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Effects of dietary DHA and α-tocopherol on bone development, early mineralisation and oxidative stress in *Sparus aurata* (Linnaeus, 1758) larvae

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Abstract

DHA deficiency has been related to skeletal malformations in fish, but high DHA levels have produced controversial results that could relate to the oxidative status of fish tissues in the different reports. In the present study, gilthead seabream (*Sparus aurata*) larvae were fed deficient, adequate or high DHA levels, or high DHA levels supplemented with the antioxidant α-tocopherol. Larvae fed deficient DHA levels tended to be smaller, and showed the highest incidence of urinary bladder calculi, lordosis and kyphosis and the lowest number of mineralised vertebrae for any given size class. Elevation of dietary DHA increased larval growth and significantly enhanced the expression of the insulin-like growth factor 1 (*IGF-1*) gene. However, a DHA level increase up to 5% raised the degree of lipid oxidation in larval tissues and deformities in cranial endochondral bones and in axial skeletal haemal and neural arches. The increase in dietary α-tocopherol supplementation in high-DHA feeds reduced again the occurrence of skeletal deformities. Moreover, the expression of genes coding for specific antioxidants such as catalase, superoxide dismutase or glutathione peroxidase, which neutralised reactive oxygen substances formed by increased dietary DHA, was significantly decreased in larvae fed high α-tocopherol levels. These results denoted the importance of DHA for early bone formation and mineralisation. Low dietary DHA levels delay early mineralisation and increase the risk of cranial and axial skeletal deformities. Excessive DHA levels, without an adequate balance of antioxidant nutrients, increase the production of free radicals damaging cartilaginous structures before bone formation.

Key words: Essential fatty acids: Cranial abnormalities: Vertebral column anomalies: Bone mineralisation

Many genetic and epigenetic factors have been linked to skeletal abnormalities in cultured teleost fish[1]. Among the nutritional factors, several vitamins and minerals have recently been recognised to influence the occurrence of skeletal malformations[2–4]. Other nutrients such as dietary lipids have also been related to skeletal anomalies[5–8]. For instance, dietary phospholipids reduce the incidence of skeletal abnormalities in ayu larvae (*Plecoglossus altivelis*)[9] and in European sea bass (*Dicentrarchus labrax*)[5]. In particular, DHA has been related to skeletal malformations that occur in early larval stages of marine fish[10]. Increased DHA levels markedly reduced the occurrence of malformed fish (20·1% in larvae fed DHA-rich rotifers *v.* 51% in larvae fed control rotifers) in red porgy (*Pagrus pagrus*)[6] and milkfish (*Chanos chanos*)[11]. However, elevation of both DHA and EPA levels over 2% in the neutral lipid fraction of compound diets led to skeletal abnormalities in European sea bass[12]. Betancor *et al.*[13] also described the deleterious effects of excessive DHA contents in microdiets for sea bass. A high dietary DHA content increases peroxidation risks. Consequently, the deleterious effects caused by high DHA contents could be related to the proliferation of free radicals, derived from this fatty acid, and to the formation of toxic oxidised compounds that subsequently negatively affect bone development[10].

To control the formation of these reactive oxygen species, the fish antioxidant system includes a series of compounds that together neutralise reactive oxygen species. Components of the antioxidant system are vitamins C and E and several enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPX)[14]. Thus, reactive

Abbreviations: CAT, catalase; DH, DHA high; DHE, DHA high + α-tocopherol; DL, DHA low; DM, DHA medium; dph, days post-hatching; dw, dry weight; GPX, glutathione peroxidase; IGF-1, insulin-like growth factor 1; SOD, superoxide dismutase.

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oxygen species, such as $O_2^{\cdot-}$, are converted to H_2O_2 by SOD, and then to O_2 and water by several enzymes including CAT or GPX[15]. Differential expression of genes encoding these enzymes is considered as an effective method of denoting oxidative stress caused by an imbalance of oxidant/antioxidant compounds[16], following excessive dietary DHA. Other genes potentially affected by DHA and related to bone-forming tissues are insulin-like growth factors (*IGF*). Dietary PUFA may up-regulate or down-regulate the expression of such genes in fish tissues by modulating PG production as well as the expression of IGF-binding proteins. IGF-1, in particular, functions as both systemic and local growth factors for different tissues by stimulating cell proliferation and differentiation[17]. In mammalian bone, locally produced IGF-1 stimulates new bone cell formation and matrix production[18].

Since many malformations originate early during fish development, a better understanding of the processes of bone formation would allow a better control of the occurrence of bone anomalies[19]. Most bony elements of the endoskeleton have a cartilaginous precursor that is replaced by bone in the frame of endochondral bone formation[20]. Typical, mammalian-like, endochondral bone formation is often lacking in teleost fish. Especially, in fish larvae, bone develops around cartilaginous elements (perichondral bone). The cartilage inside the bone collar can remain intact or can be replaced by adipose tissue. Indeed, rather than simply bone and cartilage, fish skeletal tissue is often best described as a continuous spectrum ranging from the connective tissue to the cartilage and to the bone[20]. Bone is a specialised vascularised connective tissue consisting of cells and a mineralised extracellular matrix. Before being mineralised, the extracellular matrix is composed mainly of collagen type I called osteoid that subsequently becomes mineralised through the osteoblast-mediated deposition of hydroxyapatite[20]. Cartilage is an avascular skeletal tissue composed of chondrocytes that are embedded in an extracellular matrix primarily composed of collagen type II and proteoglycans. Chondroid bone is an intermediate tissue that is found, for example, in the mandibular and maxillary tissue of teleost fish and has intermediate characteristics of both bone and cartilage, containing both collagen types I and II, and may be mineralised. In teleosts, such as gilthead seabream (*Sparus aurata*), the formation of the vertebral body centrum is initiated by direct mineralisation of the notochordal sheath. In a second step, intramembranous bone is deposited around the mineralised notochord sheath. Different from most basal bonefish and tetrapods, teleost vertebral body development does not pass through a cartilaginous stage[21,22].

