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PHYSIOLOGICAL PATHWAYS INVOLVED IN NUTRITIONAL MUSCLE DYSTROPHY AND HEALING IN EUROPEAN SEA BASS (*Dicentrarchus labrax*) LARVAE

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Abbreviations: α-TOH: α-tocopherol; AOE: Antioxidant enzymes; Calpn: μ-calpain; CAT: Catalase; dph: Days post hatching; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; FID: Flame ionization detector; GPX: Glutathione peroxidase; IGF: insulin-like growth factors; MDA: Malonaldehyde; MyHC: Myosin heavy chain; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances; PUFA: Polyunsaturated fatty acids; LC-PUFA: Long chain PUFA

Abstract

The potential muscle regeneration after nutritional dystrophy caused by high dietary DHA contents in fish and the physiological pathways involved are still unknown. To better understand this process, an experiment was conducted for 3 weeks in 14 day-old European sea bass larvae using different DHA ratios (1 or 5%). After this period, part of the larvae fed 5% DHA diet was switched to 1% DHA diet ("wash-out") for another 2 weeks. Larvae fed 5% DHA diet showed altered oxidative status as indicated by the highest TBARS values, antioxidant enzymes (AOE) expression and incidence of muscular lesions. Accordingly, "washed-out" larvae showed lower dry weight and α-TOH content. IGF-I gene expression was elevated in 5% DHA larvae at 35 dph, suggesting increased muscle mitogenesis that was corroborated by the increase in myosin heavy chain expression. It can be concluded that high dietary DHA contents alter the oxidative status and causes muscular lesions in European sea bass larvae, with morphological and molecular aspects of mammalians muscular degenerative disease.

1-Introduction

In order to improve growth and development, marine fish larvae require high contents of long-chain polyunsaturated fatty acids (LC-PUFA), such as docosahexaenoic acid (DHA; 22:6n-3). These high requirements are due, in part, to the limited capacity of marine fish species to synthesize these fatty acids when their precursors are included in the diet (Sargent et al., 1995; Izquierdo, 1996). Nevertheless, fish larvae appear to posses higher specific requirements for DHA than juveniles or adults, due to their elevated growth rate (Watanabe et al., 1989; Takeuchi, 1997). Therefore, high contents of long chain-polyunsaturated fatty acids must be included in marine fish larvae diets. However, as DHA is highly unsaturated, the susceptibility of this fatty acid to be oxidized by reactive oxygen species (ROS) is higher than that of other fatty acids (Nagaoka et al., 1990). To protect DHA from ROS attack, adequate quantities of antioxidants must be included in larval diets. Vitamin E (α-tocopherol; α-TOH) is a powerful antioxidant that also stabilizes biological membranes (Wang and Quinn, 2000). An interaction exists between α-TOH and the dietary levels of highly unsaturated fatty acids in marine fish larvae (Betancor et al., 2011; Hamre, 2011), indicating that increasing contents of LC-PUFA must be accompanied by increased levels of α-TOH.

Apart from low weight antioxidant molecules, an array of antioxidant enzymes (AOE) helps to protect organisms from ROS attack. The AOE comprise a series of enzyme scavengers of oxyradicals and other free radicals, including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX). SOD catalyzes dismutation of superoxide radicals to hydrogen peroxide and oxygen; CAT catalyzes the breakdown of hydrogen peroxide to water and molecular oxygen, and GPX decomposes

peroxides (Halliwell, 2006). Most of the studies on the activity of AOE deal with pollutant detoxification (Ji et al., 2011; Kim et al., 2010) or fish developmental aspects (Peters and Livingstone, 1996; Mourente et al., 1999). Limited information is available on the effect of dietary components on the activity and AOE´s gene expression during early developmental stages in European sea bass larvae (Tovar-Ramírez et al., 2010).

Whenever there is an imbalance between the generation and removal of ROS by cellular defences, a state of oxidative stress is initiated. This status may lead to the oxidation of various cellular constituents like lipids, proteins or DNA. For instance, European sea bass larvae muscle appears to be very sensitive to ROS attack, as severe dystrophic lesions in the epaxial musculature have been reported due to *in vivo* lipid peroxidation (Betancor et al., 2011; Betancor et al., in press). The term regeneration refers to a process that allows an organism to regain the function of an organ or structure damaged by injury or disease (Stoick-Cooper et al., 2007). In adult zebrafish (*Danio rerio*), an exceptionally high capability for regeneration has been reported (Lien et al., 2006; Yoshinari et al., 2009). Therefore, it can be hypothesized that at younger stages an activation of muscle repair process in a situation of oxidative stress will take place. However, information about muscle regeneration in fish has been rarely described and only related to mechanical injury or bacterial infection (Rowlerson et al., 1997; Ingerslev et al., 2010) and there is a complete lack of studies describing muscle regeneration process in marine fish larvae or regeneration after a nutritional dystrophy.

Myosin and actin are the major muscle proteins, where myosin is the major structural component of striated muscle. Both myosin chains, the heavy (MyHC) and the light (MyLC), exist as multiple isoforms that are tissue and/or developmental stage-specific (Funkenstein et al., 2007; Ikeda et al., 2007). MyHC gene expression has been highly correlated with muscular protein accretion (Hevrøy et al., 2006). Moreover, the

103 effect that nutritional status has on muscle growth can be determined by monitoring the
104 expression patterns of this marker gene (Overturf and Hardy, 2001). On the other hand,
105 a higher immunolocalization of this protein has been observed during regeneration
106 processes in the muscle of gilthead sea bream (*Sparus aurata*) juveniles after
107 mechanical injury (Rowlerson et al., 1997). Actins are highly conserved proteins that
108 play a key role in maintaining the cytoskeletal structure, cell motility and division, as
109 well as intracellular movements and contractile processes. Different isoforms of actins
110 exist in fish, being α-actin expressed after MyHC during the period of somite formation
111 in carp (Watabe, 2001).

112 Furthermore, cellular proliferation is an important event necessary for muscle
113 regeneration (Chargé and Rudnicki, 2004) and growth factors expected to be
114 upregulated during this process. The insulin-like growth factors I and II are two
115 myogenic regulatory factors capable of inducing satellite cell proliferation and
116 differentiation in fish (Goldspink et at., 2001; Bower et al., 2008). Moreover, IGF-II
117 was upregulated during zebrafish heart regeneration, denoting an increase of DNA
118 synthesis (Lien et al., 2006).

119 Calpains are Ca^{2+}-dependent cytoplasmic cysteine proteases that can be expressed
120 ubiquitously or in a tissue-specific way. In mammals, the calpains have received a great
121 deal of attention due to their role in muscle protein turnover and growth, as well as *post*
122 *mortem* proteolysis. However, studies of these enzymes in fish have been mainly
123 focussed on their involvement in *post mortem* muscle tenderization and texture (Chéret
124 et al., 2007; Caballero et al., 2009; Cleveland et al., 2009; Terova et al., 2011), while
125 limited information is available on the regulatory role of calpains in fish larvae and on
126 the effect of larvae nutrition on their expression levels.

In our previous studies (Betancor et al., 2011; Betancor et al., in press), when European sea bass larvae were fed high contents of DHA, α-TOH alone did not seem to be able to counteract ROS, leading to the appearance of axial muscular lesions. To better understand the molecular pathways involved in fish larvae muscle dystrophy and healing, the present study generated muscular lesions in European sea bass larvae by feeding a diet containing 5% DHA during 3 weeks (negative control diet), followed by a "wash-out" period of two weeks when larvae were switched to a diet containing only 1% DHA (positive control diet). Growth, survival, TBARS, fatty acid profile, α-TOH contents and mRNA expression levels of CAT, SOD, GPX, IGF-I, IGF-II, MyHC, α-actin and μ-calpain (Capn1) genes were studied in order to achieve this objective.

2- Methods

2.1- Fish and diets

The experiment was carried out at the *Instituto Canario de Ciencias Marinas* (ICCM; Telde, Canary Islands, Spain). European sea bass (*Dicentrarchus labrax*) larvae were obtained from natural spawnings from the *Instituto de Acuicultura de Torre de la Sal* (Castellón, Spain). Prior to the start of the feeding experiment, larvae were fed enriched yeast-fed rotifers (DHA Protein Selco®, INVE, Belgium) until they reached 14 days post hatching (dph; total length 8.58 ± 0.64 mm, dry body weight 0.36 ± 0.0 mg). Larvae were randomly distributed into experimental tanks (170 L light grey colour cylinder fibreglass tanks) at a density of 1000 larvae/tank and fed one of two experimental diets for 35 days, at a water temperature of 19.5 to 21.0ºC. Two experimental groups were defined, consisting of either four tanks for the positive control diet (1% DHA) or eight tanks for the negative control diet (5% DHA). Three

152 weeks after the start of trial (35 dph), larvae from each tank were individually counted
153 and 200 larvae per tank removed for analytical sampling with the remaining larvae
154 placed into three tanks per treatment. In addition, the remaining larvae from the 5%
155 DHA group were divided into two groups (3 tanks per treatment), with one group
156 continuing to be fed with the same diet and the other group switched to a diet containing
157 1% DHA (5%+1% DHA; "wash-out") for a further two weeks until the end of the
158 experiment (49 dph).

