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PII: S0044-8486(23)00092-3

DOI: https://doi.org/10.1016/j.aquaculture.2023.739319

Reference: AQUA 739319

To appear in: *aquaculture*

Received date: 20 September 2022

Revised date: 23 January 2023

Accepted date: 31 January 2023

Please cite this article as: Y. Tseng, K.M. Eryalçın, U. Sivagurunathan, et al., Effects of the dietary supplementation of copper on growth, oxidative stress, fatty acid profile and skeletal development in gilthead seabream (Sparus aurata) larvae, *aquaculture* (2023), https://doi.org/10.1016/j.aquaculture.2023.739319

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Effects of the dietary supplementation of copper on growth, oxidative stress, fatty acid profile and skeletal development in gilthead seabream (*Sparus aurata*) larvae

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Abstract

The effect of dietary copper (Cu) levels on growth, oxidative stress, fatty acid profile and bone health of gilthead seabream larvae (*Sparus aurata*) was studied in 47 days-post-hatching fish fed 4 experimental diets containing 17, 18, 19 and 25 mg Cu/kg diet. After 21 days of feeding, larval growth and survival were not affected by the dietary Cu levels. However, larvae fed the non-supplemented diet (17 mg Cu/kg) showed clear symptoms of Cu deficiency, including increased lipid peroxidation and reduced bone mineralization. Increased peroxidation risk caused a reduction in essential fatty acids and n-3 PUFA (polyunsaturated fatty acids) contents in whole body lipids, whereas the poor vertebral body mineralization in seabream larvae was related to a marked increase in abdominal vertebrae anomalies. On the contrary, dietary Cu supplementation raised whole-body contents in DHA, EPA and n-3 PUFA and reduced MUFA, TBARS and the incidence of severe, cranial, branchiostegal rays and abdominal vertebral anomalies. Polynomial regression models applied to all these parameters suggested optimum dietary Cu levels to be 21.5-22.6 mg/kg in gilthead seabream larvae microdiets supplemented with $CuSO_4.5H_2O$. These levels (21.5-22.6 mg/kg) are in the range of the Cu contents in copepods (12-38 mg/kg) and on the verge of the dietary levels authorized by EU Commission in feeds for cultured fish species (25 mg Cu/kg diet).

Keywords: Gilthead seabream (*Sparus aurata)* larvae, bone health, Copper requirement

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1. Introduction

Copper (Cu) is one of the essential trace elements for survival, development, and growth of fish (Lall, 2003). It plays important roles in metabolism, taking part in several enzymatic complexes such as superoxide dismutase, and regulating lipid metabolism or hematopoiesis (NRC, 2011; Lall and Kaushik, 2021). Additionally, a Cu-dependent enzyme, lysyl-oxidase mediates the biosynthesis of collagen and normalizes the deposition of calcium and phosphorus in bones (Tomaszewska et al., 2017). The deficiency of dietary Cu causes growth reduction, increased oxidative stress, reduced appetite, anemic condition, and low Cu content in tissues of fish juveniles (Abdel-Hameid et al., 2017; Lin et al., 2010, 2008; Moazenzadeh et al., 2020). On the contrary, excessive levels of dietary Cu may cause toxicity, leading to alterations in several physiological functions, hepatic damage and cholestasis (Domínguez et al., 2019). Moreover, exposure of fish during the embryonic stages to high Cu water contents induces different types of skeletal anomalies, such as vertebral anomalies in mummichog (*Fundulus heteroclitus*) (Mochida et al., 2008), spinal anomalies (mainly kyphosis and lordosis) in Japanese rice fish (*Oryzias latipes*) (Barjhoux et al., 2012) and vertebral curvatures in ide (*Leuciscus idus*) (Witeska et al., 2014). In fish larvae, a micro-mineral-free diet (contained low Cu: 17 mg /kg) reduced growth and the antioxidant enzymes gene expression copper-zinc-superoxide dismutase (*cuznsod*) (Eryalçın et al., 2020) in comparison with Cu supplemented group (21 mg Cu/kg), similar Cu deficiency symptoms were also found in post-larvae (Song et al., 2021). However, the effect of several dietary levels of Cu have been scarcely studied in larvae of marine fish.

