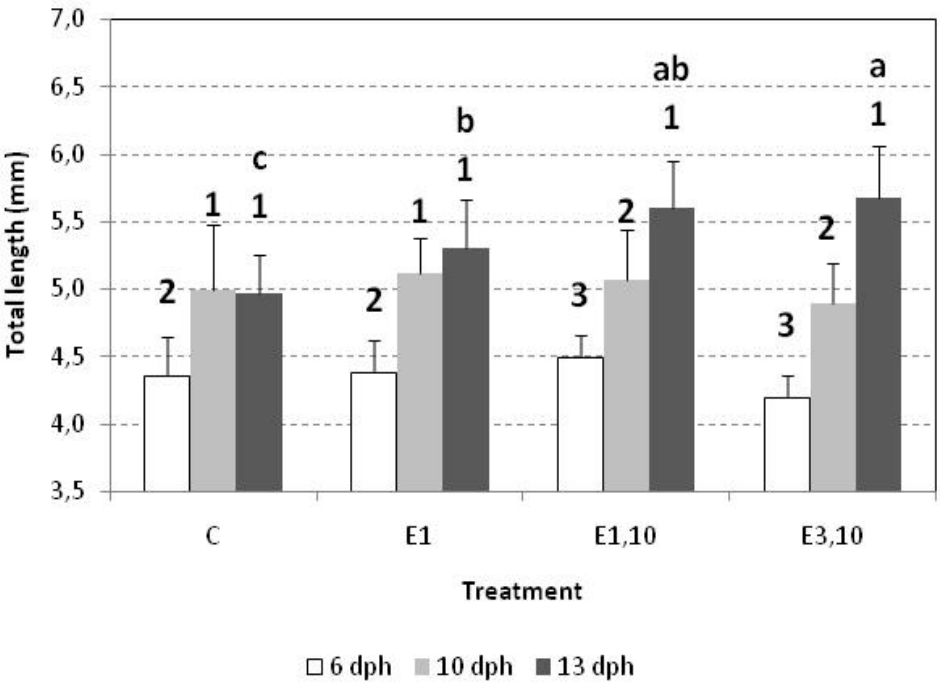

Figure 13. Total length (mm) of greater amberjack larvae, fed with rotifers enriched with commercial (C) and experimental (E1, E1,10 and E3,10) emulsions at 6, 10 and 14 dph. Values are mean ± SD, n=3. Different numbers indicate a significant difference within treatments at each age. Different letters indicate significant difference between treatments at each age (ANOVA (P ≤0.05); Tukey's HSD).

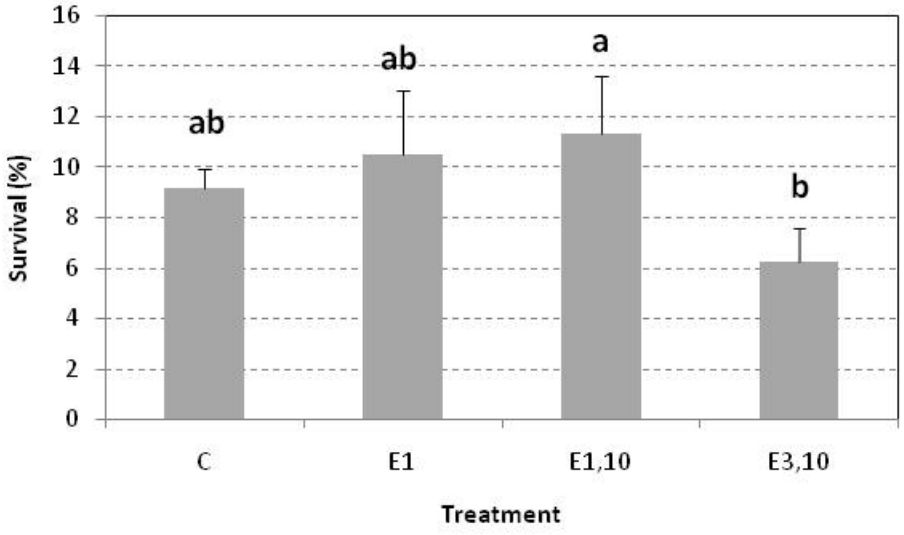

Figure 14. Final survival percentage (14 dph) of greater amberjack larvae, fed with rotifers enriched with commercial (C) and experimental (E1, E1,10 and E3,10) emulsions. Values are mean ± SD (n=3). Different letters indicate a significant difference between treatments (ANOVA (P ≤0.05); Tukey's HSD).