In order to better understand the dose-related effect of dietary DHA on early bone formation and on the development of skeletal anomalies, in the present study, gilthead seabream larvae were fed four different types of rotifers containing either deficient, adequate or excessive levels of this dietary fatty acid. A fourth type of rotifer contained the high DHA level and increased supplementation of α-tocopherol to determine whether the negative effects of excess DHA can be prevented by an increasing level of a nutritional antioxidant.

Experimental methods

Eggs from a spontaneous spawning of a genetically characterised seabream broodstock of the Grupo de Investigación en Acuicultura (Canary Islands, Spain) were seeded at a concentration of 120 eggs/l in sixteen 170-litre cylindrical conical tanks. The tanks were provided with fluorescent light at 12 h dark–12 h light and an air flow of 170 ml/min. Temperature, dissolved O_2 and pH recorded along the study were 20·66 ± 0·65°C, 5·87 ± 0·31 parts per million and 8·26 ± 0·08, respectively. Determination of egg quality[23] showed 98 % viability in terms of morphologically normal eggs, 80·5 % hatching rate and 89·06 % survival of larvae 3 d after hatching (days post-hatching; dph).

The four enrichment treatments were prepared containing three different levels of DHA and two different levels of vitamin E (Table 1). A low DHA enrichment (DHA low, DL) was prepared with DHA Protein Selco® (Inve) where fatty

Table 1. Ingredients and analysed composition of the enrichment products used to feed rotifers to attain low (DL), medium (DM) or high (DH) DHA contents and high DHA contents with an extra supplement of α-tocopherol (DHE)

(Mean values and standard deviations, *n* 3)

	DL		DM		DH		DHE	
Ingredients	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DHA Protein Selco®	–		100		90		90	
Defatted DHA Protein Selco®	90		–		–		–	
Lipid source*	10		–		–		–	
MorDHA†	–		–		10		10	
α-Tocopherol (mg/kg)‡	6000		6000		6000		10000	
Analysed nutrients								
Lipids (% dw)	10·93[a]	0·11	21·36[b]	0·17	31·11[c]	0·23	31·71[c]	0·35
Ash (% dw)	8·29	0·04	8·25	0·04	6·42	0·03	6·40	0·04
Protein (% dw)	34·23	3·21	30·66	2·75	25·62	5·06	24·28	4·84
DHA (% dw)	0·17[a]	0·04	3·20[b]	0·15	13·15[c]	0·30	13·84[c]	0·55
α-Tocopherol (mg/kg)	6393[a]	134	6325[a]	122	6149[a]	161	11758[b]	378

dw, Dry weight.
[a,b,c] Mean values with unlike superscript letters were significantly different ($P<0·05$).
* Lipid source: EPA50 and arachidonic acid (CRODA) and oleic acid (Sigma-Aldrich).
† MorDHA omega-3 I.Q. (Minami Nutrition).
‡ DL-α-Tocopherol acetate (Sigma-Aldrich).

acids had been extracted by washing three times with chloroform (3:1, v/v, chloroform:enrichment) and substituted by a mixture of lipids with a similar fatty acid composition except for the low DHA content (EPA50 and arachidonic acid; CRODA and oleic acid; Sigma-Aldrich) and included α-tocopherol (DL-α-tocopherol acetate; Sigma-Aldrich) to reach a concentration of 6000 mg/kg. A second enrichment (DHA medium, DM) contained the original DHA Protein Selco® plus α-tocopherol to reach a concentration of 6000 mg/kg. The third enrichment (DHA high, DH) contained DHA Protein Selco®, a DHA-rich oil (MorDHA omega-3 I.Q.; Minami Nutrition) and α-tocopherol to reach a concentration of 6000 mg/kg. Finally, the fourth enrichment (DHA high + α-tocopherol, DHE) was equal to the previous one, but increasing the α-tocopherol content up to 10000 mg/kg. These enrichments were kept at +4°C along the trial. Rotifers were enriched for at least 8 h to allow the incorporation of DHA from either ethyl ester (DH and DHE) or TAG forms (DL and DM) into the TAG and phospholipid fractions of rotifers, in agreement with previous studies[6]. After enrichment, rotifers contained 0·25, 2·39, 5·24 and 5·27 % DHA dry weight (dw) for the DL, DM, DH and DHE enrichments, respectively (Table 2).

The feeding regimen resemble that of commercial hatcheries and was as follows: from 3 to 25 dph, larvae were fed rotifers enriched with one of the four different enrichment mediums described: DL, DM, DH or DHE at a concentration of 10 rotifers/ml; from 15 to 34 dph, larvae were also fed enriched (DHA Easy Selco®; Inve) *Artemia* sp. (0·25–0·5 nauplii/ml) and from 24 to 34 dph with a weaning dry feed (Gemma Micro; Skretting). Therefore, feeding regimens only differed in the enrichment medium of rotifers between 3 and 25 dph, although larvae were reared until bone mineralisation at 34 dph for skeleton and mineralisation studies. For each treatment, four replicates were tested until 15 dph, when, in order to determine the effect of rotifer feeding on fish biochemical composition before feeding with *Artemia* sp., larvae from one tank per treatment were collected for biochemical analysis. After 15 dph, three replicates were kept for each treatment until the end of the trial at 34 dph.

The time of sampling was as follows: rotifers from each feeding treatment were sampled every 3 d for biochemical analysis; to determine the effect of rotifer feeding on larval growth, larvae were sampled at 3 (first feeding), 15 (before *Artemia* sp. feeding), 20 (end of maximum rotifer intake by the larvae) and 34 dph (end of the trial), standard length of thirty larvae per tank was measured by a profile projector (PJ-A3000; Mitutoyo) and dry body weight of thirty larvae per tank was obtained by drying them at 105°C until constant weight; to determine feed ingestion, the abdominal cavity of five larvae per tank was photographed every 3 d until the end of the experiment; to determine swim bladder inflation, twenty larvae per tank were sampled at 7 and 12 dph (period of first inflation of seabream swim bladder); to determine the effect of rotifer feeding on larval biochemical composition and thiobarbituric acid reactivity, all the larvae from one tank per treatment were collected at 15 dph (before *Artemia* sp. feeding), and analyses performed in two subsamples of that population; for gene expression studies at this age (15 dph), fifty larvae per tank were sampled; to determine the larval welfare status, fifteen larvae per tank were sampled at 20 dph (end of maximum rotifer intake by the larvae) and subjected to an acute stress of handling them out of the water for 60 s and returning them to a bucket with aerated fresh water to determine larval survival 24 h later[24]; to determine the occurrence of skeleton anomalies and mineralisation, 150 larvae per treatment were sampled