In fact, the information about mineral nutrition in marine fish larvae is still very scarce, particularly in relation to copper. Among various live preys, rotifer (*Brachionus sp.*) is the most common feed for the cultured marine larvae during the first-feeding stage for its adequate size for the mouth of marine fish larvae and simple batch production techniques and high reproduction rate (Holt, 2011). However, rotifers are not the main food for wild marine fish larvae, whereas copepods are part of their natural diet. Indeed, rotifers are deficient in several nutrients including microminerals (Hamre et al., 2008). Thus, the Cu content in rotifer (3.4 mg/kg dry weight) may be 7.3 times lower than that of copepods (12-38 mg/kg dry weight), suggesting that rotifers may not fulfill the Cu requirement for marine fish larvae (Hamre et al., 2008). Moreover, Cu contents in rotifer are even lower than the dietary levels required by some juvenile marine fish (NRC, 2011).

Many malformations, particularly in bone, may appear during larval development. In fact, skeletal anomalies are a major problem for the quality of juveniles produced, since they negatively impact swimming ability, growth, feed efficiency, and survival, leading to reduced production and profitability (Boglione et al., 2013, 2001). Moreover, a high incidence of skeletal anomalies severely reduces larval quality and increase mortality. An optimal micro-mineral supplementation reduces the incidence of skeletal anomalies. For instance, increase in dietary levels of manganese (Mn) (90 mg/kg) and zinc (Zn) (130 mg/kg) reduced vertebral malformations in Senegalese sole post-larvae (*Solea senegalensis*) and improved their survival (Viegas et al., 2021). Besides, dietary supplementation with Zn regulates the

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expression of bone related genes in zebrafish (*Danio rerio*) larvae, such as *runx2,* the principal transcription factor involved in the control of osteoblast differentiation and osteocalcin (*oc*) (an effective molecular marker of bone mineralization) secretion, was up-regulated, which indicating that an appropriate level of Zn can promote the bone formation (Roberto et al., 2018). Furthermore, in gilthead seabream larvae, a combined supplementation with Zn, Mn and Se in the organic form can promote body weight, mineralization and reduce branchial arches anomalies (Izquierdo et al., 2017). Similarly, Cu plays an important role in bone metabolism by being a cofactor for lysyl-oxidase, one of the principal enzymes involved in collagen cross-linking (Tomaszewska et al., 2017). Besides, the antioxidant role of Cu may prevent the formation of free radicals, which could damage the developing bone (Beer and Wegener, 2011; Izquierdo et al., 2013), hence this micronutrient is also essential for the skeletal development. Gilthead seabream juveniles fed a non-Cu-supplemented diet (5.5 mg Cu/kg) showed a high prevalence of skeletal anomalies (Domínguez et al., 2019). In a previous larval study, feeding gilthead seabream larvae a diet without Cu supplementation (17 mg Cu/kg) led to a reduction in whole body contents of Cu, zinc (Zn), or iron (Fe), however the potential effect of dietary Cu deficiency on skeletal anomalies was not studied (Eryalçın et al., 2020). Moreover, such study only compared two dietary Cu levels. Thus, it would be interesting to determine the effect of graded dietary Cu levels on the incidence of skeletal anomalies in gilthead seabream larvae.

Therefore, this study aimed at investigating the effects of several dietary Cu levels on growth performance, oxidative stress, and skeletal development in gilthead seabream (*Sparus aurata*) larvae.

2. Materials and methods

All the animal experiments were performed according to the European Union Directive (2010/63/EU) and Spanish legislation (Royal Decree 53/2013) on the protection of animal for scientific purposes at ECOAQUA Institute of University of Las Palmas de Gran Canaria (Canary Island, Spain).

2.1 Experimental diets

Four experimental microdiets were prepared to be isonitrogenous (66.6%) and isolipidic (17%), and formulated to contain different Cu levels (Table 1). The negative control diet was not supplemented with Cu and contained 17 mg Cu/kg (diet Cu17), whereas the other three diets were supplemented with copper sulfate pentahydrate $CuSO_4 \cdot 5H_2O$ and contained 18, 19 and 25 mg Cu/kg (diets Cu18, Cu19 and Cu25, respectively). Each experimental microdiet was tested in triplicate (n=3). All the ingredients were grounded (Braun, Suderm, Germany) and sieved (Filtra, Barcelona) <125 μm prior to micro diet preparation (Eryalçın et al., 2017). Briefly, water soluble components were mixed following an increasing order of ingredient content and then, the lipid source and fat-soluble vitamins were added. Finally, distilled water with dissolved gelatin was added. The paste was further mixed until a stiff dough was obtained and then passed through a mincer (Severin, Suderm, Germany). The obtained strands were dried in the oven (Ako, Barcelona, Spain) at 37°C for 24 h. After drying, the strands were grounded and sieved again into two different particle sizes, < 250 μm and 250-500 μm, and then stored at −80°C until use.

