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Dietary modulation of arachidonic acid metabolism in senegalese sole (*Solea Senegalensis*) broodstock reared in captivity

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ABSTRACT

Previous studies have shown higher levels of arachidonic acid (20:4n-6, ARA) in testis, liver, and muscle of wild Senegalese sole (*Solea senegalensis*) compared to fish reared in captivity (first generation, G1). The present study was conducted to establish the optimal level of dietary ARA for G1 Senegalese sole broodstock, using as a reference the fatty acid profile of wild broodstock (gonads, liver and muscle). A total of 120 Senegalese sole broodstock were randomly distributed into 12 tanks (1:1 male and female) and fed in duplicate with six experimental diets containing increasing amounts of ARA (0.7%, 1.6%, 2.3%, 3.2%, 5.0%, and 6.0 % of total fatty acids) for 9 months. The relative ARA levels in liver, muscle and male and female gonads at the end of the feeding period increased in a dose dependent manner. Dietary ARA was mainly incorporated and stored in testis or ovary, followed by liver and muscle. Fish fed 2.3% and 3.2% ARA showed no differences in the ARA content of testis, ovary and liver when compared to wild fish. In male fish, a significant increase in the levels of 22:4n-6 and 22:5n-6 fatty acids was also observed, which was consistent with the up-regulation of fatty acyl elongase (*elovl5*) and desaturase (*d4fad*) transcript levels in the liver of fish fed 0.7%, 2.3% and 6% ARA. These results suggest that dietary inclusion of 3.2% ARA during periods shorter than 9 months, or of 2.3% ARA for prolonged periods, can maintain optimal levels of tissue ARA in captive Senegalese sole broodstock. In addition, the data indicate that male Senegalese sole is able to elongate and desaturate ARA to 22:4n-6 and 22:5n-6, suggesting that these fatty acids may be important for male reproduction.

1. Introduction

One of the most important nutritional factors for successful fish reproduction, and among the most studied, is the fatty acid arachidonic acid (20:4n-6, ARA) (Alorend, 2004; Furuita et al., 2003; Mazorra et al., 2003; Meunpol et al., 2005; Sargent et al., 1999; Tocher, 2010). Arachidonic acid is the main precursor for production of 2-series prostaglandins (PGs) (Smith et al., 2002; Tocher, 2003), which stimulate ovarian and testicular steroidogenesis, triggering oocyte maturation in females and milt production in males, and are involved in female sexual behavior (Mercure and Van der Kraak, 1995; Sorbera et al., 2001; Sorensen and Stacey, 2004; Van der Kraak and Chang, 1990; Wade and Van der Kraak, 1993). Arachidonic acid itself and its metabolites regulate cholesterol (CHOL) transfer from the outer to inner mitochondrial membrane where the P450 enzyme resides to initiate steroid hormone synthesis (Mercure and Van der Kraak, 1995; Wang and Stocco, 2005). Moreover, ARA had differential effects on steroid biosynthesis. Although it stimulates testosterone production by elevating cAMP levels in a dose-dependent manner, ARA at high doses can also inhibit steroidogenesis by affecting the availability of CHOL (Mercure and Van der Kraak, 1995, 1996).

Senegalese sole (*Solea senegalensis*) is a promising species for aquaculture in Southern Europe. However, an important problem encountered in this species is the fact that first generation (G1) of reared fish often fail to spawn viable eggs, contrary to wild animals, which produce eggs of sufficient quality and quantity after variable times of acclimation in captivity (Carazo et al., 2011). This is hindering the expansion of Sole aquaculture and hence several studies have been recently performed in an attempt to understand the underlying causes. Studies on wild sole broodstock showed higher levels of ARA and ARA-derived fatty acids in different tissues compared to