Table 2. Proximate (% dry weight (dw)) and fatty acid (% total fatty acid) composition of total lipids of rotifers fed enrichments with low (DL), medium (DM) or high (DH) DHA contents and high DHA contents with an extra supplement of α-tocopherol (DHE)*

(Mean values and standard deviations, *n* 8)

	DL		DM		DH		DHE	
Fatty acids	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14 : 0	1·54^{a}	0·12	1·75^{a}	0·03	1·26^{b}	0·02	1·22^{b}	0·02
16 : 0	11·47^{a}	0·67	22·07^{b}	0·92	14·25^{c}	0·36	13·07^{a,c}	0·66
18 : 0	2·92^{a}	0·26	4·17^{b}	0·07	2·99^{a}	0·43	3·17^{a}	0·10
18 : 1*n*-9	40·61^{a}	0·95	16·66^{b}	0·43	12·06^{c}	0·44	12·38^{c}	0·57
20 : 4*n*-6	1·33^{a}	0·09	1·26^{a,b}	0·04	1·13^{a,b}	0·04	1·09^{b}	0·03
20 : 5*n*-3	6·14^{a}	0·50	5·99^{a,b}	0·11	6·87^{b}	0·10	7·02^{b}	0·11
22 : 6*n*-3	1·68^{a}	0·28	12·34^{b}	0·24	27·04^{c}	0·71	27·01^{c}	0·63
Saturated†	17·85^{a}	0·57	29·48^{b}	2·65	19·92^{a}	1·64	18·91^{a}	2·17
Monounsaturated‡	60·91^{a}	0·79	33·63^{b}	2·96	28·69^{b}	2·63	29·60^{b}	2·48
n-3§	11·38^{a}	0·70	23·13^{b}	0·77	39·80^{c}	1·66	39·96^{c}	1·74
n-6‖	6·03^{a}	0·27	10·91^{b}	0·45	8·65^{c}	0·38	8·68^{c}	0·60
Lipids (% dw)	14·71^{a}	2·56	18·87^{a,b}	3·33	19·33^{b}	2·80	19·43^{b}	4·09
Ash (% dw)	2·03^{a}	0·38	2·00^{a}	0·35	1·79^{a}	0·64	1·96^{a}	0·50
Protein (% dw)	40·92^{a}	8·24	42·82^{a}	7·71	52·11^{a}	9·06	53·89^{a}	9·84

^{a,b,c} Mean values with unlike superscript letters were significantly different ($P<0·05$).

* Sampling along the rotifer feeding period every 3 d.

† Includes 12 : 0, 14 : 0, 15 : 0, 16 : 0, 17 : 0, 18 : 0, 20 : 0, 22 : 0 and 24 : 0.

‡ Includes 14 : 1*n*-5, 14 : 1*n*-7, 16 : 1*n*-9, 16 : 1*n* -7, 16 : 1*n*-5, 18 : 1*n*-9, 18 : 1*n*-7, 18 : 1*n*-5, 20 : 1*n*-9, 20 : 1*n*-7, 20 : 1*n*-5, 22 : 1*n*-11, 22 : 1*n*-9 and 22 : 1*n*-7.

§ Includes 16 : 2*n*-3, 16 : 3*n*-3, 16 : 4*n*-3, 18 : 3*n*-3, 18 : 4*n*-3, 20 : 3*n*-3, 20 : 4*n*-3, 20 : 5*n*-3, 22 : 4*n*-3, 22 : 5*n*-3 and 22 : 6*n*-3.

‖ Includes 16 : 2*n*-6, 18 : 2*n*-6, 18 : 3*n*-6, 18 : 4*n*-6, 20 : 2*n*-6, 20 : 3*n*-6, 20 : 4*n*-6, 20 : 5*n*-6, 22 : 3*n*-6, 22 : 4*n*-6 and 22 : 5*n*-6.

at 34 dph (earliest moment to determine complete mineralisation in seabream larvae), fixed and stored in buffered (10 % phosphate) formaldehyde after a light sedation with 10 % clove oil solution. The experiments of the present study were designed according to the Animal Welfare Ethics Committee guidelines of Las Palmas University.

Fixed larvae were stained with alizarin red and immediately photographed and examined for the occurrence of skeletal anomalies[25]. The different regions of the vertebral column were divided according to Boglione *et al.*[26]. Vertebrae were numerated from 1 to 24 using Roman numerals in a cranial to caudal direction. The presence of supernumerary vertebral bodies, and the presence of urinary calculus and anomalies different from the ones described by Boglione *et al.*[26] were analysed separately. The effects of the different enriched rotifers on axial skeleton mineralisation were evaluated considering the total number of completely mineralised vertebral bodies within a larval size class (standard length).

Proximate and fatty acid composition of rotifers and larvae were analysed. Total lipids were extracted with the chloroform–methanol mixture[27] and fatty acid methyl esters obtained by transmethylation of total lipids[28]. Fatty acid methyl esters were separated by GLC, quantified by a flame ionisation detector (GC Termo Finnigan Fucus GC; Thermo Fisher Scientific Inc.) under the conditions described in Izquierdo *et al.*[29]. Crude protein, moisture and ash content were analysed following the Association of Official Analytical Chemists methods[30]. α-Tocopherol content was analysed by HPLC. Thiobarbituric acid reactivity was determined by an adaptation of the Burk *et al.*[31] method as reported in Tocher *et al.*[32]. Values were expressed as μg malonaldehyde/g sample.

