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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

28 **Abstract**

29

30 The potential muscle regeneration after nutritional dystrophy caused by high dietary 31 DHA contents in fish and the physiological pathways involved are still unknown. To 32 better understand this process, an experiment was conducted for 3 weeks in 14 day-old 33 European sea bass larvae using different DHA ratios (1 or 5%). After this period, part of 34 the larvae fed 5% DHA diet was switched to 1% DHA diet ("wash-out") for another 2 35 weeks. Larvae fed 5% DHA diet showed altered oxidative status as indicated by the 36 highest TBARS values, antioxidant enzymes (AOE) expression and incidence of 37 muscular lesions. Accordingly, "washed-out" larvae showed lower dry weight and α-38 TOH content. IGF-I gene expression was elevated in 5% DHA larvae at 35 dph, 39 suggesting increased muscle mitogenesis that was corroborated by the increase in 40 myosin heavy chain expression. It can be concluded that high dietary DHA contents 41 alter the oxidative status and causes muscular lesions in European sea bass larvae, with 42 morphological and molecular aspects of mammalians muscular degenerative disease.

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53 **1-Introduction**

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

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

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

83 Whenever there is an imbalance between the generation and removal of ROS by
84 cellular defences, a state of oxidative stress is initiated. This status may lead to the
85 oxidation of various cellular constituents like lipids, proteins or DNA. For instance,
86 European sea bass larvae muscle appears to be very sensitive to ROS attack, as severe
87 dystrophic lesions in the epaxial musculature have been reported due to *in vivo* lipid
88 peroxidation (Betancor et al., 2011; Betancor et al., in press). The term regeneration
89 refers to a process that allows an organism to regain the function of an organ or
90 structure damaged by injury or disease (Stoick-Cooper et al., 2007). In adult zebrafish
91 (*Danio rerio*), an exceptionally high capability for regeneration has been reported (Lien
92 et al., 2006; Yoshinari et al., 2009). Therefore, it can be hypothesized that at younger
93 stages an activation of muscle repair process in a situation of oxidative stress will take
94 place. However, information about muscle regeneration in fish has been rarely
95 described and only related to mechanical injury or bacterial infection (Rowlerson et al.,
96 1997; Ingerslev et al., 2010) and there is a complete lack of studies describing muscle
97 regeneration process in marine fish larvae or regeneration after a nutritional dystrophy.

98 Myosin and actin are the major muscle proteins, where myosin is the major
99 structural component of striated muscle. Both myosin chains, the heavy (MyHC) and
100 the light (MyLC), exist as multiple isoforms that are tissue and/or developmental stage-
101 specific (Funkenstein et al., 2007; Ikeda et al., 2007). MyHC gene expression has been
102 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.

127 In our previous studies (Betancor et al., 2011; Betancor et al., in press), when
128 European sea bass larvae were fed high contents of DHA, α-TOH alone did not seem to
129 be able to counteract ROS, leading to the appearance of axial muscular lesions. To
130 better understand the molecular pathways involved in fish larvae muscle dystrophy and
131 healing, the present study generated muscular lesions in European sea bass larvae by
132 feeding a diet containing 5% DHA during 3 weeks (negative control diet), followed by a
133 "wash-out" period of two weeks when larvae were switched to a diet containing only
134 1% DHA (positive control diet). Growth, survival, TBARS, fatty acid profile, α-TOH
135 contents and mRNA expression levels of CAT, SOD, GPX, IGF-I, IGF-II, MyHC, α-
136 actin and μ-calpain (Capn1) genes were studied in order to achieve this objective.

137

138 **2- Methods**

139 **2.1- Fish and diets**

140

141 The experiment was carried out at the *Instituto Canario de Ciencias Marinas*
142 (ICCM; Telde, Canary Islands, Spain). European sea bass (*Dicentrarchus labrax*) larvae
143 were obtained from natural spawnings from the *Instituto de Acuicultura de Torre de la*
144 *Sal* (Castellón, Spain). Prior to the start of the feeding experiment, larvae were fed
145 enriched yeast-fed rotifers (DHA Protein Selco®, INVE, Belgium) until they reached 14
146 days post hatching (dph; total length 8.58 ± 0.64 mm, dry body weight 0.36 ± 0.0 mg).
147 Larvae were randomly distributed into experimental tanks (170 L light grey colour
148 cylinder fibreglass tanks) at a density of 1000 larvae/tank and fed one of two
149 experimental diets for 35 days, at a water temperature of 19.5 to 21.0ºC. Two
150 experimental groups were defined, consisting of either four tanks for the positive
151 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