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those observed in G1 fish (Norambuena et al., 2012a), similarly to that has been reported in other fish species (Aslan et al., 2007; Blanchet et al., 2005; Cejas et al., 2003, 2004; Harrell and Woods, 1995; Lund et al., 2008; Rodríguez et al., 2004; Salze et al., 2005; Sheikh-Eldin et al., 1996; Silversand et al., 1996). High accumulation of ARA in sperm has also been reported in rainbow trout (*Oncorhynchus mykiss*) fed diets low in docosahexaenoic acid (22:6n-3, DHA) (Vassallo-Agius et al., 2001), and in wild European seabass (*Dicentrarchus labrax*) (Bell et al., 1996). In addition, previous studies on Senegalese sole showed that differences in ARA tissue content resulted in differences in cyclooxygenase (COX-2) gene expression, which was significantly up-regulated in the sperm-duct, oviduct and gills of males from wild origin compared to G1 fish (Norambuena et al., 2012b). Thus, wild fish showed significantly higher levels of 2-series PGs compared to cultured fish, especially in testis, whereas G1 Senegalese sole, with a lower ARA tissue content, exhibited significantly higher levels of 3-series PGs and lower levels of CHOL (Norambuena et al., 2012a), the precursor of steroid hormones in vertebrates (Baron and Hylemon, 1997). On the other hand, Senegalese sole fed artificial diets formulated with graded ARA levels showed an increase in ARA levels in circulating blood, which in turn may induce an increase in CHOL and steroid production, especially in males (Norambuena, 2012). Higher levels of ARA in the tissues of wild fish and increased levels in the blood of G1 fish previously fed ARA-enriched diets resulted in an increase in ARA-derived fatty acids, 22:4n-6 and 22:5n-6 (Norambuena, 2012). A similar increase in these n-6 long-chain polyunsaturated fatty acids (LC-PUFA) was observed in the sperm of wild European seabass (Bell et al., 1996). These fatty acids are present in the cells of reproductive (i.e., seminiferous tubules, sperm) and nervous tissues in larger quantities (human, bull, boar and rabbit) (Ahaluwalia and Holman, 1969; Lenzi et al., 1996; Picardo et al., 1990; Tinoco, 1982) than those reported in any other fish tissue and mammals (Ayala et al., 1973; Bridges and Coniglio, 1970). On the other hand, although the physiological function of these LC-PUFAs in sperm is not well known, in mammals they are considered indicators of normal testicular development, spermatogenesis, germ cell populations and fertility (Furland et al., 2007; Leat et al., 1983; Lenzi et al., 1996, 2000; MacDonald et al., 1984) as well as in sperm formation and transportation in the rat testicle (Ayala et al., 1973; Lenzi et al., 1996).

Biologically active essential fatty acids such as ARA, eicosapentaenoic acid (20:5n-3, EPA) and DHA can be synthesized to some extent by some mammals and freshwater fish through elongation and desaturation of dietary shorter chain precursors. Marine fish have only negligible biosynthetic capacity and hence require preformed LC-PUFA in the diet (Sprecher, 2000; Tocher, 2010). However, it was recently demonstrated that desaturation of 22:4n-6 to 22:5n-6 may be carried out by a direct pathway involving a delta 4 desaturase in both a marine herbivorous fish (Li et al., 2010), as well as in Senegalese sole larvae (Morais et al., 2012). This indicates that there is more than one possible pathway for the synthesis of 22:5n-6 and DHA in vertebrates, i.e., not only the classical "Sprecher pathway" (Sprecher, 2000).

In the present study we conducted a nine-month feeding trial on broodstock G1 Senegalese sole using a standard commercial feed formulation with six graded levels of dietary ARA. The objectives were: (1) to determine the optimal dietary level of ARA for G1 Senegalese sole, using as a reference the fatty acid profile in gonads, liver and muscle of wild broodstock (Norambuena et al., 2012a); and (2) to investigate the regulation of fatty acyl desaturase (*d4fad*) and elongase (*elovl5*) gene expression in the liver of G1 sole fed different amounts of ARA.

2. Materials and methods

Research involving animal experimentation conformed to the principles for the use and care of laboratory animals, in agreement with the Spanish and European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA).