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(Insert **Table 1** & **Table 2**)

2.2 Fish, experimental conditions, and procedure

Larvae were obtained from natural spawns from the gilthead seabream broodstock at Grupo de Investigación en Acuicultura (GIA). Before the trial, larvae were fed rotifers (*Brachinous plicatilis*) enriched with Ori-Green (Skretting, France). Larvae (initial dry weight 0.46 ± 0.09 mg, total length 7.9 ± 1.61 mm, mean ± SD) at 26 days post-hatching (dph) were randomly distributed into 12 fiberglass cylinder tanks with conical bottoms (170 L), at a density of 2100 larvae per tank. All tanks were supplied with filtered seawater (37‰ salinity) that entered from the bottom and outflowed from the top at an increasing rate of 0.3–1.0 L/min. Fish larvae were rearing under the flow-through system during the entire experiment. Water was continuously aerated (125 mL/min) attaining 5–8 g/L dissolved O_2, and saturation ranged between 60% and 80%. Water temperature was daily determined in each tank at h. 14:00 and the average temperature was 20.4 ± 0.4°C during the entire trial. Dissolved Cu concentrations (nM) and free inorganic Cu (pM) were very low, 0.58 nM, 0.16 pM, respectively (Arnone et al., 2022). Photoperiod was kept at 12h light: 12h dark. Tanks were daily siphoned in the afternoon. Larvae were hand fed every 45 minutes from 8:00 am to 8:00 pm for 21 days until 47 dph. The total daily amount of diet (size < 250 µm)/tank was 3 g during the first week of feeding, and then was increased to 4 and later 5 g (size 250-500 µm)/tank during the second and third week of feeding, respectively. After the fifth day of feeding, larvae were checked under a stereomicroscope (Leica, M125, Germany) to determine the feed acceptance. Larvae mortalities were daily registered by individually counting the dead larvae.

2.3 Growth determination

Prior to each of sampling point at initial (26 dph), intermediate samplings (33, 40 dph) and at the end of trial (47 dph), larvae were anesthetized using iced seawater and cleaned with distilled water. Growth performance was determined by measuring the total length of 30 larvae per tank under the profile projector (Mitutoyo, PJ-A3000, Japan), and dry weight was registered by drying the larvae in an oven at 105°C until constant weight. Final survival rate was calculated by individually counting all the live larvae at the end of experiment. Biomass was calculated by multiplying the dry weight of the larvae by the number of remaining live larvae. Then those remaining live larvae were anesthetized using iced seawater, washed with distilled water, and kept at -80°C for further analysis.

2.3 Proximate composition, fatty acid profiles and lipid peroxidation

Feed and whole-body samples were weighed and analyzed for moisture, ash, and crude protein contents according to AOAC (2000). Total lipids were extracted using a chloroform:methanol solution (2:1) (Folch et al., 1957). Fatty acid methyl esters were obtained by transmethylation of lipids as described by Christie (1989), separated by gas chromatography (GLC), quantified by flame ionization detection (FID) (GC-14A; Shimadzu, Tokyo, Japan) following the conditions described in Izquierdo (1989) and identified by comparison with previously characterized standards.

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Thiobarbituric acid reactive substances (TBARS) were analyzed in triplicate samples of total lipid from whole-body (Burk et al., 1980). Briefly, 200 µL of lipids were added to 50 uL of 0.2% (w/v) BHT and 0.5 mL of 10% (w/v) trichloroacetic acid (TCA). A freshly prepared solution, 0.5 mL of 0.288% (w/v) thiobarbituric acid (TBA), was added before mixing in a vortex and heating in a water bath at 100°C for 20 min. After cooling, the precipitate was removed by centrifugate (Sigma 2-16KL, Germany) at 2000 *g.* The supernatant was read in a spectrophotometer (Evolution 300; Thermo Scientific, Cheshire, UK) at 532 nm against a blank sample. The concentration of TBARS, expressed as nmol malondialdehyde (MDA)/g lipid was calculated using the absorption coefficient 0.156 μM^{-1} cm^{-1}.