159 All tanks were supplied with filtered sea water (34 g/L salinity) at an increasing
160 rate of 1.0 - 1.5 L/min during the feeding trial. Water entered the tank from bottom to
161 top; water quality was tested daily with no deterioration observed. Water was
162 continuously aerated (125 ml/min), attaining 5-8 g/L dissolved O_2 and saturation
163 ranging between 60 and 80%.

164 Two isonitrogenous and isolipidic experimental microdiets (pellet size<250 μm)
165 similar in their EPA content and different in DHA content were formulated (Table 1)
166 using concentrated fish oils EPA50 and DHA50 (CRODA, East Yorkshire, England,
167 UK), as sources of EPA and DHA and DL- α- Tocopheryl Acetate (Sigma-Aldrich,
168 Madrid, Spain) as source of α-TOH. Diets were chosen based on previous trials results
169 (Betancor et al., 2011; in press). A positive control diet was formulated to include 1 g
170 DHA/100 g DW and 150 mg α-TOH/100 g DW (diet 1% DHA). The negative control
171 diet consisted of 5 g DHA/100 g DW and 150 mg α-TOH/100 g DW (diet 5% DHA).
172 The protein source, squid meal, was defatted 3 consecutive times with a
173 chloroform:squid meal ratio of 3:1 to allow complete control of the fatty acid profile of
174 the microdiet. The microdiet was based on defatted squid meal (2.4% lipid content) with
175 EPA50 and DHA50 added in different quantities to obtain the desired ratios. Oleic acid
176 (Merck, Darmstadt, Germany) was added to equalize the lipid content in each diet

(Table 1). The microdiets were prepared according to Liu et al. (2002) by first mixing the squid powder and water soluble components followed by the lipid and fat soluble vitamins and, finally, warm water dissolved gelatine. The paste was pelleted and oven dried at 38ºC for 24 h. Pellets were ground and sieved to obtain a particle size below 250 µm. Diets were analyzed for proximate and fatty acid composition on a dry weight basis and manually supplied; fourteen times per day every 45 min from 9:00-19:00. Daily feed supplied was 2, 2.5 and 3 g/tank during the first, second and third week of feeding respectively.

2.2- Growth and survival

Final survival was calculated by individually counting live larvae at the beginning, middle and end of the experiment. Growth was determined by measuring dry body weight (105ºC for 24 hours) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish/tank at the beginning (14 dph), middle (35 dph) and final (49 dph) of the experimental trial.

2.3- Biochemical analysis

At 35 dph, larvae from the two treatments were manually counted and 150 larvae removed from each tank, washed with distilled water and kept at -80ºC for biochemical analysis. Similarly, at the end of the experimental trial (49 dph), following a 12 hour starvation period, all remaining larvae per tank were washed with distilled water, sampled and kept at -80ºC until analysis for biochemical composition, fatty acid methyl

esters (FAMEs), α-TOH and TBARS. Moisture, protein (A.O.A.C., 1995) and lipid (Folch et al., 1957) contents of larvae and diets were analyzed.

2.3.1- Total lipid fatty acid analysis

Fatty acid methyl esters (FAMEs) were obtained by transmethylation of total lipids as described by Christie (1982). FAMEs were separated by GLC and quantified by a flame ionization detector (FID; GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo et al. (1992) and identified by comparison to previously characterized standards and by GLC-MS.

2.3.2- Determination of α-TOH *content*

α-TOH concentrations were determined in diets and total larvae by using HPLC. Samples were weighed, homogenised in pyrogallol and saponified as described by McMurray et al. (1980) for diets and to Cowey et al. (1981) for larval tissues. HPLC analysis was performed using 150 x 4.60 mm reverse phase Luna 5µm C18 column (Phenomenox, California, USA). The mobile phase was 98% methanol supplied at a flow rate of 1.0 ml/min, the effluent from the column was monitored at a wavelength of 293 nm and quantification achieved by comparison with (+)-α-tocopherol (Sigma-Aldrich, Madrid, Spain) as external standard.

2.3.3- Measurement of thiobarbituric acid reactive substances (TBARS)

The measurement of TBARS in triplicate samples was performed using a method adapted from Burk et al. (1980). Approximately 20-30 mg of larval tissue per sample were homogenized in 1.5 ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% butylated hydroxytoluene in methanol. To this, 2.95 ml of freshly prepared 50mM

thiobarbituric acid solution was added before mixing and heating for 10 minutes at 100ºC. After cooling protein precipitates were removed by centrifugation (Sigma 4K15, Osterode am Harz, Germany) at 2000 x *g* and the supernatant read in a spectrophotometer (Evolution 300, Thermo Scientific, Cheshire, UK) at 532 nm. The absorbance was recorded against a blank at the same wavelength. The concentration of TBA-malondialdehyde (MDA) expressed as µmol MDA per g of tissue was calculated using the extinction coefficient 0.156 μM^{-1} cm^{-1}.

2.4- Histopathological sampling

Thirty larvae from each tank were collected every seven days from the beginning of the feeding trial for histopathological analysis. Larvae were fixed in 10% buffered formalin, dehydrated through graded alcohols, then xylene, and finally embedded in paraffin wax. Six paraffin blocks containing 5 larvae per tank were sectioned at 3 µm, and stained with Hematoxylin and Eosin (H&E; Martoja and Martoja-Pearson, 1970).

Additionally, ten larvae per tank were fixed for 24 hours at 4ºC in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). Samples were then rinsed in phosphate buffer and post-fixed for 1 hour in 2% osmium tetra oxide in 0.2 M potassium ferrocyanide. Each larva was then embedded in an Eppon/Araldite resin block. Serial transverse and longitudinal larvae thick sections were cut at 1 µm, stained with toluidine blue and examined under light microscopy (Hoffman et al, 1983).

2.5- Gene expression analysis

2.5.1- Total RNA extraction and quantitative real time RT-PCR

Molecular biology analyses were carried out at the University of Insubria (Varese, Italy) using 14, 26, 35 and 49 dph larvae. Total RNA was extracted from European sea bass larvae (≈200 mg; pool per tank) using PureYield RNA Midiprep System (Promega, Italy). The quantity and purity of RNA was assessed by spectrophotometer. Visualization on 1% agarose gel stained with ethidium bromide showed that RNA was not degraded. After DNAse treatment (Invitrogen, Milan, Italy), 3 µg of total RNA was reverse transcribed into complementary DNA (cDNA) in a volume of 12 µl, including 1 µl of oligo dT16 primer (50 pmol) and 1 µl of 10 mM deoxynucleotide triphosphates (dNTPS). This mix was heated at 65ºC for 5 minutes, chilled on ice before 4 µl of 5X reverse transcription buffer, 2 µl 0.1M DTT, 1 µl RNAse out and 1 µl of Moloney murine leukemia virus reverse transcriptase (M-MLVRT) were added. After incubation at 37ºC for 50 minutes, the reaction was stopped by heating at 75ºC for 15 minutes.

2.5.2- Generation of in vitro-transcribed mRNAs for standard curves

We quantified the transcript copies of each target gene (CAT, SOD, GPX, IGF-I, IGF-II, α-actin, MyHC and Capn1) in *D. labrax* larvae by using the method of standard curve also known as the absolute method of real time quantification. Calibration curves are highly reproducible and allow the generation of highly specific, sensitive and reproducible data. The standard curves we used, were based on known concentrations of mRNAs, synthesized *in vitro* for each target gene. For this, a forward and a reverse primer were designed based on each gene's mRNA sequence (Table 2). These primer pairs were used to create templates for the *in vitro* transcription of either CAT, SOD, GPX, IGF-I, IGF-II, α-actin, MyHC, or Capn1 mRNAs: the forward primers were engineered to contain a T3 phage polymerase promoter gene sequence to its 5′ end and

used together with the reverse primer in a conventional RT-PCR of total sea bass larvae RNA. RT-PCR products were then evaluated on a 2.5% agarose gel stained with ethidium bromide, cloned using pGEM®-T cloning vector system (Promega, Italy), and subsequently sequenced in T7 and SP6 directions.

In vitro transcription was performed using T3 RNA polymerase and other reagents supplied in the Promega RiboProbe *In Vitro* Transcription System kit according to the manufacturer's protocol.