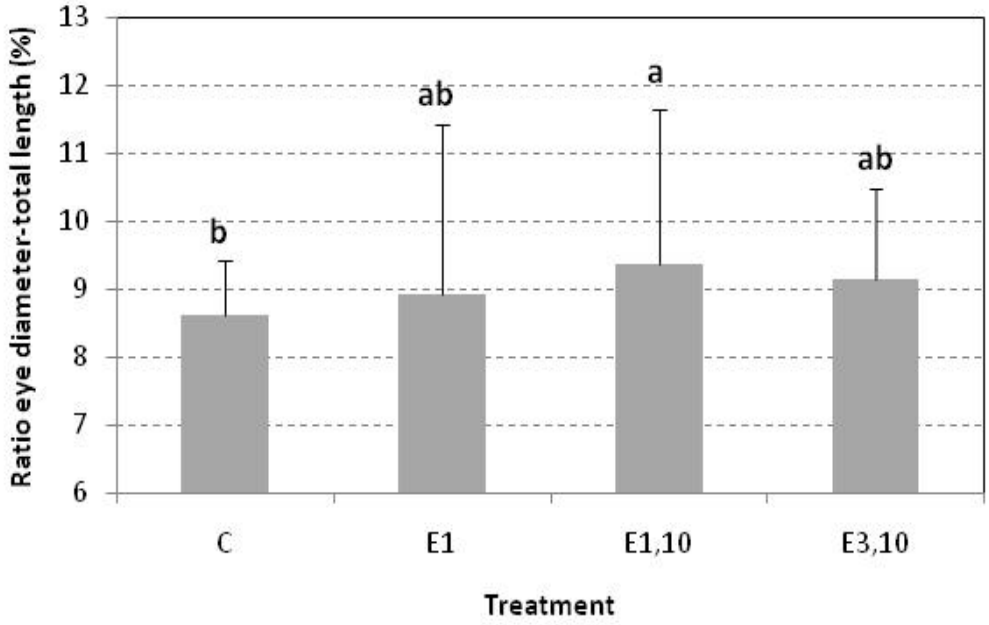

Figure 15. Eye diameter to total length ratio (%) at 14 dph of greater amberjack larvae, fed with rotifers enriched with commercial (C) and experimental (E1, E1,10 and E3,10) emulsions. Values are mean ± SD, n=3. Different letters indicate significant differences between treatments (ANOVA (P ≤0.05); Tukey's HSD).

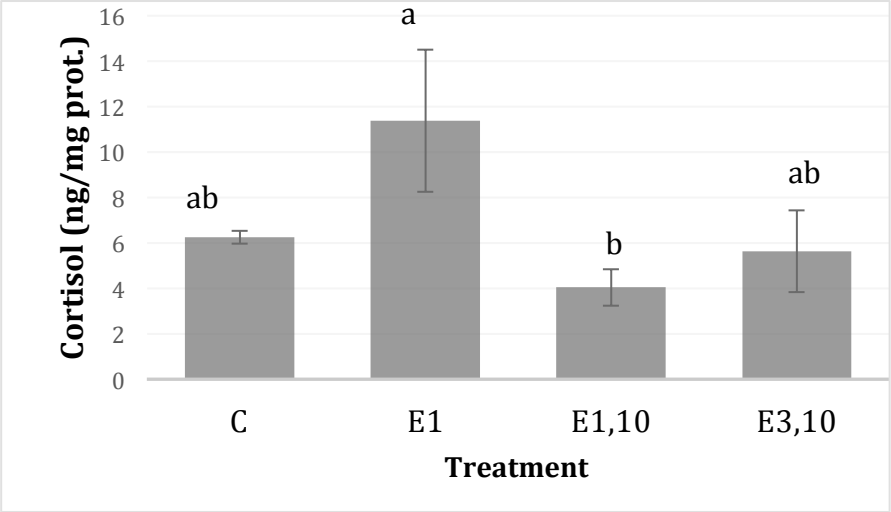

Figure 16. Whole body cortisol (ng/mg prot) levels in 14 dph greater amberjack larvae fed with rotifers enriched with commercial (C) and experimental (E1, E1,10 and E3,10) emulsions. Values are mean ± SD, n=3. Different letters indicate significant differences between treatments (ANOVA ($P \leq 0.05$); Tukey's HSD).