177 (Table 1). The microdiets were prepared according to Liu et al. (2002) by first mixing
178 the squid powder and water soluble components followed by the lipid and fat soluble
179 vitamins and, finally, warm water dissolved gelatine. The paste was pelleted and oven
180 dried at 38ºC for 24 h. Pellets were ground and sieved to obtain a particle size below
181 250 µm. Diets were analyzed for proximate and fatty acid composition on a dry weight
182 basis and manually supplied; fourteen times per day every 45 min from 9:00-19:00.
183 Daily feed supplied was 2, 2.5 and 3 g/tank during the first, second and third week of
184 feeding respectively.
185
186 **2.2- Growth and survival**
187
188 Final survival was calculated by individually counting live larvae at the beginning,
189 middle and end of the experiment. Growth was determined by measuring dry body
190 weight (105ºC for 24 hours) and total length (Profile Projector V-12A Tokyo, Nikon) of
191 30 fish/tank at the beginning (14 dph), middle (35 dph) and final (49 dph) of the
192 experimental trial.
193
194 **2.3- Biochemical analysis**
195
196 At 35 dph, larvae from the two treatments were manually counted and 150 larvae
197 removed from each tank, washed with distilled water and kept at -80ºC for biochemical
198 analysis. Similarly, at the end of the experimental trial (49 dph), following a 12 hour
199 starvation period, all remaining larvae per tank were washed with distilled water,
200 sampled and kept at -80ºC until analysis for biochemical composition, fatty acid methyl

201 esters (FAMEs), α-TOH and TBARS. Moisture, protein (A.O.A.C., 1995) and lipid
202 (Folch et al., 1957) contents of larvae and diets were analyzed.
203
204 *2.3.1- Total lipid fatty acid analysis*
205 Fatty acid methyl esters (FAMEs) were obtained by transmethylation of total
206 lipids as described by Christie (1982). FAMEs were separated by GLC and quantified
207 by a flame ionization detector (FID; GC -14A, Shimadzu, Tokyo, Japan) under the
208 conditions described in Izquierdo et al. (1992) and identified by comparison to
209 previously characterized standards and by GLC-MS.
210
211 *2.3.2- Determination of* α-TOH *content*
212 α-TOH concentrations were determined in diets and total larvae by using HPLC.
213 Samples were weighed, homogenised in pyrogallol and saponified as described by
214 McMurray et al. (1980) for diets and to Cowey et al. (1981) for larval tissues. HPLC
215 analysis was performed using 150 x 4.60 mm reverse phase Luna 5μm C18 column
216 (Phenomenox, California, USA). The mobile phase was 98% methanol supplied at a
217 flow rate of 1.0 ml/min, the effluent from the column was monitored at a wavelength of
218 293 nm and quantification achieved by comparison with (+)-α-tocopherol (Sigma-
219 Aldrich, Madrid, Spain) as external standard.
220
221 *2.3.3- Measurement of thiobarbituric acid reactive substances (TBARS)*
222 The measurement of TBARS in triplicate samples was performed using a method
223 adapted from Burk et al. (1980). Approximately 20-30 mg of larval tissue per sample
224 were homogenized in 1.5 ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1%
225 butylated hydroxytoluene in methanol. To this, 2.95 ml of freshly prepared 50mM

226 thiobarbituric acid solution was added before mixing and heating for 10 minutes at
227 100ºC. After cooling protein precipitates were removed by centrifugation (Sigma 4K15,
228 Osterode am Harz, Germany) at 2000 x *g* and the supernatant read in a
229 spectrophotometer (Evolution 300, Thermo Scientific, Cheshire, UK) at 532 nm. The
230 absorbance was recorded against a blank at the same wavelength. The concentration of
231 TBA-malondialdehyde (MDA) expressed as µmol MDA per g of tissue was calculated
232 using the extinction coefficient 0.156 μM^{-1} cm^{-1}.