The molecular weight (MW) of the *in vitro*-transcribed mRNAs for each gene was calculated according to the following formula:

MW= (n° of A bases × 329.2) + (n° of U bases × 306.2) + (n° of C bases × 305.2) + (n° of G bases × 345.2) + 159

2.5.3- Generation of standard curves for target genes

The mRNAs produced by *in vitro* transcription were then used as quantitative standards in the analysis of mRNA copies for each target gene in sea bass larvae. For this, defined amounts of *in vitro* transcribed mRNAs (up to nine orders of magnitude from $<10^2$ to $>10^{10}$ start molecules) and total RNA extracted from the sea bass samples, were analyzed together (in the same 48 wells plate) *via* Taqman® real-time RT-PCR using One-step TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Italy), including 1×Taqman buffer, 3 mM $Mn(OAc)_2$, 0.3 mM dNTP except dTTP, 0.6 mM dUTP, 0.3 μM forward primer, 0.3 μM reverse primer, 0.2 μM FAM-6 (6-carboxyfluorescein-labeled probe), 5 units rTH DNA polymerase, and 0.5 units AmpErase® UNG enzyme in a 25-μL reaction. AmpErase® uracil-N-glycosylase (UNG) is a 26-kDa recombinant enzyme encoded by the Escherichia coli uracil-N-glycosylase gene. UNG acts on single-and double-stranded dU-containing DNA, hydrolyzing uracil-

glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA. For TaqMan® assays, AmpErase® UNG treatment can prevent the reamplification of carry over PCR products from previous PCR reactions. When dUTP replaces dTTP in PCR amplification, AmpErase UNG treatment can remove up to 200,000 copies of amplicon per 50 µL reaction. RT-PCR conditions were: 2 min at 50 °C, 30 min at 60 °C, and 5 min at 95 °C, followed by 40 cycles consisting of 20 s at 92 °C, 1 min at 62 °C. The Ct values obtained by amplification were used to create standard curves for target genes.

2.5.4- Target genes transcripts quantification by one-step TaqMan® real-time RT-PCR

Total RNA (100 ng) extracted from European sea bass larvae samples were analyzed *via* TaqMan® real-time PCR, in parallel to triplicates of 10-fold-diluted defined amounts of standard mRNAs, under the same experimental conditions as those used to establish the standard curves. Real-time Assays-by-Design SM PCR primers and TaqMan® gene-specific fluorogenic probes were designed by Applied Biosystems (Table 2).

TaqMan® RT-PCR was performed on a StepOne Real Time PCR System (Applied Biosystems). To reduce pipetting errors, master mixes were prepared to set up triplicate reactions (3×30 µL) for each sample.

Data from the TaqMan® RT-PCR runs were collected with StepOne™ Software v2.0. Cycle threshold (CT) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were used to create standard curves to calculate the absolute amounts of mRNA in total RNA extracted from sea bass larvae.

326

327 **2.6- Statistical analysis**

328 Statistical analysis was performed using SPSS software (SPSS for Windows 14.0;
329 SPSS Inc., Chicago, IL, USA, 2005). Survival, growth, fatty acids methyl esters and
330 molecular biology data were tested for normality and homogeneity of variances with
331 Levene´s test. Where necessary data were log or arcsin transformed before statistical
332 analysis. A Chi-squared test was employed for incidence of muscular lesions and
333 TBARS content. Survival, growth and biochemical analysis data were treated using
334 one-way ANOVA and molecular biology results by general linear model (GLM) with
335 means compared by Duncan's test (Sokal and Rolf, 1995). Results are presented as
336 means and standard deviation. Tank was considered the experimental unit, except for
337 the estimation of the incidence of muscular lesions, where each individual larvae was
338 considered as a unit. For analysis of one-way ANOVA the following general linear
339 model was used:

340 $$Y_{ij} = \mu + \alpha_i + \varepsilon_{ijk}$$

341 where Y_{ijk} is the mean value of the tank, μ is the mean population, α_i is the fixed effect
342 of the diet and ε_{ij} is the residual error. For analysis of molecular biology data a two
343 variables GLM was used to analyze possible interactions between treatment and time:

344 $$Y_{ijk} = \mu + \alpha_i + \delta_j + (\alpha\delta)_{ij} + \varepsilon_{ijk}$$

345 Where Y_{ij} is the mean value of the tank, μ is the mean population, α_i is the fixed effect
346 of the diet, δ_j is the fixed effect of the time, $(\alpha\delta)_{ij}$ is the interaction between diet and
347 time and e_{ij} is the residual error. Significance was accepted at $P \leq 0.05$.

348

349 **3- Results**

350

3.1 Growth and survival

All experimental diets were well accepted by larvae. Dietary DHA increases did not have any effect on growth at 35 dph. However, at 49 dph a higher dry weight was observed in 5% DHA fed larvae (Table 3). No significant differences were observed in either total length or larval survival at any of the sampling points (Table 3).

3.2 Biochemical analysis

Diet composition was similar among treatments (Table 4). Fatty acids analysis revealed a higher percentage of total n-3, in particular and n-3 LC-PUFA, in the 5% DHA diet due to the increased DHA content, whereas the 1% diet had a higher monounsaturated level, primarily n-9, due to a higher oleic acid content (Table 4).

At 35 dph larval FA composition closely reflected that of the microdiets fed. Thus, levels of 22:6n-3, n-3 and n-3 LC-PUFA were significantly lower ($P<0.05$) in larvae fed the 1% DHA diet (Table 5). At 49 dph, 2 weeks after starting the "wash-out" period, 5%+1% DHA larvae were similar in 18:4n-3, 18:1n-9, 22:6n-3, monoenoics, n-6 and n-9 contents compared to larvae fed 1% DHA diet (Table 5). However, total n-3 content and n-3 LC-PUFA were similar to those of larvae fed both 1% and 5% DHA diets.

The highest TBARS content (µmol/g larval tissues), an indicator of lipid peroxidation, was detected with an increase in DHA level at 35 dph ($P=0.001$; Table 3). TBARS increased from 35 to 49 dph in all treatments, with a higher value in 5% DHA larvae ($P=0.025$; Table 3). Although larvae fed 5%+1% DHA diet showed higher TBARS content than larvae fed 1% DHA diet, no significant differences were found ($P=0.539$; Table 3).

The α-tocopherol content of European sea bass larvae increased throughout the experimental period, increasing by five times by 49 dph compared to 14 dph for all treatments. At 35 dph, larvae fed the 5% DHA diet did not reflect dietary content of α-TOH, but showed a lower α-TOH value compared to larvae fed 1% DHA diet ($P<0.05$; Table 3). However, no differences in α-TOH content (in dry weight) were observed at 49 dph between larvae fed 1% and 5% DHA diet ($P>0.05$), with the lowest α-TOH value found in larvae fed 5%+1% DHA diet.

3.3 Histopathological evaluation

Histopathological examinations revealed the presence of lesions affecting European sea bass larvae axial musculature. These lesions showed the typical features of necrotic degeneration of muscle, characterized by marked eosinophilia, loss of striations and adjacent nucleus. The incidence of muscular lesions increased with DHA dietary content (Table 3), two times higher in 5% DHA than 1% DHA fed larvae by day 35. The change of diet decreased the incidence of muscular lesions by 49 dph. However, the lowest incidence of muscular lesions was found in larvae fed the low DHA diet (1% DHA). Conversely, incidence of muscular lesions decreased from 35 to 49 dph.

More detailed features of these muscular lesions could be observed in semithin sections. In severely damaged fibres a coagulation of the muscular proteins could be observed as a darkening of affected fibres due to hypercontraction (Figure 1.A). In initial-mild stages of the condition, an increase in the presence of vacuoles within fibres was observed, together with the shape loss of muscular fibres, alteration of sarcoplasmic membranes, and variation in the diameter of fibres (Figure 1.A), especially if compared to the non injured muscular fibres (Figure 1.B).

3.4 Gene expression analysis

The general pattern of antioxidant enzymes gene expression in all groups of European sea bass larvae was characterised by a rapid increase between 14 and 26 dph, followed at 35 dph by a decrease to levels slightly higher than those observed at 14 dph (Figure 2). CAT and SOD mRNA copies were higher in larvae fed 5% DHA diet than in those fed 1% DHA at 26 and 35 dph ($P<0.05$; Figure 2.A and 2.B). GPX expression levels varied throughout the experimental period: at 26 dph, no differences were observed ($P=0.551$; Figure 2.C), but a rapid decrease was detected in 5% DHA fed larvae at 35 dph ($P=0.012$). "Wash-out" influenced the AOE expression in European sea bass larvae: expression of SOD and GPX was increased in 5%+1% DHA ($P<0.05$) fed larvae. Conversely, CAT expression remained similar in "washed-out" larvae compared to larvae fed 1% DHA or 5% DHA diet ($P=0.166$; Figure 2.A).

IGF-I expression levels showed an initial up regulation (from 14 to 26 dph) in both 1% DHA and 5% DHA larvae, with significantly higher values in larvae fed 5% DHA diet ($P=0.008$). At 35 dph, IGF-I mRNA copies showed a further increase in larvae fed 5% DHA diet (Figure 3.A), whereas it decreased in larvae fed 1% DHA diet ($P=0.018$). Conversely, at 49 dph a higher mRNA abundance was found in 1% DHA fed larvae ($P=0.002$). At the same sampling point, "washed-out" larvae showed an expression pattern similar to that observed in 5% DHA fed larvae.