Total RNA was extracted from seabream larvae (approximately 200 mg; pool per tank), using the PureYield RNA Midiprep System. The quantity and purity of RNA were assessed by a spectrophotometer. Visualisation on 1 % agarose gel stained with ethidium bromide showed that RNA was not degraded. After DNase treatment (Invitrogen), 3 μg of total RNA were reverse transcribed into complementary DNA in a volume of 12 μl, including 1 μl of oligo-dT16 primer (50 pmol) and 1 μl of 10 mM-deoxynucleotide triphosphates. This mix was heated at 65°C for 5 min and chilled on ice, and then 4 μl of 5 × reverse transcription buffer, 2 μl of 0·1 M-dithiothreitol, 1 μl RNase out and 1 μl of Moloney murine leukaemia virus were added. After incubation at 37°C for 50 min, the reaction was stopped by heating at 75°C for 15 min. The PCR primer sequences used for the PCR amplification of complementary DNA of target genes such as *CAT*, *SOD*, *GPX* and *IGF-1* are shown in Table 3. A total of thirty PCR amplification cycles (eight touchdown) were performed for all primer sets, using an automated thermal cycler (MyCycler; BioRad). An aliquot of each sample was then electrophoresed on 1 % agarose gel and bands were detected by ethidium bromide staining. The PCR products from each primer set amplification were cloned using the pGEM®-T Easy Vector (Promega) and subsequently sequenced in both directions (T7 and SP6). TaqMan® real-time RT-PCR was performed on a StepOne Real Time PCR System (Applied Biosystems) using Assays-by-Design℠ PCR primers (Applied Biosystems)

Table 3. Gene abbreviations, GenBank accession numbers and PCR forward primer sequences for the analysed reference genes

Genes	Accession no.	Component	Forward primer (5′–3′)
SOD	FJ860004	Forward Reverse Taqman® probe	GTTGGAGACCTGGGAGATGT CTCCTCATTGCCTCCTTTTC CAGGAGGAGATAACATTG
CAT	FJ860003	Forward Reverse Taqman® probe	ATGGTGTGGGACTTCTGGAG AGTGGAACTTGCAGTAGAAAC CAGACACTCAGGCCTCA
GPX	FM013606	Forward Reverse Taqman® probe	AGTTAATCCGGAATTCGTGAGA TGAGTGTAGTCCCTGGTTGTTG AATGGCTGGAAACGTG
IGF-1	AY996779	Forward Reverse Taqman® probe	GCAGTTTGTGTGTGGAGAGAGA GACCGCCGTGCATTGG CTGTAGGTTTACTGAAATAAAA

SOD, superoxide dismutase; *CAT*, catalase; *GPX*, glutathione peroxidase; *IGF-1*, insulin-like growth factor 1.

and gene-specific fluorogenic probes (Table 3). Data from TaqMan® PCR runs were collected with ABI's Sequence Detector Program. Cycle threshold (C_t) values corresponded to the number of cycles at which fluorescence emission monitored in real time exceeded the threshold limit. The C_t values were used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA in total RNA. To reduce pipetting errors, master mixes were prepared to set up triplicate reactions (3 × 30 μl) for each sample.

All values presented as percentage (skeletal anomalies, mineralisation of the column, total survival and survival after the activity test) were arc cosine transformed. Statistical differences were checked with one-way ANOVA for multiple comparisons of means. In the case of statistical differences among the treatment groups, a Duncan *post hoc* test was applied in order to evaluate inter-group differences. If variances were not homogeneous, a parametric test was applied (homogeneity of the variance with Levene's test, data normality with Shapiro–Wilk test). The significance level was fixed at 95%.

Results

The proximate composition of rotifers enriched with the different DHA and α-tocopherol levels was not significantly different in terms of crude protein and ash content ($P>0·05$; Table 2). However, the lipid content was lower in rotifers fed the lowest DHA level (DL rotifers; $P<0·05$) than in those fed the highest DHA level (DH and DHE rotifers) (Table 2). Regarding the fatty acid profiles (Table 2), DL rotifers showed the lowest ($P<0·05$) DHA contents (0·25% dw), together with lower *n*-3, *n*-6 and SFA, particularly 16:0 and 18:0. On the contrary, these rotifers showed the highest percentage of MUFA, mainly oleic acid (18:1*n*-9). DM rotifers showed medium contents of DHA levels (2·39% dw) and the highest percentages of SFA, principally myristic (14:0), palmitic (16:0) and stearic (18:0) acids (Table 2). Rotifers fed the highest DHA level (DH and DHE rotifers) showed significantly ($P<0·05$) higher levels of DHA (5·2% dw) than DL and DM rotifers. Thus, an increase in DHA content in the enrichment products proportionally raised the DHA content in rotifers (R 0·96, $P<0·05$).

Lipid contents in 15 dph larvae were about 14% dw, being slightly higher in fish fed the highest DHA content (DH larvae) (Table 4). Crude protein and ash contents were significantly lowest in seabream fed DL rotifers. Regarding the fatty acid profiles (Table 4), while the total content of SFA was similar among larvae fed the different types of rotifers, monoenoic acid contents were significantly highest in DL fish, principally due to a higher percentage of oleic acid (18:1*n*-9). As expected, total *n*-3 fatty acids and, particularly, DHA contents were significantly lowest in DL larvae, gradually increasing in DM, DH and DHE larvae as a consequence of higher dietary DHA levels. Thus, a significant correlation between the DHA content in total lipids of rotifers and larvae was found ($y = 0·4502x + 1·1023$, R^2 0·9182). The degree of lipid oxidation in larval tissues, expressed as the malonaldehyde content (μmol/g) in gilthead seabream, was proportionally increased with the elevation of DHA in rotifers, the significantly lowest peroxidation level ($P<0·05$) being found in DL and DHE larvae (Table 4).