233

234 **2.4- Histopathological sampling**

235

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

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

248

249 **2.5- Gene expression analysis**

250

251 *2.5.1- Total RNA extraction and quantitative real time RT-PCR*

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

264

265 *2.5.2- Generation of in vitro-transcribed mRNAs for standard curves*

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

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

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

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

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

287

288 *2.5.3- Generation of standard curves for target genes*

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

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

310

311 *2.5.4- Target genes transcripts quantification by one-step TaqMan® real-time RT-PCR*

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

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

321 Data from the TaqMan® RT-PCR runs were collected with StepOne™ Software v2.0.
322 Cycle threshold (CT) values corresponded to the number of cycles at which the
323 fluorescence emission monitored in real time exceeded the threshold limit. The Ct
324 values were used to create standard curves to calculate the absolute amounts of mRNA
325 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

351 *3.1 Growth and survival*

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

356

357 *3.2 Biochemical analysis*

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

362 At 35 dph larval FA composition closely reflected that of the microdiets fed.
363 Thus, levels of 22:6n-3, n-3 and n-3 LC-PUFA were significantly lower (P<0.05) in
364 larvae fed the 1% DHA diet (Table 5). At 49 dph, 2 weeks after starting the "wash-out"
365 period, 5%+1% DHA larvae were similar in 18:4n-3, 18:1n-9, 22:6n-3, monoenoics, n-
366 6 and n-9 contents compared to larvae fed 1% DHA diet (Table 5). However, total n-3
367 content and n-3 LC-PUFA were similar to those of larvae fed both 1% and 5% DHA
368 diets.

369 The highest TBARS content (µmol/g larval tissues), an indicator of lipid
370 peroxidation, was detected with an increase in DHA level at 35 dph (P=0.001; Table 3).
371 TBARS increased from 35 to 49 dph in all treatments, with a higher value in 5% DHA
372 larvae (P=0.025; Table 3). Although larvae fed 5%+1% DHA diet showed higher
373 TBARS content than larvae fed 1% DHA diet, no significant differences were found
374 (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).

400 *3.4 Gene expression analysis*

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

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

419 With regards to IGF-II expression, 1% and 5% DHA larvae showed a similar
420 pattern from 14 to 35 dph (Figure 3.B). At 49dph, there was a 1.2 fold increase in IGF-
421 II expression of 1% DHA fed larvae compared to 35 dph. The expression levels of IGF-
422 II at this sampling point were significantly higher than those found in 5% DHA fed
423 larvae (P=0.001). "Washed-out" larvae displayed the same IGF-II expression pattern of
424 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≤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

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

508 From 14 to 35 dph, when European sea bass larvae were exposed to high DHA
509 dietary contents (5%), the induction of AOE genes coincided with increases in TBARS
510 contents. This is in agreement with previous studies in this species (Betancor et al., in
511 press) and Manchurian trout (*Brachymystax lenok*) (Zhang et al., 2009), in which young
512 fish fed high lipid levels had higher TBARS contents, inducing an antioxidant response
513 noticeable as an increase in the activity of AOE. Moreover, in the present study, a
514 marked increase in the expression of each AOE was observed from 14 to 26 dph,
515 whereas exposure to a high dietary DHA content caused a significant increase in CAT
516 and SOD gene expression in larvae fed the 5% DHA diet at both 26 and 35 dph.
517 Similarly, juvenile rainbow trout (*Oncorhynchus mykiss*) fed with high PUFA contents,
518 displayed significantly higher SOD activities (Trenzado et al., 2009). Nevertheless, at
519 49 dph CAT and SOD were less induced, suggesting an adjustment in the expression of
520 these two genes at the last sampling point, after the supra-induced expression found in
521 the former sampling at 35 dph. This would also explain why “washed out” larvae
522 showed the highest mRNA copies of SOD and GPX, as larvae may still be adapting to
523 the new dietary treatment. Equally, CAT and SOD activities would be expected to

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

542 AOE and low molecular weight antioxidants are known to form a primary
543 defence against lipid peroxidation, being mainly preventive (Girotti, 1998). Among
544 AOE, GPX seems to be the only enzyme able of detoxifying fatty acid hydroperoxides,
545 thereby acting as a secondary defence line. Thus, a high GPX gene expression in larvae
546 fed high DHA diets was expected, as this fatty acid is being oxidized. However, a low
547 GPX expression was found in larvae fed a 5% of DHA. It must be noted that other
548 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