With regards to IGF-II expression, 1% and 5% DHA larvae showed a similar pattern from 14 to 35 dph (Figure 3.B). At 49dph, there was a 1.2 fold increase in IGF-II expression of 1% DHA fed larvae compared to 35 dph. The expression levels of IGF-II at this sampling point were significantly higher than those found in 5% DHA fed larvae ($P=0.001$). "Washed-out" larvae displayed the same IGF-II expression pattern of that showed by 1% DHA fed larvae (Figure 3.B).

425 The expression pattern of genes encoding muscular proteins, showed an increase
426 between 14 and 26 dph, followed by a decrease at 35 dph in both 1% and 5% DHA fed
427 larvae (Figure 4). α-actin expression did not show differences in its expression through
428 time (P>0.05; Figure 4.A). Conversely, one-way ANOVA of α-actin mRNA copies at
429 day 49 indicated that the "washed-out" larvae exhibited the lowest expression levels of
430 this gene (P=0.005). MyHC expression levels showed a higher value in larvae fed diet
431 1% DHA by 26 dph (P=0.049; Figure 4.B), whereas no differences were found at 35
432 (P=0.083) or at 49 dph (P=0.166) between 1% and 5% DHA fed larvae. Similarly to α-
433 actin expression, MyHC mRNA copies in "washed-out" larvae were lowest at 49 dph
434 (P=0.0017).
435 In contrast to MyHC, the "wash-out" period increased Capn1 mRNA copies to
436 levels comparable with larvae fed 1% DHA (Figure 4.C). No differences were observed
437 between 1% and 5% DHA fed larvae at 26 dph. However, a higher expression
438 (P=0.045) was found by day 35 post hatching in larvae fed 5% DHA diet.
439 GLM analysis denoted a marked interaction between dietary treatments and
440 sampling points ($P \leq 0.01$) for all the target genes, with the exception of SOD and α-
441 actin, which, conversely, did not show differences among diets and sampling points
442 (Table 6).
443 **3- Discussion**
444 Feeding European sea bass larvae with high dietary DHA contents, without the
445 adequate combination of antioxidants, has deleterious effects on axial musculature,
446 leading to the appearance of muscle dystrophy, as has been previously described
447 (Betancor et al., 2011; Betancor et al., in press). However, there is limited information
448 about the molecular response of larval muscular tissue to this pathological alteration. In
449 the present study, the interrelations between the gene expression of MyHC and α-actin,

450 the most abundant proteins in skeletal muscle, μ-calpain, a Ca^{+2}-activated proteolytic
451 enzyme and IGFs was examined together with AOE gene expression.

452 Although α-TOH contents of all diets were the same (150 mg/100 g DW), at 35
453 dph the highest α-TOH levels were found in larvae fed the lowest level of DHA,
454 indicating that α-TOH is influenced by dietary DHA ratio. This suggests that larvae fed
455 higher DHA levels utilized more α-TOH as an antioxidant to protect their tissue lipids
456 from the increased oxidation risk. These results are similar to previous studies where α-
457 TOH concentration in juvenile or adult fish were lower when high contents of n-3 LC-
458 PUFA were included in diets (Puangkaew et al., 2005). However, at 49 dph α-TOH
459 contents in European sea bass larvae fed diets with 1 and 5% of DHA became similar.
460 This could be related to the dependency of younger larvae on low molecular weight
461 antioxidants, such as α-TOH to defend from free radicals, as their AOE systems are still
462 poorly developed (Rudneva, 1999). Therefore, from 49 dph an enhancement in AOE
463 activity could effectively quench ROS, without the need of oxidizing α-TOH.
464 Conversely, an inverse relationship was observed between TBARS and α-TOH
465 contents, with an increase of this aldehyde observed at 49 dph. Similar findings have
466 been described in Senegalese sole (*Solea senegalensis*) larvae (Solé et al., 2004). These
467 results may indicate that despite of increase in AOE, this was not enough to counteract
468 ROS formation.

469 As expected, the different dietary fatty acid compositions were reflected in the
470 fatty acid compositions of larvae. Thus, at 35 dph larvae fed the high DHA diet resulted
471 in increased body levels of total n-3 LC-PUFA, particularly DHA, whereas the low
472 dietary DHA larvae resulted in lower levels of total n-3 LC-PUFA and higher levels of
473 18:1n-9. Therefore, the potential for lipid peroxidation was theoretically higher in larvae

474 fed a diet with a 5% of DHA. This is in agreement with the high TBARS values found
475 in larvae fed high DHA diets, being more than 3 times higher at 35 dph.

476 The second phase of the experiment involved feeding larvae which were
477 previously fed for three weeks with 5% of DHA diet with the diet containing 1% of
478 DHA for a period of two weeks. This action lowered 22:6n-3, n-6 and n-3 LC-PUFA
479 levels, and increased 18:1n-9, monoenoics and n-9 levels similar to values found in
480 larvae fed the 1% DHA diet. This result proves for the first time that using a "wash-out"
481 period of only two weeks, changing from a 5 to 1% DHA diet, was enough for the
482 larvae to reflect the diet content. This short "wash-out" period contrasts with previous
483 studies in juvenile fish where "wash-out" periods of 12-18 weeks were necessary to
484 restore fatty acid compositions in Atlantic salmon (Bell et al., 2003a,b), 8-12 weeks in
485 gilthead sea bream (Izquierdo et al., 2005) or longer than 8 weeks in sunshine bass
486 (*Morone chrysops* ♀ x *M. saxatilis* ♂; Trushenki et al., 2008).

487 Nevertheless, although TBARS values were still higher in larvae fed 5% DHA at
488 49 dph, this difference was less than 2 times higher than larvae fed 1% DHA with no
489 significant differences in α-TOH levels between larvae fed both diets. Similarly, the
490 incidence of muscular lesions decreased from 35 to 49 dph in 5% DHA larvae. It must
491 be noted that, in contrast to our previous studies, larvae from current investigation were
492 fed from 14 to 49 dph with an experimental dry basis diet only, levels not previously
493 tested for such a long period of time. It has been shown that the time or duration of
494 feeding is an important additional factor to consider in relation to determining the
495 biochemical responses to oxidative stress (Mourente et al., 2002). On the basis of the
496 present results, it seems that from 35 dph, a diet containing 5% of DHA could be
497 suitable for European sea bass larvae weaning, in combination with the adequate
498 amounts of antioxidant nutrients. In agreement with this, larvae switched to 1% DHA

diet exhibited a lower dry weight and α-TOH content compared to larvae fed 5% DHA diet, indicating that it would be appropriate to feed larvae on DHA contents of 1% during the first five to six weeks after hatching and increasing the DHA content to 5% after this period, as diminished negative effects associated to an altered oxidative status were observed in 5% DHA larvae by 49 dph. Similarly, Villeneuve et al. (2006) found that the earlier that European sea bass larvae were fed with a high marine phospholipid content microdiet, the greater its negative effects upon larval growth, with larvae more resistant to this treatment at 40 dph. Accordingly, in 5% DHA larvae CAT and GPX expression decreased to levels comparable to larvae fed 1% DHA diet at 49 dph.

From 14 to 35 dph, when European sea bass larvae were exposed to high DHA dietary contents (5%), the induction of AOE genes coincided with increases in TBARS contents. This is in agreement with previous studies in this species (Betancor et al., in press) and Manchurian trout (*Brachymystax lenok*) (Zhang et al., 2009), in which young fish fed high lipid levels had higher TBARS contents, inducing an antioxidant response noticeable as an increase in the activity of AOE. Moreover, in the present study, a marked increase in the expression of each AOE was observed from 14 to 26 dph, whereas exposure to a high dietary DHA content caused a significant increase in CAT and SOD gene expression in larvae fed the 5% DHA diet at both 26 and 35 dph. Similarly, juvenile rainbow trout (*Oncorhynchus mykiss*) fed with high PUFA contents, displayed significantly higher SOD activities (Trenzado et al., 2009). Nevertheless, at 49 dph CAT and SOD were less induced, suggesting an adjustment in the expression of these two genes at the last sampling point, after the supra-induced expression found in the former sampling at 35 dph. This would also explain why "washed out" larvae showed the highest mRNA copies of SOD and GPX, as larvae may still be adapting to the new dietary treatment. Equally, CAT and SOD activities would be expected to

parallel each other based on the known action mechanisms of these two enzymes together with the fact that superoxide anions are efficiently scavenged by α-TOH in biological systems (Cay and King, 1980). Thus, the increase in SOD expression observed in 5%+1% DHA larvae at 49 dph, accompanied by a decrease in α-TOH content, could indicate an increase in the production of superoxide anion radical and the attempt of these two antioxidant mechanisms to quench such ROS. Conversely, GPX expression differed from the other AOEs, being significantly higher in larvae fed 1% DHA by 35 dph. Both CAT and GPX have the capability to remove hydrogen peroxide, thus it would be expected to see a certain relationship between these two enzymes as that observed in *Dentex dentex* larvae (Mourente et al., 1999). Accordingly, in the present study, GPX expression was reduced, whereas CAT gene expression was increased in larvae fed high DHA levels. It must be noted that the reduced GPX expression observed would reduce the larval tissues capability to cope with hydrogen peroxide, leading to a likely increase in CAT activity as an adaptation process (Mahfouz and Kummerow 2000). SOD gene expression was also affected by elevated dietary DHA content. This suggests that larval tissues would be able to convert more superoxide radicals to hydrogen peroxide, which is in agreement with the high CAT expression found.