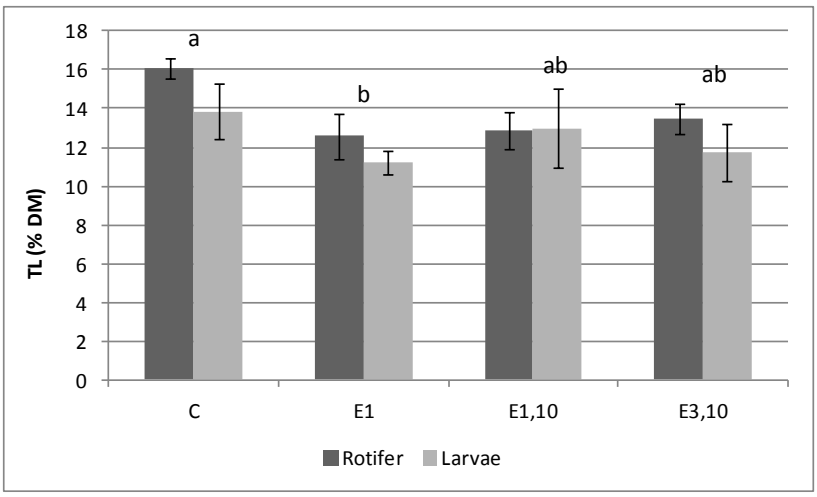

Figure 17. Total lipid content (% DM) of rotifers and larvae enriched with the control (C) or one of three experimental emulsions (E1; E1,10; E3,10).

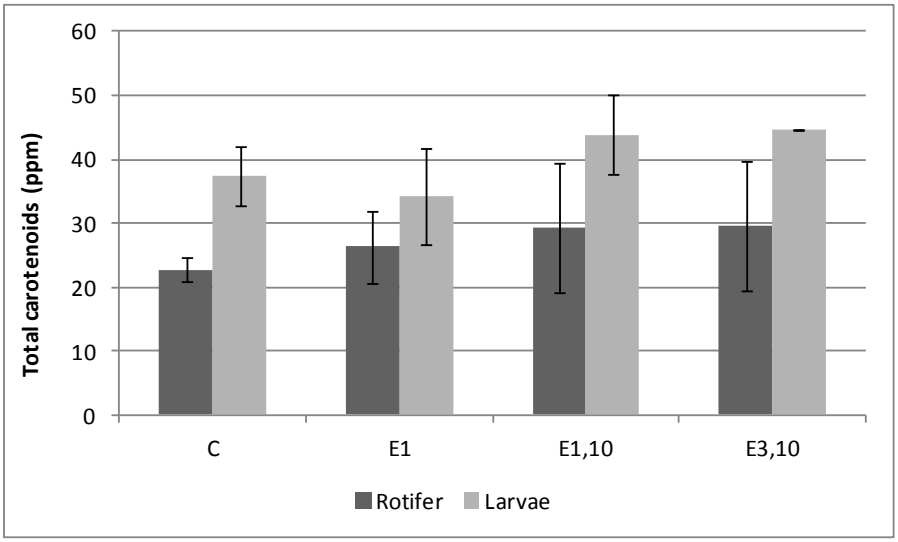

Figure 18. Total carotenoids content (ppm) of rotifers and larvae enriched with the control (C) or one of three experimental emulsions (E1; E1,10; E3,10).