598 transcription control and regulate the expression of both IGF forms (Canalis et al.,
599 1991). From 35 dph on there is a marked decrease in both IGFs expression in larvae the
600 fed 5% DHA diet, in which a high expression of this gene would be expected due to
601 regeneration processes. No information is available about the molecular process of
602 regeneration in fish larvae after a chronic insult, like ROS attack. It must be noticed that
603 IGFs exert their effects on cells through the binding to the IGF receptors (IGF-R). An
604 increase in IGF-IR receptors has been described in cultured trout muscle cells in
605 response to cell differentiation (Castillo et al., 2004). Nonetheless, a critical element in
606 the action of IGF and their receptors are the role of IGF binding proteins (IGFBPs),
607 which influence IGF function by enhancing or inhibiting their action (Reinecke et al.,
608 2005). Conversely, in mammals, it is known that certain cytokines, such as tumour
609 necrosis factor (TNF), produced as a result of any inflammatory process like muscle
610 injury, can inhibit the action of IGF and has been demonstrated in chronic muscular
611 diseases (Grounds et al., 2008; Gebski, 2009). A full understanding of IGFs function in
612 fish muscle regeneration requires further work.

613 Regarding α-actin expression, no differences between the 1% and 5% DHA fed
614 larvae were observed, nor was any correlation to MyHC or IGF-I, indicating that it
615 could not be associated with regeneration process. Although it has been stated that IGF-
616 I induces sarcomeric actin filament formation in mammals (Takano et al., 2010), a
617 relationship between both molecules could not be proved in the present study. However,
618 it must be noted that several α-actin isoforms have been described in fish (Morita,
619 2000), thus, the reduction of the transcript content observed may reflect the switch to
620 the expression of a different actin isoform that could not be detected in this study.
621 "Wash-out" had a negative effect (decreased transcript levels) on genes encoding for
622 myofibrillar proteins expression such as MyHC and α-actin.

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

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

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

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Figure legends:

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).

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. Different superscript letters 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. Different superscript letters denote significant differences between treatments within a sampling point ($P<0.05$).

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975 Figure 4: α-actin, MyHC and μ-calpain expression levels measured by real-time PCR in
976 *Dicentrarchus labrax* larvae when were fed diets 1% DHA (○), 5% DHA (■) or 5+1%
977 DHA (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total
978 RNA. Different superscript letters denote significant differences between treatments
979 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.