Survival of larvae from hatching until metamorphosis at 34 dph did not significantly differ among larvae fed the different enriched rotifers, being on average 22% very good for this type of trial and similar to that obtained by commercial hatcheries of this species. There were no significant differences in rotifer ingestion, swim bladder inflation or survival

Table 4. Proximate (% dry weight (dw)) and fatty acid (% dw) composition from total lipids and thiobarbituric acid-reactive (TBAR) contents of gilthead seabream larvae (15 d post-hatching) fed rotifers containing low (DL), medium (DM) or high (DH) levels of DHA and high DHA levels with an extra supplement of α-tocopherol (DHE)

(Mean values and standard deviations, *n* 2)

	DL		DM		DH		DHE	
Fatty acids	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	0·20^{a}	0·02	0·12^{b}	0·01	0·13^{b}	0·06	0·13^{b}	0·01
16:0	2·43^{a}	0·29	2·28^{a}	0·02	2·37^{a}	0·13	2·41^{a}	0·12
18:0	1·18^{a}	0·08	1·11^{a}	0·04	1·29^{a}	0·15	1·17^{a}	0·02
18:1*n*-9	2·09^{a}	0·19	1·66^{a,b}	0·32	1·76^{a,b}	0·22	1·35^{b}	0·15
20:4*n*-6	0·55^{a}	0·03	0·48^{a}	0·04	0·51^{a}	0·03	0·48^{a}	0·05
20:5*n*-3	1·47^{a}	0·18	1·14^{b}	0·09	1·31^{a,b}	0·13	1·22^{a,b}	0·11
22:6*n*-3	0·97^{a}	0·11	2·49^{b}	0·41	3·62^{c}	0·34	3·12^{c}	0·18
Saturated†	4·02^{a}	0·38	3·72^{a}	0·16	4·02^{a}	0·11	3·93^{a}	0·02
Monoenoic‡	4·37^{a}	0·26	3·56^{b}	0·22	3·63^{b}	0·29	3·24^{b}	0·18
n-3§	3·34^{a}	0·41	4·46^{b}	0·60	5·80^{c}	0·25	5·35^{b,c}	0·50
n-6‖	1·32^{a}	0·51	1·36^{a}	0·10	1·41^{a}	0·50	1·18^{a}	0·16
Crude lipids (% dw)	13·39^{a}	0·99	13·53^{a}	0·93	15·63^{a}	1·26	14·03^{a}	0·75
Ash (% dw)	1·47^{a}	0·05	1·96^{c}	0·05	1·97^{b,c}	0·32	1·53^{a,b}	0·24
Protein (% dw)	73·86^{a}	2·12	77·38^{b}	2·86	75·85^{b}	3·97	77·90^{b}	2·88
TBAR (μg malonaldehyde/g)	0·20^{a}	0·04	8·93^{b}	1·20	11·04^{b}	1·50	0·23^{a}	0·06

^{a,b,c} Mean values with unlike superscript letters were significantly different ($P<0·05$).

† Includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0.

‡ Includes 14:1*n*-5, 14:1*n*-7, 16:1*n*-9, 16:1*n*-7, 16:1*n*-5, 18:1*n*-9, 18:1*n*-7, 18:1*n*-5, 20:1*n*-9, 20:1*n*-7, 20:1*n*-5, 22:1*n*-11, 22:1*n*-9 and 22:1*n*-7.

§ Includes 16:2*n*-3, 16:3*n*-3, 16:4*n*-3, 18:3*n*-3, 18:4*n*-3, 20:3*n*-3, 20:4*n*-3, 20:5*n*-3, 22:4*n*-3, 22:5*n*-3 and 22:6*n*-3.

‖ Includes 16:2*n*-6, 18:2*n*-6, 18:3*n*-6, 18:4*n*-6, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:5*n*-6, 22:3*n*-6, 22:4*n*-6 and 22:5*n*-6.

after the activity test (85 %). Regarding growth, during the first 2 weeks of pure rotifer diet, the total length of larvae increased proportionally to the DHA levels of rotifers (mean total length: DL 4·78 (SD 0·34), DM 4·97 (SD 0·53), DH 5·02 (SD 0·26) and DHE 5·15 (SD 0·22), $P<0·05$, n 120). Nevertheless, lipid and, hence, gross energy contents were also increased in rotifers with higher DHA levels. Once *Artemia* and the weaning diet were fed to all larvae, growth became similar among the treatments and, thus, no significant differences among larvae were observed at the end of the trial (Fig. 1). Trend curves of the standard length increase in larvae fed rotifers with the lowest DHA content (DL) reflected the lowest growth in these larvae. Similar trends were also found in larval dry body weight, which, at 34 dph, reached 0·92 (SD 0·24), 1·20 (SD 0·27), 1·22 (SD 0·32) and 1·16 (SD 0·25) mg for DL, DM, DH and DHE larvae, respectively.

The lowest occurrence of total skeletal deformities was found in larvae fed DM rotifers ($P<0·05$) (Table 5). Either the reduction of DHA contents in DL rotifers or its increase in DH rotifers significantly raised the percentage of total anomalies (Table 5). The types of deformities differed according to the level of DHA. Thus, DL larvae showed significant increases in the occurrence of lordosis and kyphosis (cranial–pre-haemal *v.* II–III vertebrae or haemal *v.* XX–XXI vertebrae) in comparison with DM larvae. Concurrently, DH larvae showed a significantly higher incidence of maxillary or mandibular anomalies (maxillary prognatism), as well as a higher occurrence of haemal or neural spine abnormalities (curled, wrong directed or bifurcated, frequently in cranial (*v.* II), pre-haemal (*v.* V) or haemal vertebrae). On the contrary, increasing α-tocopherol contents in DHE rotifers significantly reduced the occurrence of total deformities in DHE larvae in comparison with DH larvae (Table 5). Thus, the incidence of both maxillary/mandibular or haemal/neural spines in DHE larvae was similar to that in DM larvae. Opercular anomalies were found only in one specimen.

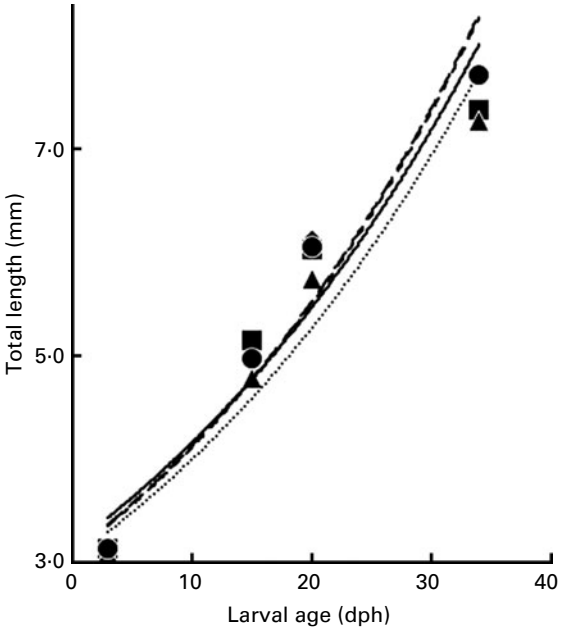

Fig. 1. Development of the standard length of gilthead seabream fed rotifers low (DL, ▲; $y=3·1619e^{0·0274x}$, R^2 0·92325), medium (DM, ●; $y=3·0722e^{0·0292x}$, R^2 0·95291) or high (DH, ◆; $y=3·083e^{0·0292x}$, R^2 0·94394) in DHA and high in DHA with an extra supplement of α-tocopherol (DHE, ■; $y=3·0317e^{0·0276x}$, R^2 0·95628) (n 90). dph, Days post-hatching.