5.- Discussion and conclusions

Discussion

In the present study, larval amberjack survival (over 20%) was higher than that reported in Japanese hatcheries (2-9%, Sawada et al., 2002), and similar to that obtained in Canary Islands under semi-intensive conditions in this (Roo et al., 2013) and other *Seriola* species (longfin yellowtail, *Seriola rivoliana*, Roo et al., 2014). The highest greater amberjack growth was obtained when larvae (17-35 dah) were fed *Artemia* containing DHA in a range of 5-8 %TFA, with a maximum around 7% (1.5 g 100 g^{-1} DHA dw). These results are in agreement with the optimum dietary EFA levels determined for younger amberjack larvae (3-10 dah) (Matsunari et al., 2013), suggesting that EFA requirements are similar during rotifer and *Artemia* feeding as reported for larvae of other marine fish species (Izquierdo et al., 2000; Bransden et al., 2005). Requirements of amberjack larvae for DHA (1.5 g.100g^{-1} DHA DW) were higher than those found in other marine fish species (Hamre et al., 2013) and similar to those for other fast growing species. For instance, yellowtail (*Seriola quinqueradiata)* or striped jack (*Pseudocaranx dentex*) larvae require 1.3–2.6 g 100 g^{-1} DHA and 1.6–2.2 g 100 g^{-1} DHA (Furuita et al., 1996; Takeuchi et al., 1996, 1998), respectively, for maximum survival and handling stress resistance. In agreement, the lowest dietary DHA levels significantly reduced larval greater amberjack survival, whereas increases in DHA tend to improve larval resistance to handling. DHA has been found to be particularly important for handling resistance in fast growing species such as striped jack (Arakawa et al., 1987), Pacific blue fin tuna *Thunnus orientalis* (Seoka et al., 2007) or longfin yellowtail larvae (Roo et al., 2014). Increase in dietary DHA has been also found to improved stress tolerance in larvae of other species, such as red seabream (*Pagrus major*, Kanazawa et al., 1997) or red porgy (*Pagrus pagrus*, Roo et al., 2009). This effect has been related to a higher efficiency in oxygen transport and better gill membrane fluidity properties, to recover normal oxygen levels after the stress test, in larvae fed higher DHA.

Emulsion enrichment protocols frequently result in high *Artemia* neutral lipid (NL) levels that can be excessive (Morais et al., 2006). In fact, DHA seems to be better utilized by cod (*Gadus morhua*) larvae when they are fed rotifers with a high dietary n-3 HUFA content, particularly DHA, provided within the phospholipids fraction than when supplied as triacylglicerides (TAG) (Olsen et al., 2014). In the present

study, even the highest DHA levels in the emulsion (70% DHA in TFA) resulted in reduced incorporation of DHA into *Artemia* lipids (11% DHA in TFA). The difficulties to boost DHA in *Artemia* has been extensively studied from early-mid 1990s by different authors (Rainuzzo et al., 1994; McEvoy et al., 1996; Evjemo et al.,1997; Navarro et al., 1999). These studies revealed that most *Artemia* strains are characterized by elevated content of EPA in comparison to DHA, which clearly differs from copepods, the main natural live prey for marine fish larvae (Van der Meeren et al., 2008). In addition, in enriched *Artemia* neutral lipids are predominant over phospholipid classes and when high DHA PL are fed to *Artemia*, an active translocation of DHA to *Artemia* NL was identified (Monroig et al., 2006; Guinot et al., 2013).