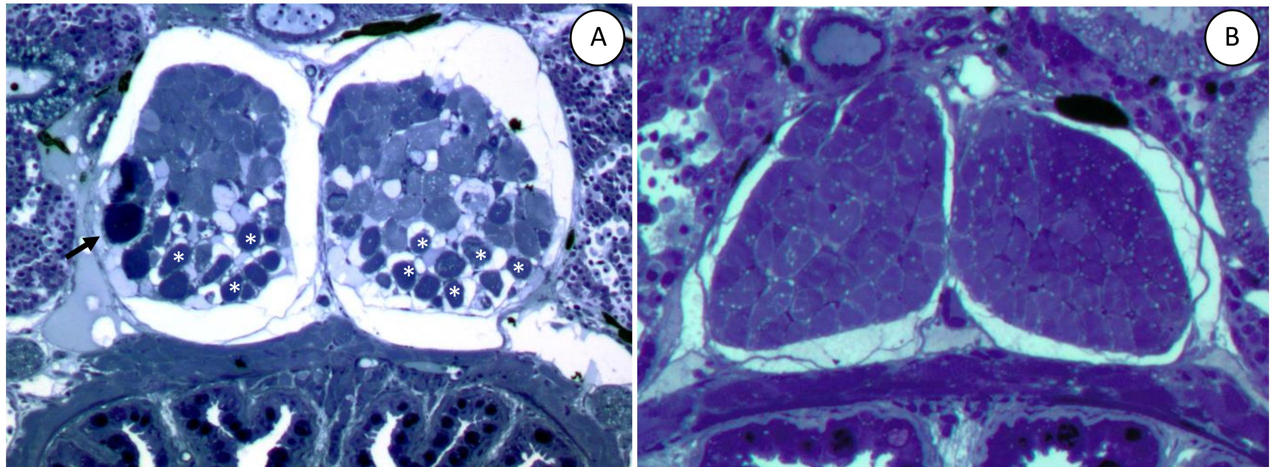
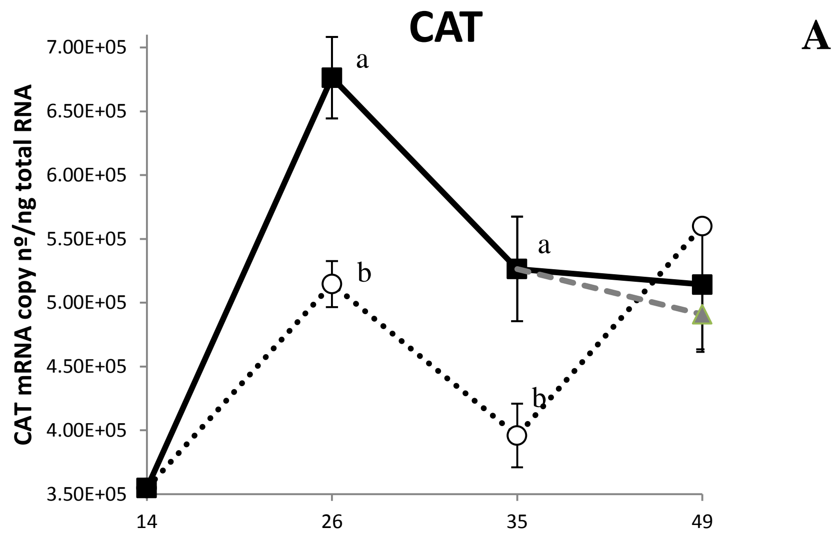
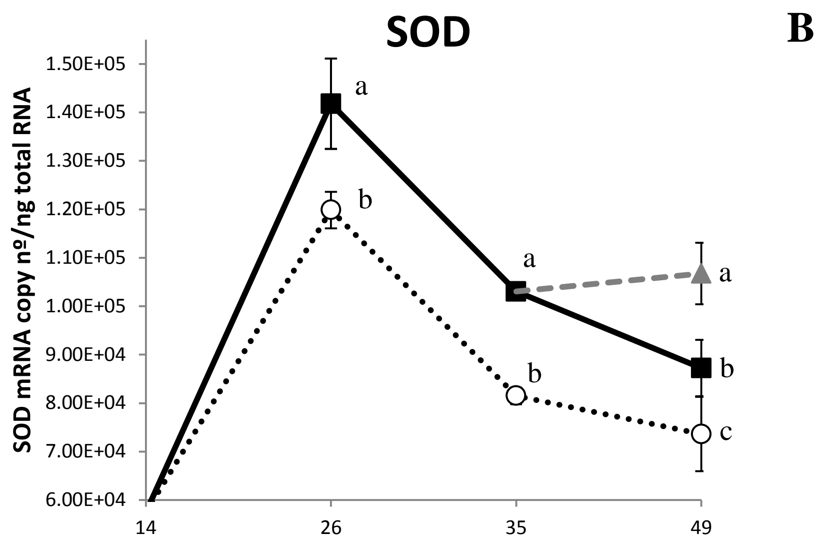


Figure 1- Transversal semithin sections on the same selected morphological area of 49 dph sea bass larvae fed 5% DHA (A) or 1% DHA (B) diets. (A) Damaged muscular fibres showing hypercontraction of the myofilaments (*) and coagulation of proteins, observed as darkening of the fibre (arrow). Besides,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