AOE and low molecular weight antioxidants are known to form a primary defence against lipid peroxidation, being mainly preventive (Girotti, 1998). Among AOE, GPX seems to be the only enzyme able of detoxifying fatty acid hydroperoxides, thereby acting as a secondary defence line. Thus, a high GPX gene expression in larvae fed high DHA diets was expected, as this fatty acid is being oxidized. However, a low GPX expression was found in larvae fed a 5% of DHA. It must be noted that other enzymes, different from GPX, with peroxidative activity have been implicated in lipid

549 hydroperoxide detoxification. Phospholipid hydroperoxide glutathione peroxidase
550 (PHGPX or GPX 4) can act directly on phospholipid hydroperoxides in membranes
551 (Ursini et al., 1991), whereas other GPXs are unreactive unless *sn*-2 fatty acyl bonds are
552 cleaved to liberate fatty acid hydroperoxides (van Kuijka et al., 1987). In our case,
553 cytosolic GPX or GPX 1 expression has been determined, indicating that this enzyme
554 may not be as active as PHGPX in case of DHA *in vivo* oxidation.

555 The role of IGF-I and –II in regulating growth and the profound effect of the
556 nutritional status on the IGF system in fish has received much attention in the recent
557 years (Carnevali et al., 2006; Terova et al., 2007; Mazurais et al., 2008; Enes et al.,
558 2010; Hevrøy et al., 2011; Darias et al., 2011; Fernández et al., 2011). However, there
559 are no studies on the implication of IGFs in the healing of musculoskeletal tissue in fish,
560 particularly marine fish larvae. The progressive increase of IGF-I expression in 1%
561 DHA larvae in the present study was in agreement with the high cell proliferation rate,
562 and/or the increase in specific cell activity in different tissues during larval
563 morphogenesis (Perrot et al., 1999; Patruno et al., 2008; Fernández et al., 2011).
564 However, an abrupt increase in the expression of IGF-I was observed in larvae fed 5%
565 DHA diet, which also showed the highest incidence of muscular lesions. In mammals,
566 IGF-I levels are upregulated in skeletal muscle undergoing regeneration (Chargé and
567 Rudnicki, 2004), suggesting that the increase of this peptide could be associated to
568 regenerative processes in European sea bass larvae. Indeed, in a previous study
569 (Betancor et al., 2011) abundant satellite cells, which are increased during the first
570 phase of muscle regeneration, were detected in injured muscle of European sea bass
571 larvae. In fine flounder (*Paralichthys adspersus*) (Fuentes et al., 2011), an
572 overexpression of IGF-I, resulted in greater skeletal mass, thus, the increase in the

573 expression of IGF-I observed in European sea bass larvae could be related to a
574 compensatory muscle reaction in response to the injuries caused by ROS.

575 Contrastly, an increase in MyHC expression has been described in mammalian
576 muscle undergoing regeneration processes (Järva et al., 1997). Initially, muscular
577 regeneration is dominated by a proliferative phase during which satellite cells and
578 fibroblasts increase in number (Schultz and McCormick, 1994). Satellite cells then fuse
579 to form myotubes regenerating the muscle, and transcription of muscle-specific genes,
580 such as myosin and actin, takes place in the new muscle fibres. Therefore, the higher
581 MyHC expression in 5% DHA fed larvae from 26 to 49 dph would indicate that a
582 regeneration process is taking place. This was confirmed by the high IGF-I expression
583 found in 5% DHA larvae from day 26, as this peptide potently activates cell
584 proliferation and DNA synthesis *via* mitogen-activated protein kinase (MAPK) and
585 phosphatidylinositol 3-kinase (PI3 kinase) as reported in zebrafish (Pozios et al., 2001).
586 Additionally, IGF-I has been showed to increase MyHC protein in denervated skeletal
587 muscle in mouse (Shanely et al., 2009). However, the highest number of MyHC mRNA
588 copies were found in 1% DHA larvae at 26 dph, probably mirroring muscular growth
589 and larval development at this stage, as the myosin transcripts abundance has reported
590 to be a potential biochemical marker for growth in rainbow trout (Overturf and Hardy,
591 2001), and spotted wolffish (*Anarhichas minor*; Imsland et al., 2006).

592 In contrast to IGF-I, IGF-II expression was constant and decreased in larvae
593 from both dietary treatments until 35 dph. Transcript levels of this gene were much
594 higher than those for IGF-I, coinciding with the results in sea bream larvae (Radaelli et
595 al., 2003), suggesting that IGF-II acts earlier than IGF-I in myogenesis. IGFs expression
596 in "washed-out" larvae showed irregular patterns, decreasing for IGF-I and increased
597 for IGF-II, supporting the idea that different hormonal signals and mechanisms of gene

transcription control and regulate the expression of both IGF forms (Canalis et al., 1991). From 35 dph on there is a marked decrease in both IGFs expression in larvae the fed 5% DHA diet, in which a high expression of this gene would be expected due to regeneration processes. No information is available about the molecular process of regeneration in fish larvae after a chronic insult, like ROS attack. It must be noticed that IGFs exert their effects on cells through the binding to the IGF receptors (IGF-R). An increase in IGF-IR receptors has been described in cultured trout muscle cells in response to cell differentiation (Castillo et al., 2004). Nonetheless, a critical element in the action of IGF and their receptors are the role of IGF binding proteins (IGFBPs), which influence IGF function by enhancing or inhibiting their action (Reinecke et al., 2005). Conversely, in mammals, it is known that certain cytokines, such as tumour necrosis factor (TNF), produced as a result of any inflammatory process like muscle injury, can inhibit the action of IGF and has been demonstrated in chronic muscular diseases (Grounds et al., 2008; Gebski, 2009). A full understanding of IGFs function in fish muscle regeneration requires further work.

Regarding α-actin expression, no differences between the 1% and 5% DHA fed larvae were observed, nor was any correlation to MyHC or IGF-I, indicating that it could not be associated with regeneration process. Although it has been stated that IGF-I induces sarcomeric actin filament formation in mammals (Takano et al., 2010), a relationship between both molecules could not be proved in the present study. However, it must be noted that several α-actin isoforms have been described in fish (Morita, 2000), thus, the reduction of the transcript content observed may reflect the switch to the expression of a different actin isoform that could not be detected in this study. "Wash-out" had a negative effect (decreased transcript levels) on genes encoding for myofibrillar proteins expression such as MyHC and α-actin.

One of the first alterations observed in European sea bass larvae muscle is due to the attack of ROS against the sarcoplasmic membrane, causing dysregulation of cell volume and massive intracellular increase in Ca^{2+} (Cotran et al., 2004). Similarly, elevated intracellular Ca^{2+} concentrations have been found in muscular dystrophies and other muscle pathologies in mammals, with this elevated Ca^{2+} concentration stimulating calpain activity (Mongini et al., 1988; Hopf et al., 1996). Accordingly, a higher expression level of μ-calpain was found in 5% DHA fed larvae, which also showed the highest incidence of muscular lesions. In agreement with this, high levels of calpain were found in rapidly atrophying muscles in rabbits fed diets deficient in α-TOH (Dayton et al., 1979). Moreover, some authors indicate that calpain activity is required for myoblasts to progress in the mitotic cycle (Zhang et al., 1997) as well as for myoblast fusion (Kwak et al., 1993; Temm-Grove et al., 1999), suggesting that decreased calpain activity during muscle development may be associated with an increased number of myoblasts. However, whole larval tissues were used for the gene expression analysis, therefore, the expression obtained does not refer exclusively to the muscle and the response of other tissues could be affecting the gene expression.

In conclusion, an increased oxidative stress in European sea bass larvae fed high dietary DHA contents may account for the high occurrence of muscular lesions observed in 5% DHA larvae. Supplementation with 150 mg/100g of α-TOH did not counteract the negative effects of oxidative stress when 5 g/100 g of DHA were included in the microdiets. European sea bass larval antioxidant defence enzymes appeared to respond strongly to high DHA contents, shown by the high expression of CAT and SOD, although elevated lipid oxidation products were observed. High MyHC and IGF-I mRNA copies might indicate muscle regeneration. However α-actin and IGF-II expression did not support these results, suggesting the implication of different

regulation mechanisms of these gene´s transcription. Calpn1 transcript levels were elevated in 5% DHA larvae as pointed out by muscle dystrophy in mammals. Thus, high DHA contents in European sea bass larvae diets leads to an alteration of the oxidative status and to the appearance of muscular lesions, with the morphological and molecular aspects of mammal muscular degenerative disease. Moreover, the "wash-out" period indicated that DHA contents of 5% would suit European sea bass larvae requirements from 35 dph, probably due to a maturation of their antioxidant defence systems. Further studies are needed in order to improve our understanding of the molecular pathways underpinning regeneration processes in marine fish larvae.