Marked differences among larvae fed the different types of rotifers were also found regarding the mineralisation of the vertebral centra (Fig. 2). Since the number of mineralised centra increased with larval growth, the number of mineralised vertebrae in the different larval groups was compared within the size classes (Fig. 2). For each given size class between 6 and 9 mm, DL larvae always showed a significantly ($F=6·11>F_{crit}=2·54$, $T=2·36$) lower number of mineralised vertebrae, whereas the elevation of dietary DHA (DM, DH and DHE larvae) markedly increased mineralisation (Fig. 2). In particular, from 7·085 up to 8·085 mm standard length, larvae from the DHE group showed twenty completely mineralised vertebral centra, whereas the reduction in dietary α-tocopherol in DH larvae limited this number to seventeen to twenty. The reduction in dietary DHA in DM and DL larvae further decreased the number of completely mineralised vertebrae to fourteen to twenty and ten to twenty, respectively. As a consequence, the size at which all larvae had completed the mineralisation of the vertebral column was delayed until 9·09 mm standard length in DL larvae, in comparison with larvae fed higher DHA (8·58 mm in DM and DH larvae) or α-tocopherol (8·09 mm standard length in DHE larvae). Although the presence of calculi in the urinary bladder of gilthead seabream did not significantly differ among the treatments, the highest value was found in fish fed the DHA-deficient diet (9·3 % of the population) in comparison with those larvae fed DHA-enriched rotifers (2·0, 5·3 and 0·7 % for DM, DH and DHE, respectively).

Regarding the expression of the target genes (Fig. 3), the *IGF-1* mRNA copy number significantly increased with the elevation of dietary DHA. On the contrary, in comparison with larvae fed the highest DHA content (DH), those fed higher dietary α-tocopherol showed a significantly reduced expression of *IGF-1* mRNA. The expression of the *CAT* gene tended to increase proportionally to the elevation of DHA contents in rotifers, although no significant differences were found among these values (Fig. 4). However, the increase in dietary α-tocopherol significantly reduced the expression of both *CAT* and *SOD* genes (Fig. 4), whereas the elevation of dietary vitamin E led to an increase in the number of mRNA copies of GPX in larvae fed the DHE diet (Fig. 4).

Discussion

The contents in essential arachidonic acid (20 : 4*n*-6) and EPA (20 : 5*n*-3) in all the enriched rotifers (over 0·2 and 0·9 % dw, respectively) were enough to cover the requirements of gilthead seabream larvae for these fatty acids[33]. Additionally, DHA levels in rotifers enriched with DHA (2–5 % dw in DM, DH and DHE rotifers) were also higher than the minimum requirement determined for this species[34,35]. However, rotifers containing the lowest DHA levels (0·25 % dw in DL) represent a deficient diet in this fatty acid. They contained

Table 5. Percentage of larvae bearing each skeletal anomaly (% of total fish) and the sum of these incidences (total anomalies) in 34 d post-hatching (dph) gilthead Seabream after feeding from 2 to 25 dph with rotifers low (DL), medium (DM) or high (DH) in DHA and high in DHA with an extra supplement of α-tocopherol (DHE)

(Mean values and standard deviations, *n* 150)

	Total anomalies		Lordosis		Kyphosis		Fusions		Maxilla/ mandible		Haemal/- neural spine anomalies	
Dietary treatments	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DL	9·3[a]	1·6	4·6[a]	3·6	2·0[a]	1·1	0·7	0·6	0·7[a]	0·6	1·3[a]	1·2
DM	4·0[b]	0·8	1·0[b]	0·9	ND		ND		1·0[a]	0·9	2·0[a]	0·1
DH	10·3[a]	1·4	1·4[b]	1·2	0·7[b]	0·6	0·7	0·6	4·1[b]	3·7	2·7[a]	1·1
DHE	7·6[c]	0·5	1·4[b]	1·2	0·7[b]	0·6	1·4	1·2	2·0[a,b]	1·2	1·4[a]	1·2

ND, not detected.
[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).

only 31 % of DHA required by gilthead seabream larvae (0·8 % dw)[36]. Among the different essential fatty acids, DHA appears to be the most effective larval growth promoter. Its incorporation into larval tissues affects intercellular interaction, receptor expression, nutrient transport and signal transduction, all of which affect cell growth[33]. Accordingly, in the present study, larval growth tended to be lower when fish were fed DL rotifers with only 0·25 % DHA content. These fish also presented the lowest *IGF-1* gene expression. Moreover, these fish showed a higher incidence of lordosis and kyphosis, possibly a consequence of poor mineralisation found in these larvae. Similar types of anomalies in adult fish appear to result from inadequate adaptation of individual vertebrae to muscular load[37]. In other vertebrates, an essential fatty acid deficiency has been associated with a reduction in bone mineralisation and increased renal calcification[38]. The presence of calculi in the urinary bladder of gilthead seabream, observed in the present study, was highest in fish fed the DHA-deficient diet, although the differences were not statistically different from the other fish. The present results are in agreement with those found in mice, where increased DHA levels reduced the presence of renal calculi[39]. Moreover, DHA has been found to increase bone mineral content and bone mass in other vertebrates[38,39]. Accordingly, together with the lowest number of mineralised vertebrae, gilthead seabream larvae fed the lowest DHA level also presented a lower mineral content reflected in the lowest ash quantity in these larvae. In addition to the important role of DHA for mineral metabolism across biomembranes, this fatty acid seems to be required for bone formation, which is regulated by systemic hormones and local factors produced in bone such as eicosanoids[40] and, probably, docosanoids. Docosanoids are mono-, di- and trihydroxylated derivatives of DHA including docosatrienes, protectins and the D-series resolvins[41]. In gilthead seabream, lipoxygenase derivatives from DHA have been found to regulate cell responses in the kidney[42,43]. In turn, these compounds influence the synthesis and action of IGF, growth factors also produced locally in bones[44] that promote cell proliferation and differentiation[45]. Accordingly, in the present study, IGF-1 expression was the lowest in larvae fed the lowest DHA level. These larvae also showed the lowest growth. The increase in DHA has also been associated with higher IGF-1 expression in European seabass (*D. labrax*)[13]. IGF-1 acts as a regulator of bone cell function, as it stimulates the proliferation of pre-osteoblasts, thereby increasing the number of cells capable of producing the bone matrix[38]. Consequently, the enhanced mineralisation effect of increased DHA could be at least partly mediated by