2.4. Mineral analysis

Mineral composition of diets and whole body larvae samples were determined by inductively coupled plasma mass spectrometry (ICP-MS) at the Institute of Marine Research (IMR, Bergen, Norway). Approximately 0.20-0.25 g of samples with 0.5 mL of Milli-Q® water were digesting using 2 mL of concentrated nitric acid (HNO_3) by Milestone Ultrawave. Then digested samples were diluted to 25 mL with Milli-Q® water. Subsequently the samples were introduced into nebulizer tube of the ICP-MS (iCapQ ICP-MS, Thermo Scientific, Waltham, USA) with an auto sampler (FAST SC-4Q DX, Elemental Scientific, Omaha, USA) and the mineral compositions were determined by ICP-MS.

2.5 *Gene expression*

After 8 (33 dph), 15 (40 dph) and 21 (47 dph) days of feeding, 30 larvae from each tank were randomly collected and washed with DEPC water and stored in RNALater® (Sigma, Madrid, Spain) at -80°C until further analysis. Total RNA was extracted from the whole body tissue by using a RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. The quality and quantity of the extracted RNA were determined by using a NanoDrop spectrophotometer (Thermo Scientific,Wilmington, DE, USA). Synthesis of cDNA was conducted following instruction of iScript cDNA Synthesis Kit (Bio-Rad) in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) then was used for quantitative RT-PCR. The respective relative gene expressions were performed by using iQ5 Multicolor Real-Time PCR detection system (Bio-Rad). The final reaction mixture for Real-time PCR contained 0.6 µL (10 mM) of each forward and reverse primer, 5 µL of cDNA, 7.5 µL of SYBR Green (Bio-Rad), and 1.3 µL of MilliQ. The RT-PCR conditions were: 95 °C for 3 min 30 s, followed by 40 cycles of 95 °C for 15 s, then 30 s of annealing temperature for each primer (Table 3), and 72 °C for 30 s, 95 °C for 1 min, a final denaturing step from 58 °C to 95 °C for 10 s. To analyze the mRNA expression level, the comparative $2^{-\Delta\Delta CT}$ method was used. Expression levels were calculated using the mathematical model described by Livak and Schmittgen (2001). The used housekeeping and target gene primers were listed in Table 3.

(Insert **Table 3**)

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2.6 Bone mineralization and incidence of skeletal anomalies

A total number of 75 seabream larvae per tank were sampled at the end of trial (21 days, 47dph) to determine the mineralization of the vertebral body and the incidence of severe skeletal anomalies. The larvae samples were fixed and stored in buffered formalin (4% formalin in phosphate buffer, pH 7.2, 0.1 M) and stained with alizarin red (Vandewalle et al., 1998). Larvae were observed under the stereomicroscope (Leica, M125, Germany) and imagined by Leica DFC295 digital camera with using the Leica application suite, from the direction of cranial to the caudal part to evaluate bone mineralization and skeletal anomalies (Prestinicola et al., 2013). The effects of dietary Cu on axial skeleton mineralization were evaluated by counting the average number of vertebral bodies stained with alizarin red for each dietary group. Since skeletal mineralization is greatly dependent on fish length, fish were divided into 9 different size classes according to the total length, from 0.89 to 1.42 mm as follows 1: 0.89 to 0.94 mm; 2: 0.95 to 1.0 mm; 3: 1.01 to 1.06 mm; 4: 1.07 to 1.12 mm; 5: 1.13 to 1.18 mm; 6: 1.19 to 1.24 mm; 7: 1.25 to 1.3 mm; 8: 1.31 to 1.36 mm; 9: 1.37 to 1.42 mm.

2.7 Statistical analysis

Each experimental diet was administered to three tanks that were located according to a completely randomized design. All data were tested for normality and homogeneity of variances with Levene's test. Results were analyzed through a one-way analysis of variance by using IBM SPSS Statistics software (21.0 version; SPSS Inc., Chicago, IL, USA) and significance was set at $P < 0.05$. Multiple means comparisons were performed with Duncan's new multiple-range test. Linear and quadratic regression models were used to determine the effect of dietary Cu on different parameters.