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955 **Figure legends:**
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Figure 4: α-actin, MyHC and μ-calpain expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1% DHA (○), 5% DHA (■) or 5+1% DHA (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. Different superscript letters denote significant differences between treatments within a sampling point ($P<0.05$).

Table 1. Formulation of experimental diets

Dietary DHA/vitamin E	*1% DHA*	*5% DHA*
Defatted squid powder (g/100g)[*]	69.00	69.00
EPA g $100g^{-1}$ (DW)[†]	2.80	1.80
DHA g $100g^{-1}$ (DW) [†]	0.20	6.70
Oleic acid (%)[‡]	10.00	4.50
Soy lecithin[§]	2.00	2.00
Gelatin	3.00	3.00
Attractants	3.00	3.00
Taurin	1.50	1.50
Vitamin premix[**]	6.00	6.00
Mineral premix[††]	2.50	2.50

[*] Riber and Son, Bergen, Norway
[†] Croda, East Yorkshire, UK
[‡]Merck, Darmstadt, Germany
[§] Acrofarma, Barcelona, Spain
[**] Vitamin premix supplied per 100g diet: Cyanocobalamine, 0.030; Astaxanthin, 5.00; folic acid, 5.44; pyridoxine-HCI, 17.28; thiamine, 21.77; riboflavin, 72.53; Ca-pantothenate, 101.59; p-aminobenzoic acid, 145.00; nicotinic acid, 290.16; *myo*-inositol, 1450.90; retinol acetate, 0.180; ergocalcipherol, 3.650; menadione, 17.280; α-tocopherol acetate, 150.000
[††]Mineral premix supplied g per 100g diet: NaCl, 215.133; $MgSO_4 \cdot 7H_2O$, 677.545; $NaH_2PO_4 \cdot H_2O$, 381.453; K_2HPO_4, 758.949; $Ca(H_2PO_4) \cdot 2H_2O$, 671.610; $FeC_6H_5O_7$, 146.884; $C_3H_5O_3 \cdot 1/2Ca$, 1617.210; $Al_2(SO_4)_3 \cdot 6H_2O$, 0.693; $ZnSO_4 \cdot 7H_20$, 14.837; $CuSO_4 \cdot 5H_2O$, 1.247; $MnSO_4 \cdot H_2O$, 2.998; KI, 0.742; $CoSO_4 \cdot 7H_2O$, 10.706

Table 2. Gene abbreviations, GenBank accession numbers, PCR primer and TaqMan® probe sequences for analysed reference genes.

Gene	Accesion nº	Component	Forward primer (5´-3´)
CAT	FJ860003	Forward	ATGGTGTGGGACTTCTGGAG
		Reverse	GCTGAACAAGAAAGACACCTGATG
		TaqMan® Probe	CAGACACTCAGGCCTCA
SOD	FJ860004	Forward	TGGAGACCTGGGAGATGTAACTG
		Reverse	TCTTGTCCGTGATGTCGATCTTG
		TaqMan® Probe	CAGGAGGAGATAACATTG
GPX	FM013606	Forward	AGTTAATCCGGAATTCGTGAG
		Reverse	AGCTTAGCTGTCAGGTCGTAAAAC
		TaqMan® Probe	AATGGCTGGAAACGTG
IGF-I	AY996779	Forward	GCAGTTTGTGTGTGGAGAGAGA
		Reverse	GACCGCCGTGCATTGG
		TaqMan® Probe	CTGTAGGTTTACTGAAATAAAA
IGF-II	AY839105	Forward	TGCAGAGACGCTGTGTGG
		Reverse	GCCTA CTGAAATAGAAGCCTCTGT
		TaqMan® Probe	CAAACTGCAGCGCATCC
MyHC	DQ317302	Forward	TGGAGAAGATGTGCCGTACTCT
		Reverse	CGTGTCATTGATTTGACGGACATTT
		TaqMan® Probe	AACTGAGTGAACTGAAGACC
α-actin	FJ716131	Forward	CCTCTTCCAGCCTTCCTTCA
		Reverse	TGTTGTAGGCGGTCTCATGGATA
		TaqMan® Probe	CCAGCAGACTCCATACCGA
Capn1	FJ821591	Forward	ACTTTACAGGCGGCGTGA
		Reverse	GGCTCTGCTGATGATGTTGTAGA
		TaqMan® Probe	TCAGATCGTACATTTCCG

Table 3. Sea bass larvae performance, levels of lipid peroxidation products (TBARS) and vitamin E (α-tocopherol) content of sea bass larvae at the beginning, middle and at the end of the experimental trial.

		Diets				
	Initial	35 dph		49 dph		
		1% DHA	5% DHA	1% DHA	5% DHA	5%+1% DHA
Results of dietary trial						
Larval total length (mm)	8.58 ± 0.64	12.60 ± 0.93	12.06±1.55	12.68±1.24	12.99±1.37	13.12±1.53
Larval dry weight (mg)	0.36 ± 0.00	1.46 ± 0.47	1.26±0.12	1.76±0.25^{b}	2.72±0.40^{a}	1.95±0.05^{b}
Survival (%)	-	60.51 ± 9.10	51.30±1.99	44.42±6.94	57.42±15.42	39.26±10.26
Incidence muscular lesions (%)	-	13.33 ± 5.77	29.16±6.31	6.60±1.30	20.00±8.71	13.33±8.16
Vitamin E (α-tocopherol)						
μg g dry mass^{-1}	111.45±43.26	630.24±12.39^{a}	473.67±18.40^{b}	626.47±34.45^{a}	655.39±43.25^{a}	580.25±39.04^{b}
TBARS						
nMol MDA g dry mass^{-1}	62.85±0.61	166.62±25.08^{b}	527.08±14.71^{a}	536.35±68.57^{b}	1038.87±152.08^{a}	657.76±85.87^{b}

Table 4.- Gross composition and principal fatty acid methyl esters (% of total fatty acids) of the experimental diets fed to sea bass larvae.

	Diets	
	1% DHA	5% DHA
Gross composition		
Protein (%)	66.79	65.99
Ash (%)	4.49	4.73
Moisture (%)	10.31	9.99
Lipids (% DW)	14.98	15.06
α-tocopherol (μg g^{-1} DW)	1410.12	1449.80
Selenium (μg mg^{-1})	1.54	1.48
Main fatty acids		
Total Saturated	12.28	10.09
Total Monoenoics	61.99	39.05
18:3n-3 ALA	0.72	0.80
18:4n-3 SDA	0.83	0.97
20:5n-3 EPA	8.66	12.05
22:5n-3 DPA n-3	0.32	1.23
22:6n-3 DHA	4.58	23.64
Total n-3	15.68	39.51
Total n-3 LC-PUFA	13.96	37.58
18:2n-6 LNA	7.40	6.80
20:4n-6 ARA	0.71	1.34
22:5n-6 DPA	0.19	1.48
Total n-6	8.61	10.26
18:1n-9 OLA	55.70	33.84
22:1n-9	0.08	0.24
Total n-9	57.19	35.66

Table 5. Main fatty acid composition of total lipids from sea bass larvae at the beginning (14 dph), after three weeks (35 dph) and after five weeks (49 dph) of feeding the experimental diets (% total identified fatty acids)