In the present study, despite EPA levels in *Artemia* increased from 0.87 to 6.81 % TFA, EPA levels in greater amberjack larvae were only increased up to 5.2 % TFA, denoting a saturation process that could be associated with the fulfillment of the EPA requirements. On the contrary, DHA levels in greater amberjack larvae showed a linear increase and, whereas the two highest DHA levels provided no growth or survival improvement, the slightly lower survival after handling stress and the increase in deformities suggested the potential teratogenic effect of excess DHA. Thus, when dietary DHA levels were over 2 g 100 g^{-1}, a higher incidence of skeletal malformations, particularly those related with skull development were identified. Jaw malformations in larvae of yellowtail kingfish (*Seriola lalandi*, Cobcrof et al.,, 2004) or stripped trumpered (*Latris lineata*, Cobcrof et al., 2001) have been related to fatty acids deficiencies, or DHA: EPA ratio unbalances. In gilthead seabream low dietary DHA levels in rotifers have been associated with increased vertebral anomalies (Izquierdo et al., 2013). Besides, increased dietary DHA levels without an adequate balance of antioxidant nutrients promoted the production of free radicals damaging cartilaginous structures and increasing the risk of cranial anomalies (Izquierdo et al., 2013). In agreement, in greater amberjack skeleton anomalies including lordosis, kyphosis and vertebral anomalies were higher when dietary DHA was below or over 2 g 100 g^{-1}DHA in *Artemia*, whereas dietary DHA was linearly related to skull anomalies. The type of bone and ossification timing among different skeletal elements (vertebrae and cranium structures) can be responsible for this different response to DHA levels. Thus, skeletal elements such as maxilla or mandible that develop from a cartilaginous precursor would be more sensitive to oxidative risks and, therefore to dietary DHA elevation (Izquierdo et al., 2013). Accordingly, high dietary levels of antioxidants protected gilthead sea bream fed high DHA *Artemia* from oxidative risks (Hernández-Cruz et al., 2015). Thus, the high incidence of skeleton anomalies (66%) found in longfin yellowtail fed high DHA levels in weaning diets, in the range of those found for yellowtail kingfish (Cobcrof et al., 2013) or yellowtail (Kolkovski and Sakakura, 2004), could reflect an unbalance between antioxidant and pro-oxidant nutrients in the weaning diets. In the present study, a low incidence (8%) of skeleton anomalies was found in 35 dah greater amberjack juveniles, even at the highest DHA level tested, suggesting an adequate antioxidant protection.

It is well known that raising the ratio of PL to TL in larval feeds may enhance growth (Cahu et al., 2003). This probably reflects a limited ability of early larvae to carry through de novo synthesis of PL based on dietary TAG, and is in agreement with the finding that PL appears to be essential for fish larvae in their very early stages (Coutteau et al., 1997; Tocher et al., 2008; Cahu et al., 2009; Li et al., 2014; Olsen et al. 2014). However, levels of essential n-3 fatty acids and phospholipids are often lower in rotifers than in a natural prey such as copepods (van der Meeren et al., 2008). This can be improved by several strategies including the use of high levels of n-3 fatty acids in the form of phospholipids such as the marine lecithin LC60 used in the present study or together with other phospholipid sources such as soybean lecithin for live prey enrichment (Coutteau et al., 1997; Cahu et al., 2009; Li et al., 2014; Olsen et al., 2014). Culturing lean rotifers, which contain low levels of triacylglycerol and thus have a higher ratio of phospholipids (PL) to total lipids (TL) is also a novel approach (Hamre et al., 2013). As shown in Table 11 rotifers enriched with the marine lecithin (E1), displayed a fast incorporation of polar lipids particularly rich in DHA, compared to the other treatments, which tended to accumulate more TG and DHA in total lipids. The importance of a high dietary input of DHA and DHA/EPA ratios of 2-3/1 have been also pointed out in previous studies (Rodríguez et al., 1997; Sui et al., 2007; Sorgeloos et al., 2011) as a limiting factor for marine larval performance. In fact, the increase in dietary DHA and phospholipids effectively improves stress tolerance of red sea bream (*Pagrus major*) and marbled sole (*Limanda yokohamae*) (Kanazawa et al., 1997) or red porgy (*Pagrus pagrus*) (Roo et al., 2009). This fact has been correlated with a higher efficiency in oxygen transport

and better gill membrane fluidity properties, to recover normal oxygen levels after the stress test in those larvae fed with high levels of DHA which in turn showed a higher DHA content on these tissues. Thus, Sargent et al. (1999) reported the existence of tissue specific fatty acid compositions in its polar lipid fraction, probably related to its biological function, e.g., higher concentration of 22:6*n*-3 in neural and visual tissues. On the contrary, total fatty acid compositions including neutral and free fatty acid contents are mostly determined by the levels of fatty acids available from the diet. Therefore, not only total DHA content but also its lipid form should be considered to identify HUFA requirements. In this sense, Olsen et al. (2014) found that cod larvae fed with rotifers with a high dietary n-3 HUFA content, particularly DHA, provided within the phospholipids fraction were more efficiently incorporated to larval tissues, than those supplied as triacylglicerides (TAG) resulting in a higher larval growth and survival. These findings are particularly effective during rotifers feeding stage, since enriched rotifers closely match the proportion of DHA found in enrichment emulsion. On this regard, Li and Olsen (2015) indentified not only the relationship between the dietary DHA from PL fraction and the body composition in its total lipid in rotifers, but also the relationship with the content in the main phospholipids classes.