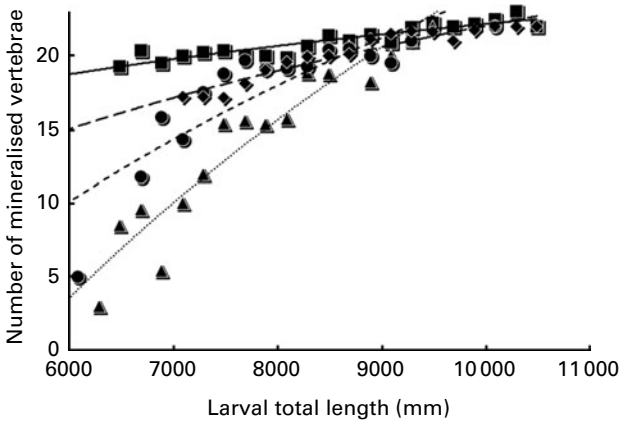

Fig. 2. Number of mineralised vertebrae per larvae of each size class of gilthead seabream (34 d post-hatching (dph)) fed from 3 to 25 dph rotifers low (DL, ▲; $y = 42·096 \ln(x) - 362·68$, R^2 0·90783), medium (DM, ●; $y = 27·511 \ln(x) - 229·27$, R^2 0·74658) or high (DH, ◆; $y = 13·777 \ln(x) - 104·85$, R^2 0·91801) in DHA or high in DHA with an extra supplement of α-tocopherol (DHE, ■; $y = 6·7165 \ln(x) - 39·698$, R^2 0·84637).

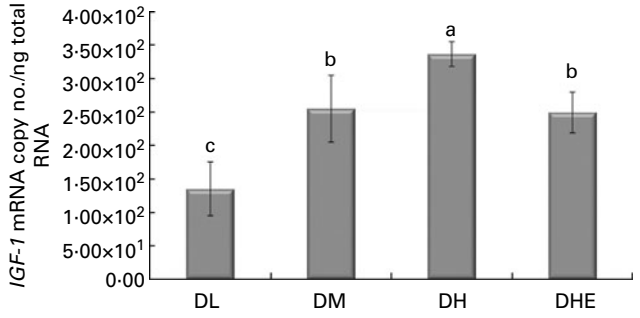

Fig. 3. Insulin growth factor 1 (*IGF-1*) gene expression levels measured by real-time PCR in *Sparus aurata* larvae (15 d post-hatching) fed exclusively rotifers low (DL), medium (DM) or high (DH) in DHA or high in DHA with an extra supplement of α-tocopherol (DHE). mRNA copy number of each gene was normalised as a ratio to 100 ng total RNA. Values are means (*n* 8), with their standard deviations represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$).