	Diets					
	14 dph	35 dph		49 dph		
		1% DHA	5% DHA	1% DHA	5% DHA	5%+1% DHA
Total Saturated	26.17±5.43	32.01±6.29	35.62±3.85	25.90±2.20	25.20±0.49	25.53±1.06
Total Monoenoics	36.62±3.61	36.73±4.51	36.80±4.09	36.28±3.80^{a}	26.06±3.09^{b}	33.84±1.86^{a}
18:3n-3 ALA	0.80±0.27	0.32±0.05^{b}	0.85±0.07^{a}	0.36±0.04	0.34±0.04	0.27±0.06
18:4n-3 SDA	0.22±0.09	0.29±0.06^{b}	0.89±0.12^{a}	0.30±0.13	0.42±0.07	0.31±0.09
20:5n-3 EPA	7.60±0.33	5.91±1.18	5.82±0.07	8.39±1.34	6.76±0.04	8.57±0.57
22:5n-3 DPA	1.58±0.33	0.64±0.26	0.92±0.16	0.97±0.15	0.76±0.01	0.76±0.02
22:6n-3 DHA	14.22±4.36	12.79±0.37^{b}	25.12±4.24^{a}	16.30±3.32^{b}	28.36±3.38^{a}	19.58±1.48^{b}
Total n-3	25.39±2.48	20.94±2.23^{b}	34.60±3.57^{a}	27.59±5.24^{b}	37.61±3.67^{a}	30.67±0.88^{ab}
Total n-3 LC-PUFA	23.74±5.94	12.92±7.45^{b}	32.27±3.84^{a}	25.91±4.89^{b}	36.21±3.65^{a}	29.16±0.97^{ab}
18:2n-6 LNA	3.75±1.14	4.23±0.08	4.27±0.35	4.49±0.50	3.62±0.46	3.67±0.28
20:4n-6 ARA	3.34±0.72	2.38±0.04	2.34±0.35	2.46±0.11	2.99±0.31	2.76±0.14
22:5n-6 DPA	0.58±0.28	1.09±0.09^{b}	0.37±0.05^{a}	0.36±0.02	1.52±0.04	0.58±0.03
Total n-6	9.70±1.22	8.45±0.97	8.18±1.27	8.24±0.73^{b}	9.26±0.26^{a}	7.92±0.18^{b}
18:1n-9 OLA	16.14±2.01	26.35±4.87	25.43±1.69	26.84±2.58^{a}	18.31±1.91^{b}	25.22±1.92^{a}
22:1n-9	0.33±0.17	0.26±0.15	0.26±0.03	0.14±0.03	0.21±0.13	0.13±0.07
Total n-9	18.81±4.97	28.44±4.72	27.52±2.77	28.93±2.79^{a}	20.25±1.98^{b}	27.10±1.85^{a}

Data are means±SD

Table 6.- Effects of the dietary treatment, time and their interaction on the global gene expression.

	DIET	TIME	D x T
CAT	**	**	**
SOD	**	**	n.s.
GPX	**	**	**
IGF-I	**	*	**
IGF-II	**	n.s.	**
MyHC	*	**	**
α-actin	**	**	n.s.
Capn1	*	**	**

Asterisks indicate significant differences as **P≤0.01, *P≤0.05. n.s. indicates non-significant differences.

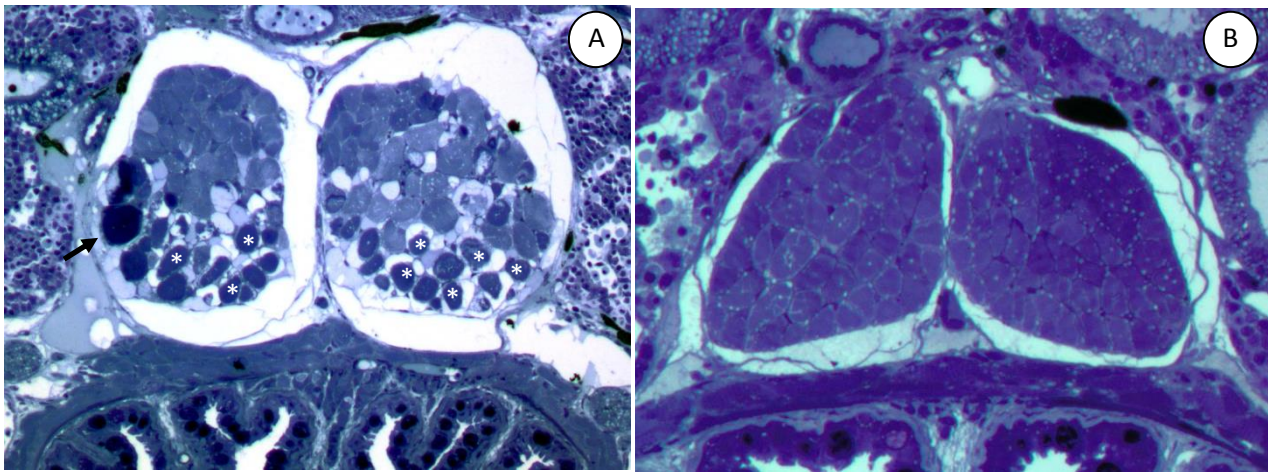


Figure 1- Transversal semithin sections on the same selected morphological area of 49 dph sea bass larvae fed 5% DHA (A) or 1% DHA (B) diets. (A) Damaged muscular fibres showing hypercontraction of the myofilaments (*) and coagulation of proteins, observed as darkening of the fibre (arrow). Besides, loss of the polyedrical structure can be observed, especially if compared to normal muscle (B).

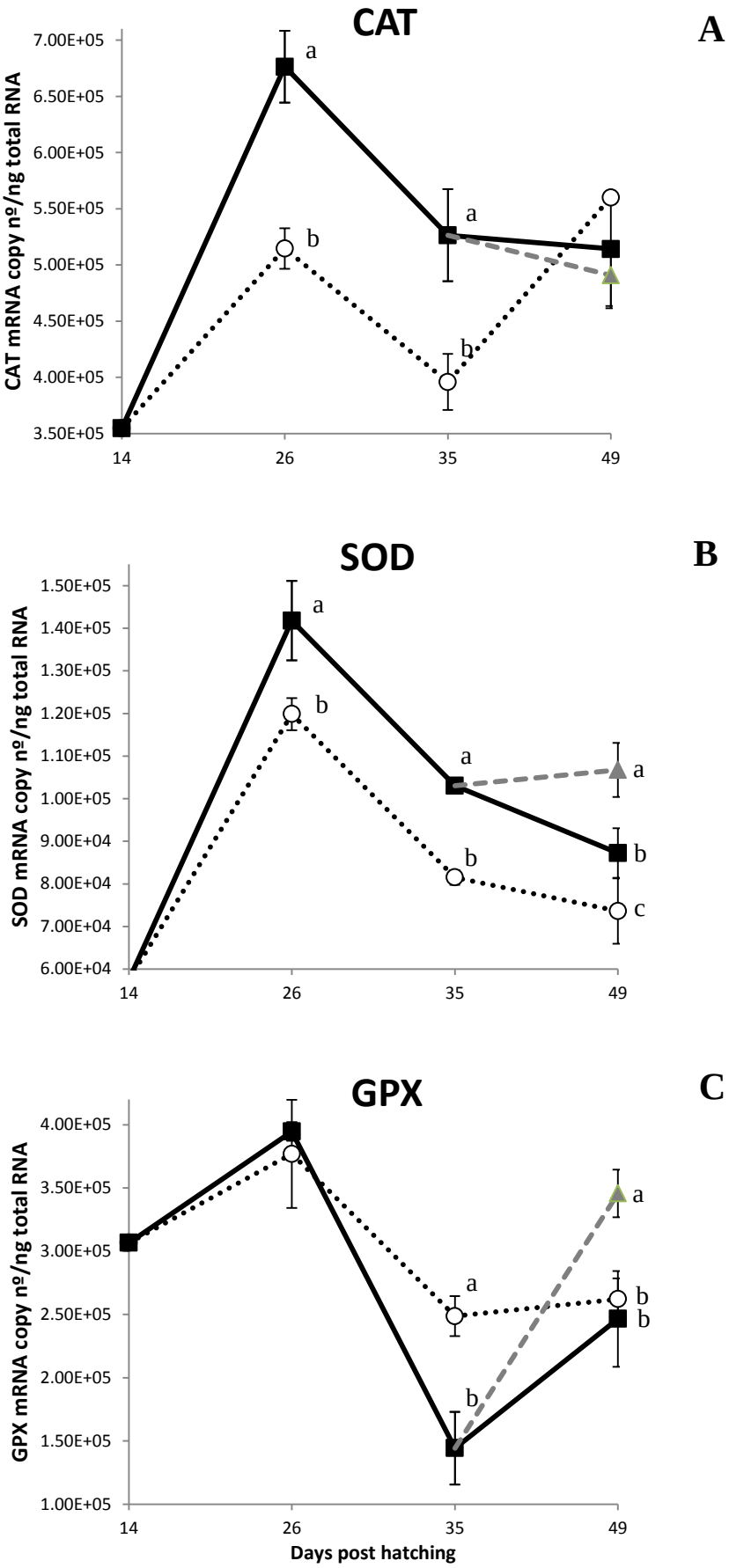

A
CAT
CAT mRNA copy nº/ng total RNA
7.00E+05
6.50E+05
6.00E+05
5.50E+05
5.00E+05
4.50E+05
4.00E+05
3.50E+05
14
26
35
49
a
b
a
b
B
SOD
SOD mRNA copy nº/ng total RNA
1.50E+05
1.40E+05
1.30E+05
1.20E+05
1.10E+05
1.00E+05
9.00E+04
8.00E+04
7.00E+04
6.00E+04
14
26
35
49
a
b
a
b
a
b
c
C
GPX
GPX mRNA copy nº/ng total RNA
4.00E+05
3.50E+05
3.00E+05
2.50E+05
2.00E+05
1.50E+05
1.00E+05
14
26
35
49
Days post hatching
a
b
a
b
b

Figure 2. CAT, SOD and GPX expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1% DHA (○), 5% DHA (■) or 5%+1% DHA (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. a, b, c denote significant differences between treatments within a sampling point ($P<0.05$).

