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# Physiological pathways involved in nutritional muscle dystrophy and healing in European sea bass (*Dicentrarchus labrax*) larvae

Mónica B. Betancor [a,*], Marisol Izquierdo [a], Genciana Terova [b], Elena Preziosa [b], Reda Saleh [a], Daniel Montero [a], Carmen María Hernández-Cruz [a], Mª. José Caballero [a]

[a] Aquaculture Research Group, University of Las Palmas de Gran Canaria & ICCM, Instituto Universitario de Sanidad Animal, Trasmontaña s/n, 35413, Arucas, Las Palmas, Canary Islands, Spain
[b] Department of Biotechnology and Life Sciences, University of Insubria, Via Dunant 3, 21100, Varese, Italy

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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 cause muscular lesions in European sea bass larvae, with morphological and molecular aspects of mammalians muscular degenerative disease.

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## 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 comprises 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 (Kim et al., 2010; Ji et al., 2011) 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 defenses, 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, 2012). The term regeneration refers to a process that allows an organism to regain the function of an organ

*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.

* Corresponding author. Tel.: +34 928 451095; fax: +34 928 451143.
*E-mail address:* monica.betancor102@doctorandos.ulpgc.es (M.B. Betancor).

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

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

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

In our previous studies (Betancor et al., 2011, 2012), 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. Materials and 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 mass 0.36 ± 0.0 mg). Larvae were randomly distributed into experimental tanks (170 L light gray color cylinder fiberglass 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 21.0 °C. 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 weeks after the start of trial (35 dph), larvae from each tank were individually counted and 200 larvae per tank removed for analytical sampling with the remaining larvae placed into three tanks per treatment. In addition, the remaining larvae from the 5% DHA group were divided into two groups (3 tanks per treatment), with one group continuing to be fed with the same diet and the other group switched to a diet containing 1% DHA (5% + 1% DHA; "wash-out") for a further two weeks until the end of the experiment (49 dph).

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

Two isonitrogenous and isolipidic experimental microdiets (pellet size<250 μm) similar in their EPA content and different in DHA content were formulated (Table 1) using concentrated fish oils EPA50 and DHA50 (CRODA, East Yorkshire, England, UK), as sources of EPA and DHA and DL-α-tocopheryl acetate (Sigma-Aldrich, Madrid, Spain) as source of α-TOH. Diets were chosen based on previous trials results (Betancor et al., 2011, 2012). A positive control diet was formulated to include 1 g DHA/100 g DW and 150 mg α-TOH/100 g DW (diet 1% DHA). The negative control diet consisted of 5 g DHA/100 g DW and 150 mg α-TOH/100 g DW (diet 5% DHA). The protein source, squid meal, was defatted 3 consecutive times with a chloroform:squid meal ratio of 3:1 to allow complete control of the fatty acid profile of the microdiet. The microdiet was based on defatted squid meal (2.4% lipid content) with EPA50 and DHA50 added in different quantities to obtain the desired ratios. Oleic acid (Merck, Darmstadt, Germany) was added to equalize the lipid content in each diet (Table 1). The microdiets were prepared according to Liu

**Table 1**
Formulation of experimental diets.

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

[a] Riber and Son, Bergen, Norway.
[b] Croda, East Yorkshire, UK.
[c] Merck, Darmstadt, Germany.
[d] Acrofarma, Barcelona, Spain.
[e] Vitamin premix supplied per 100 g diet: Cyanocobalamine, 0.030; Astaxanthin, 5.00; folic acid, 5.44; pyridoxine-HCl, 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; and α-tocopherol acetate, 150.000.
[f] Mineral premix supplied g per 100 g 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; and $CoSO_4 \cdot 7H_2O$, 10.706.

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