Carotenoids are actively mobilized into the gonads during sexual reproductive activity in aquatic animals. Although their role in the embryonic development is not very well established, there is evidence that the presence of carotenoids mitigates deleterious oxidative damage to the developing embryo and may be also present in the gonads to ensure larval visual function and adequate chromatophore responses. Specifically, carotenoids are found to be a determining factor for good egg quality in seriola (Watanabe et al., 2003). According to Miki (1991), astaxanthin displays both a strong quenching effect against singlet oxygen and a strong scavenging effect against free radicals, and it can also inhibit lipid per-oxidation mediated by these active forms of oxygen (Tejera et al., 2007). The astaxanthin ester composition of Haematococcus algae meal is similar to that of crustaceans (Foss et al., 1987), which may justify its effectiveness in fish pigmentation (Tejera e al., 2007). Additionally, the composition of Haematococcus algae meal is balanced, and supplements the normal feed with proteins and essential nutrients such as vitamins. Christiansen et al. (1995) demonstrated that Atlantic salmon fry have a definitive growth and survival requirement for astaxanthin in their diet. Fish fed diets with astaxanthin below 5.3 ppm were found to have marginal growth, whereas those fed levels above 5.3 ppm had a better performance and significantly higher lipid levels.

Rotifers enriched with polar rich emulsion containing a marine natural lecithin LC60 combined with 10 ppm of Naturose also resulted in a significant advantage in larval growth, survival and welfare compared to rotifers enriched with other emulsions. Therefore the use of the marine phospholipids combined with carotenoids may have had a beneficial effect on seriola growth and survival. According to the larval TL fatty acid composition, there were higher contents of DHA in both E1 and E1,10 treatments (24.8 ±1.3 and 22.0 ± 2.5%, respectively), whereas values of DHA for E3,10 was 17.8% ± 1.4. The same trend was even more evident for the polar lipids. Carotenoids represent a group of micronutrients that may be deficient in rotifers for adequate larval nutrition (Hamre et al., 2008). Carotenoids may perform a biological role similar to that of α-tocopherol (as a naturally occuring antioxidant), protecting tissues and reactive compounds from oxidative damage hence its importance in preventing LC-HUFA peroxidation (Guerin et al., 2003).

During chronic stress in fish culture, there are often characteristically high circulating levels of cortisol (Wendelaar Bonga, 1997). In the present study, lower levels of cortisol were observed in the larvae fed with treatment E1,10 with respect to the other treatments. The lower cortisol response coincides with the lower mortality of E1,10 larvae and with a comparable good growth. The primary stress response in fish is known to further trigger and leads to sequential secondary responses (e.g., increases in glucose, lactate, decreases in plasma chloride, sodium, potassium) (Barton et al., 2002). Whole body levels of glucose have been observed to increase at post stress. In the present study, no differences were found in whole body glucose levels but the slight increased values in the larvae from Control and E3,10 treatments could be an indication of the fact that these fish might also be under a certain degree of stress. Tissue lactate levels in fish are known also to increase at post stress (Barton et al., 2002). The increase in tissue lactate content may also be due to its involvement in osmoregulation. In the present study, whole body lactate levels were seen to increase in the larvae from control group with respect to the other treatments.

Conclusions

In summary, these results suggest that providing a 1.5 g 100 g^{-1} DHA DW is sufficient to promote fast growth in greater amberjack larvae, whereas increased levels were associated to skull anomalies.

6.- List of optimum levels and ratios of EFA and carotenoids in enrichment products

DHA in enrichment products for *Artemia* 10-17% TFA

EPA in enrichment products for *Artemia* 14-20% TFA

DHA/EPA in enrichment products for *Artemia* 1-5

DHA in enrichment products for rotifers 14% TFA

EPA in enrichment products for *Artemia* 6% TFA

DHA/EPA in enrichment products for rotifers 2.3

Carotenoids levels in enrichment products 10 ppm

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Deviations:

This deliverable was prepared according to the work described in Task 9.1, including subtasks 9.1.1. and 9.1.2. No deviations exist from DOW.

Co-funded by the Seventh Framework Programme of the European Union