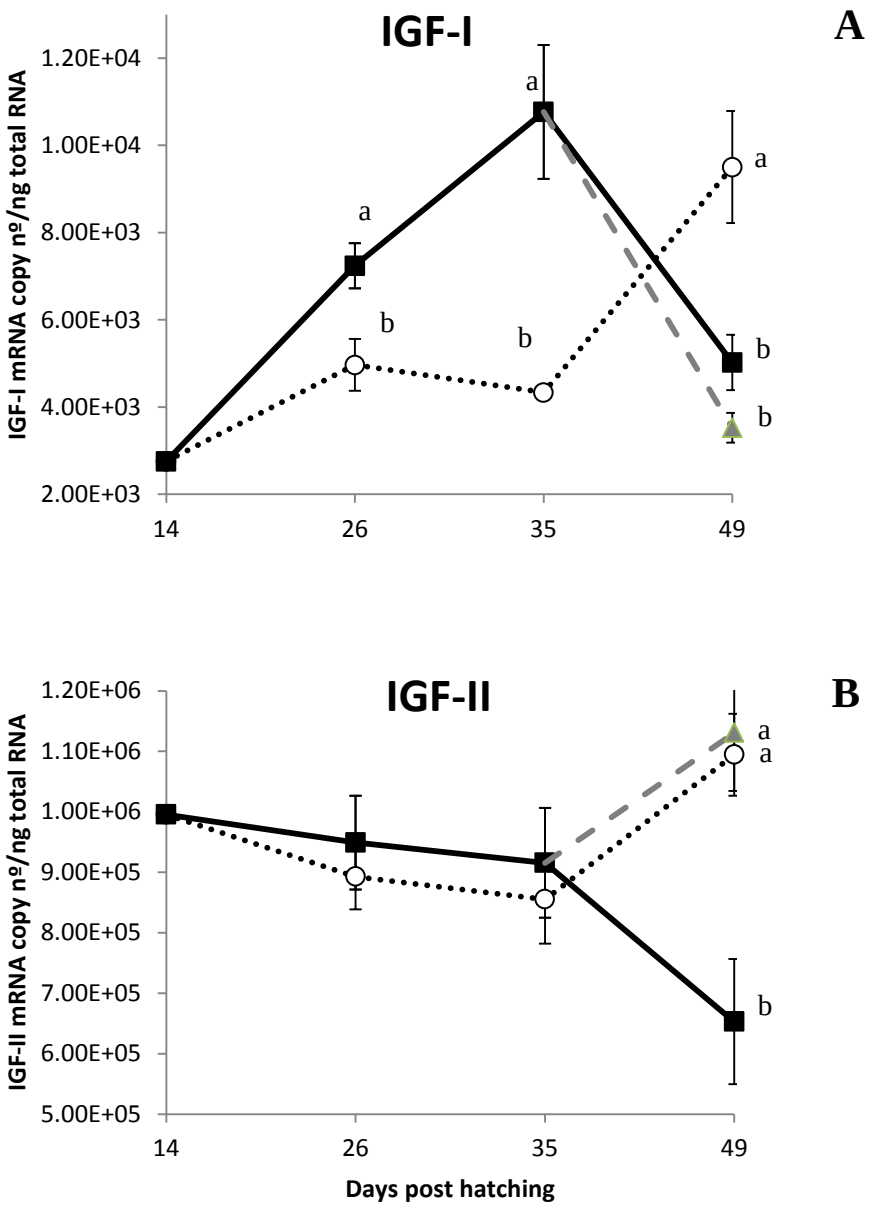
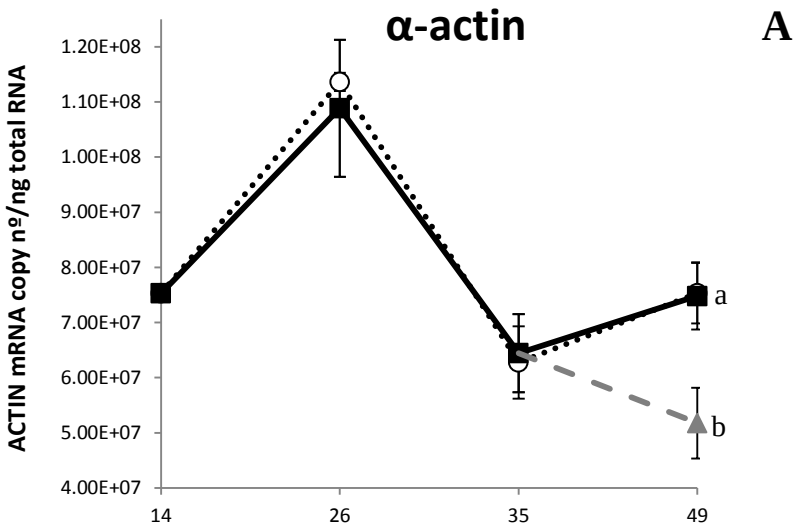
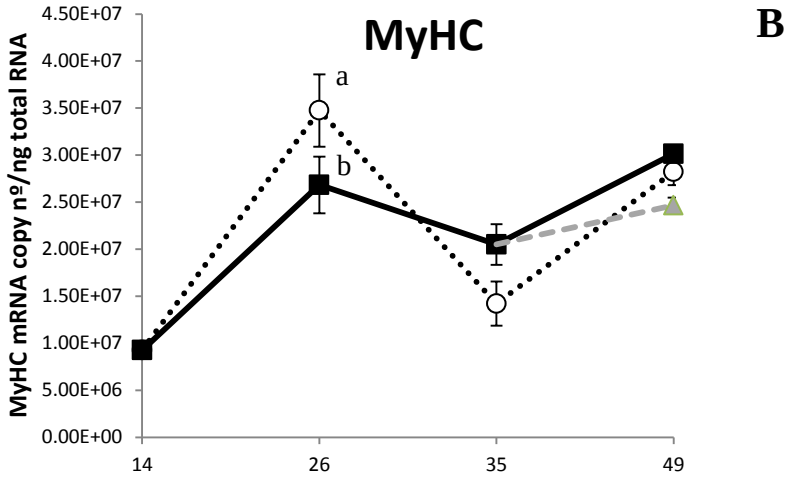


Figure 3. IGF-I and -II expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1% DHA (○), 5% DHA (■) or 5%+1% DHA (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. a, b, c denote significant differences between treatments within a sampling point ($P<0.05$).


α-actin
A
ACTIN mRNA copy nº/ng total RNA
1.20E+08
1.10E+08
1.00E+08
9.00E+07
8.00E+07
7.00E+07
6.00E+07
5.00E+07
4.00E+07
14
26
35
49
a
b


MyHC
B
MyHC mRNA copy nº/ng total RNA
4.50E+07
4.00E+07
3.50E+07
3.00E+07
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
14
26
35
49
a
b

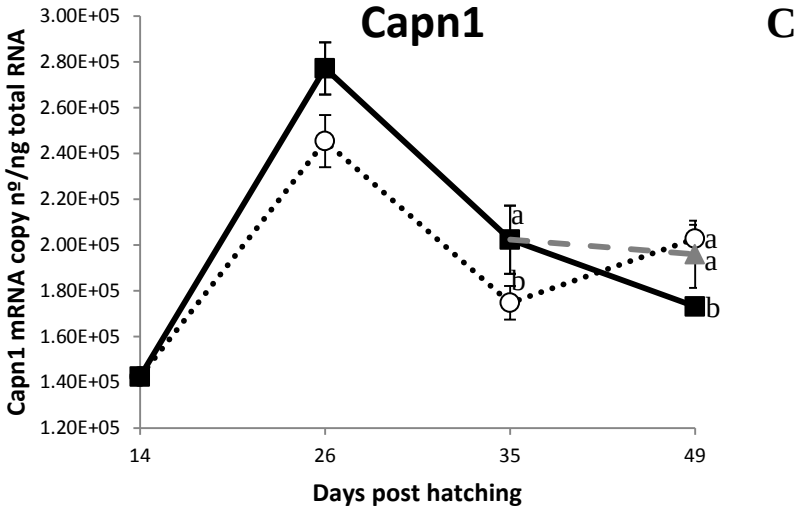

Capn1
C
Capn1 mRNA copy nº/ng total RNA
3.00E+05
2.80E+05
2.60E+05
2.40E+05
2.20E+05
2.00E+05
1.80E+05
1.60E+05
1.40E+05
1.20E+05
14
26
35
49
Days post hatching
a
b
a
a
b

Figure 4. α-actin, MyHC and μ-calpain expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1% DHA (○), 5% DHA (■) or 5%+1% DHA (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. a, b, c denote significant differences between treatments within a sampling point ($P<0.05$).