2.1. Fish and diets

One hundred and twenty Senegalese sole (four year old and 524 ± 11 g average weight), reared in captivity were PIT tagged (AVID, UK) and sexed using a heterologous vitellogenin ELISA for European seabass (*Dicentranchus labrax*) and validated for Senegaleses sole (Mañanós et al., 1994). The fish were distributed among twelve experimental tanks (10 fish per tank, 5 males and 5 females) and fed in duplicate standard commercial (extruded) diet with six graded ARA contents (Tables 1 and 2) for 9 months (from September 2009 until May 2010). The fish were held in a recirculation system with simulated natural photoperiod and temperature (40° 37′ and 40° 48′ N and between 0° 21′ and 0° 40′ E., Tarragona, Spain), with minimum temperature observed during 2 weeks in January–February (13 °C) and maximum temperature over 12 weeks during June–September (21 °C). The fish were fed 6 days per week at a daily ration of 0.15–0.3% body weight. Specific growth rate (SGR) and feed conversion rate (FCR) were calculated using the equations: $FCR = F^{*}(W_f - W_i)^{-1}$ and $SGR = ((\log W_f - \log W_i)^{*}T^{-1})^{*}100$ where F is feed supplied (g), W_i and W_f are the initial and final biomass (g) and T is the experimental time in days.

2.2. Fish sampling

In May 2010, seventy two fish were sacrificed by pithing the spinal cord (12 fish per dietary treatment, 6 males and 6 females) after anesthesia with 0.3 ml L^{-1} Aqui-S® (Scan Aqua A.S, Årnes, Norway) (Norambuena et al., 2011). Gonads, liver and muscle were collected, and 2 g of liver was frozen immediately in liquid nitrogen and subsequently stored at −70 °C until RNA extraction, whereas the rest of the tissues for lipid and fatty acid profile were stored at −20 °C. All the fish used for these analyses were in advanced stages of sexual maturation, females with vitellogenic oocytes and males containing spermatozoa in the seminiferous tubules.

2.3. Lipid and fatty acid analyses

Samples of tissues and feeds were homogenized and total lipids extracted (Folch et al., 1957) and quantified gravimetrically. Tissue samples of six males and six females for each diet treatment were analyzed, and feeds were analyzed in triplicate every 3 months during the experiment. Fatty acid methyl esters were prepared by acid-catalyzed transmethylation (Christie, 1982), and extracted and purified following Tocher and Harvie (1988). Methyl esters were separated and quantified by gas–liquid chromatography (Thermo Trace

Table 1
Ingredients and proximate composition of the experimental diets (A, B, C, D, E and F).

Ingredients (g/kg)	A	B	C	D	E	F
Fish meal[a]	645.0	645.0	645.0	645.0	645.0	645.0
Wheat gluten[b]	120.0	120.0	120.0	120.0	120.0	120.0
Wheat[c]	125.8	125.8	125.8	125.8	125.8	125.8
Fish oil[d]	80.0	76.0	71.8	67.6	63.2	59.0
Vevodar[e]	0.0	4.0	8.2	12.4	16.8	21.0
Premixes[f]	29.2	29.2	29.2	29.2	29.2	29.2
Analyzed values						
Moisture, %	8.0	7.8	8.3	8.4	8.6	8.3
Crude protein, % DM[g]	61.2	61.4	61.6	61.7	61.8	62.2
Crude fat, % DM	13.8	14.1	14.4	13.7	14.1	14.3

[a] LT fish meal, Skretting, Stavanger, Norway.
[b] Cargill Nordic, Charlottenlund, Denmark.
[c] Skretting, Stavanger, Norway.
[d] Scandinavian fish oil, Skretting, Stavanger, Norway.
[e] Contains 35% arachidonic acid, DSM Food Specialities, Delft, The Netherlands.
[f] Include micronutrients, vitamin and mineral supplementation. Trouw Nutrition, Boxmeer, Netherlands, proprietary composition Skretting ARC.
[g] Dry matter.