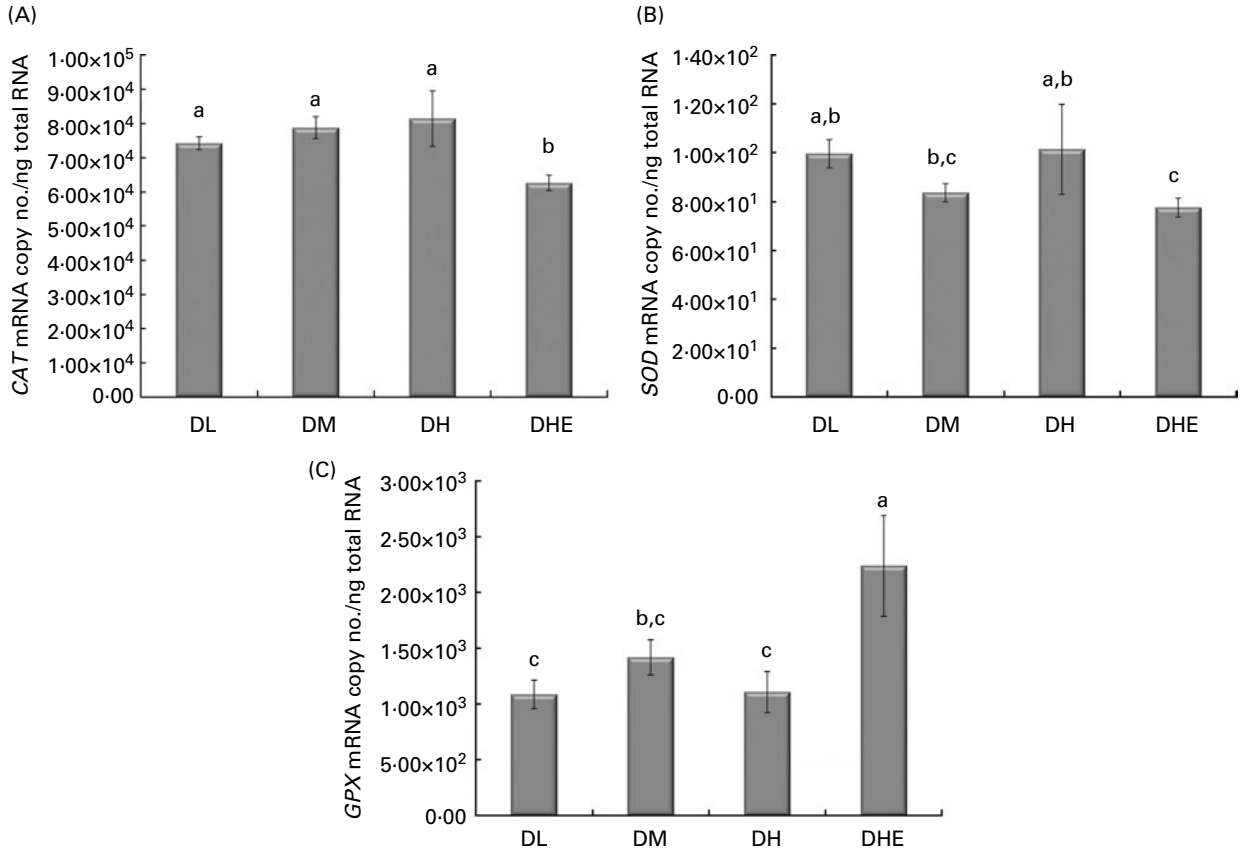

Fig. 4. Gene expression of (A) catalase (*CAT*), (B) superoxide dismutase (*SOD*) and (C) glutathione peroxidases (*GPX*) measured by real-time PCR in *Sparus aurata* larvae (15 d post-hatching) fed exclusively rotifers either low (DL), medium (DM) or high (DH) in DHA or high in DHA with an extra supplement of α-tocopherol (DHE). mRNA copy number of each gene was normalised as a ratio to 100 ng total RNA. Values are means (n 8), with their standard deviations represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$).

the significant up-regulation of IGF-1 found in the present study. Deficiencies in essential fatty acids, including DHA, have also been related to a reduction in the synthesis of the bone connective tissue matrix[46] and to the expression of osteogenic markers such as osteocalcin[47].

The increase in dietary DHA levels up to 2% (DM rotifers) significantly reduced the number of total deformed larvae, including lordosis and kyphosis, even below the mean frequency of abnormalities reported in gilthead seabream hatcheries (7–20%)[48]. However, further elevation of DHA up to 5% increased the maxillary/mandibular deformity occurrence. The detrimental effects of excessive dietary DHA have also been found in European sea bass, where severe dystrophic lesions in the epaxial musculature were observed[13,49]. An increase of both DHA and EPA in phospholipids of sea bass increased skeletal abnormalities and larval mortality[12]. However, an 8 h enrichment with the type of emulsions used in the present study increases mostly the DHA contents in the neutral lipid fraction rather than in the phospholipid fraction as demonstrated in previous studies[6], denoting the specific effect of dietary DHA, independently of EPA or phospholipid dietary levels. Thus, the elevation of dietary DHA markedly raises the risk of peroxidation and the subsequent proliferation of free radicals and toxic oxidised compounds such as fatty acid hydroxyperoxides, fatty acid hydroxides, aldehydes and hydrocarbons. The latter may be toxic and damage membrane lipids, proteins or DNA[33]. Free radicals and oxidation products have been found to induce apoptosis of mammalian bone cells. Interestingly, the increase in dietary DHA, together with an increased oxidative status of larvae, was associated with particular deformities. In the cranium, namely maxilla and mandible were affected. In the axial skeleton, haemal and neural spines were observed to be deformed. All these skeletal elements develop from a cartilaginous precursor in seabream. In contrast, skeletal elements without a cartilaginous precursor, such as the vertebral centra, were not negatively affected. Indeed, reactive oxygen species are known to actively destroy cartilaginous tissue[50], and therefore they rather affect the cartilaginous anlagen of endochondral bones than the directly mineralising acellular (anosteocytic) seabream bones.

To prevent oxidative damage, a range of effective antioxidant systems including various antioxidant enzymes, such as CAT, SOD or GPX, intercept and inactivate reactive intermediates, preventing the cascade of oxidant reactions. Together with these enzymes, dietary micronutrients such as α-tocopherol or vitamin C complete antioxidant defences in fish. Moreover, an increase in the formation of free radicals that derive from PUFA in larval tissues that would not be accompanied by an increase in α-tocopherol would reduce

the availability of ascorbic acid that recycles the oxidised α-tocopherol. Vitamin C is an essential cofactor for collagen formation, and hence a lack of vitamin C causes bone deformities in teleost fish[51]. In the present study, increased α-tocopherol in high DHA enrichments reduced the occurrence of jaw deformities and of haemal and neural spine deformities in comparison with fish fed high DHA without α-tocopherol supplementation (DH). The progressive increase in the expression of specific antioxidant genes, such as *CAT* and *SOD*, to neutralise the generated reactive oxygen substances was significantly reduced in larvae fed higher α-tocopherol (DHE), denoting the protective effect of α-tocopherol against oxidative stress. Indeed, the activities of these two enzymes would be expected to parallel each other, since superoxide anions are efficiently scavenged by α-tocopherol in biological systems. Although both GPX and CAT remove H_2O_2, GPX is more involved in the removal of organic peroxides. Disruptions in the expression of several antioxidant enzymes in fish larvae exposed to increased dietary DHA have recently been described in European sea bass[13]. These results further suggest that the type of skeletal deformities caused by high dietary DHA is related to the increased oxidative risk in larvae that damages the cartilage anlagen of endochondral bones. Besides, optimum DHA levels may also be dependent on dietary levels of other nutrients[52]. Finally, the increase in dietary α-tocopherol was also associated with a significant reduction in IGF-1, further supporting the suggestion that oxidative stress may play a role in the expression of these growth factors[13]. Nevertheless, studies on the effects of α-tocopherol on fish musculoskeletal development or malformation are scarce. The subject of malformations that are associated with oxidised lipids[19] or high levels of PUFA[49,52] deserves further investigation.

In summary, the results of the present study denote the importance of feeding amounts of dietary DHA during the first feeding of gilthead seabream larvae to better control the occurrence of skeletal anomalies. Early feeding of an adequate DHA diet is important, even if survival or growth is promoted later in development by feeding adequate DHA levels in *Artemia* and inert diets. Feeding low-DHA rotifers can delay early mineralisation and thus increase the risk of cranial and axial skeletal deformities as a consequence of the natural mechanical load that derives from swimming and feeding. On the other hand, feeding high-DHA rotifers, without an adequate balance of antioxidant nutrients, may increase the production of free radicals that damage the cartilaginous anlagen of bones and thus favour the development of skeletal deformities.

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