CAT
A
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


SOD
B
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

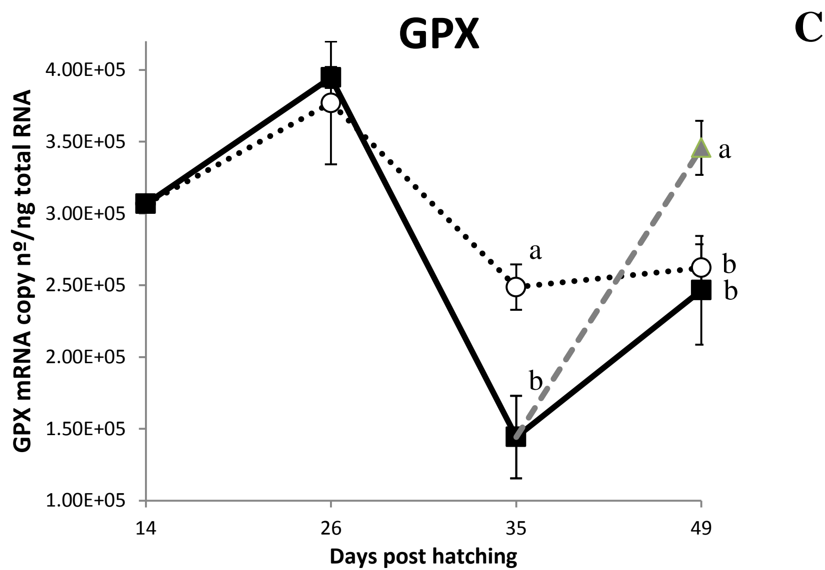

GPX
C
GPX mRNA copy nº/ng total RNA
4.00E+05
3.50E+05
3.00E+05
2.50E+05
2.00E+05
1.50E+05
1.00E+05
14
26
35
49
Days post hatching
a
a
b
b
b

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).

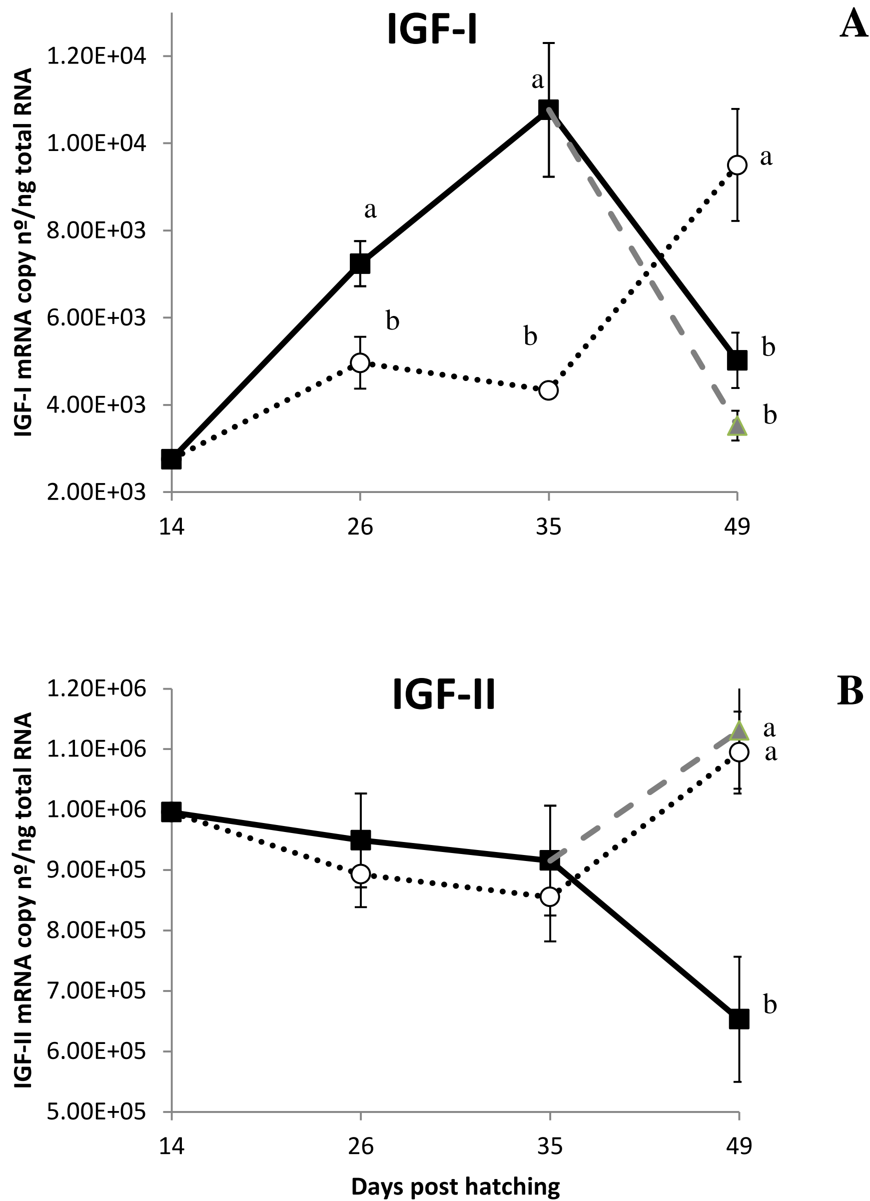
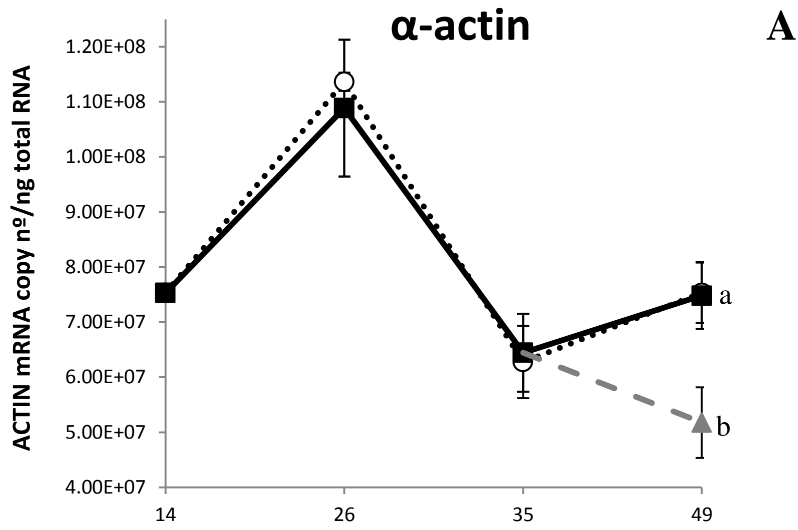
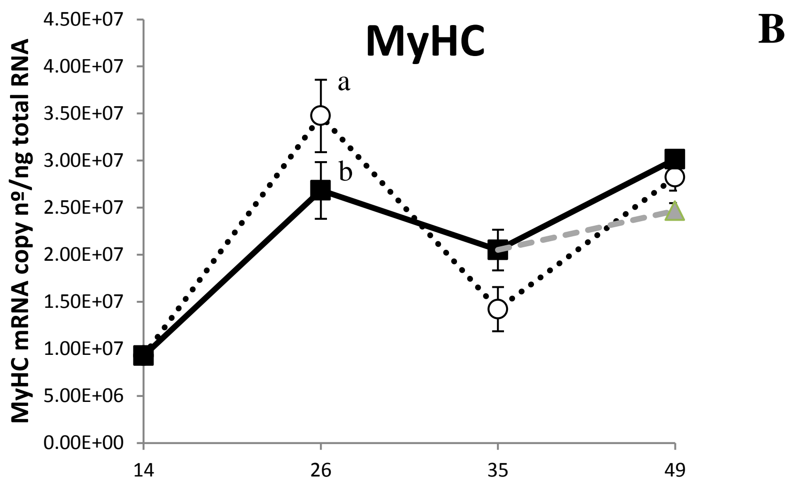


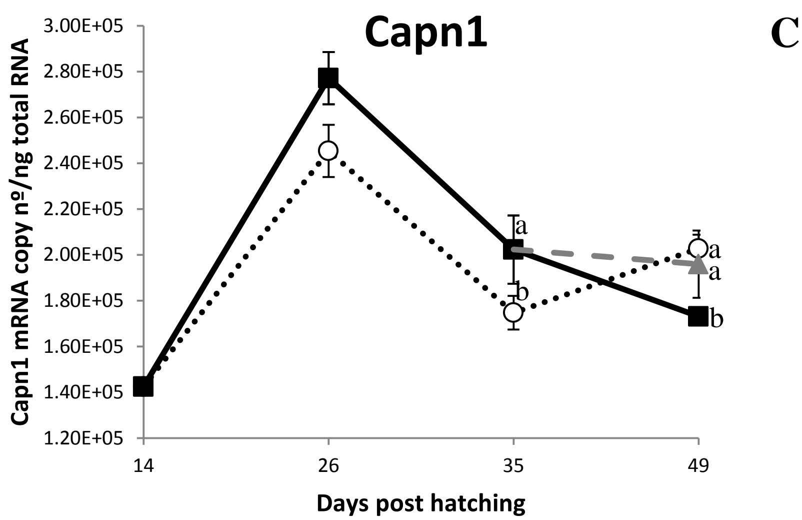
Figure 3. IGF-I and -II expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1% DHA (○), 5% DHA (■) or 5%+1% DHA (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. a, b, c denote significant differences between treatments within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