3. Results

3.1. Larval performance

All the experimental diets were well accepted by the larvae and after 21 days of feeding there were no significant differences in body weight, total length, specific growth rate or biomass among larvae fed diets with different levels of Cu (17, 18, 19 and 25 mg/kg) (Table 4). Survival rate values were lowest in fish fed diet Cu 17 and tended to increase with the dietary Cu content following a linear regression ($y = 0.74x + 53.95$, $R^2 = 0.99$), despite no significant differences among means.

(Insert Table 4)

3.2. Proximate composition, fatty acid profiles and lipid peroxidation

After 21 days of feeding, whole-body proximate compositions were not significantly (P>0.05) different among larvae fed the different diets (Table 5). However, the increase in dietary Cu significantly

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($P<0.05$) reduced larval whole-body contents in MUFA and n-9 fatty acids, particularly erucic acid (22:1n-9) or oleic acid (18:1n-9), which were the highest in larvae fed the diet non-supplemented with Cu (Table 6). On the contrary, PUFA and n-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), significantly ($P<0.05$) increased by the elevation of dietary Cu levels up to 19 mg/kg, whereas further increase in dietary Cu did not significantly raise the contents of these fatty acids (Table 6). Polynomial regression models suggested that the highest whole-body contents in DHA, EPA (Fig.1) and PUFA would occur at dietary Cu levels of 22.5 mg Cu/kg. Besides, the whole-body contents in DHA showed a high lineal regression with larval survival ($y = 1.072x - 0.61$, $R^2 = 0.77$). Interestingly, the ratio of 18:4n-3/18:3n-3 was also significantly ($P<0.05$) higher in the fish fed the Cu supplemented diets (Cu 18, Cu 19, Cu 25) (Table 6), following a polynomial regression ($y=-0.06x^2+2.62x-26.26$, $R^2 = 0.97$) with a maximum value at 22.3 mg Cu/kg.

(Insert Tables 5 and 6 and Figure 1)

Regarding the degree of lipid oxidation (TBARs), the highest malonaldehyde content (nmol/g lipid) was found in the larvae fed the diet without Cu supplementation (Cu 17), whereas the increase of dietary Cu significantly ($P<0.05$) reduced TBARs contents. A polynomial regression model suggested the lowest malonaldehyde content at dietary Cu levels of 22 mg Cu/kg (Fig 2).

(Insert Figure 2)

3.4. Mineral analysis

After 21 days of feeding the experimental diets there were no significant differences ($P>0.05$) among the mean of whole-body mineral contents (Table 7). However, whole body Se, Mn, Zn and Cr content increased with the elevation of dietary Cu followed by linear regression (Se: $R^2=0.72$; Mn: $R^2=0.67$; Cr: $R^2=0.66$; Zn: $R^2=0.80$). Interestingly, a highly correlated ($R^2=0.97$, $P=0.012$) linear regression was found between Fe content and dietary Cu levels ($y = 1.9072x + 28.916$, $R^2=0.97$, $P=0.012$). No effect of dietary Cu levels was found on Ca, P and Ca:P ratio. Noteworthily, Fe, Se and Cr content were increased with increasing EFAs (EPA, DHA, ARA) content in whole body larvae, followed by a linear regression (Fe: $R^2=0.81$, $P=0.09$; Se: $R^2=0.94$, $P=0.03$; Cr: $R^2=0.96$, $P=0.02$).

(Insert Table 7)

3.3. Gene expression

After 8 days of feeding the experimental diets there were no significant differences among mean expression of genes related to Cu or bone metabolism (Supplementary Table 1). Differences among mean gene expressions were neither found after 15 days of feeding (Supplementary Table 2). Nevertheless, the expression of the copper transporter gene (*ctr1*) in whole body of gilthead seabream larvae was progressively increased by dietary Cu levels, following a positive linear regression ($P>0.05$, $R^2 = 0.68$, Fig 3). Similarly, *runx2*, *bmp2* and *oc* expression were increased with dietary Cu levels following linear

regression at early stage (40 dph) (*bmp2*: $P>0.05$, $R^2=0.85$; *runx2*: $P<0.05$, $R^2=0.97$; *oc*: $P>0.05$, $R^2=0.55$) (Fig. 3 and 4). After 21 days of feeding, the highest dietary Cu levels tested (25 mg/kg, diet Cu 25) significantly ($P<0.01$) up-regulated *cat* expression (Table 8). Besides, increase in dietary Cu up to 19 mg/kg tended to up-regulate *cuznsod* expression, which according to a polynomial regression model showed the highest values at 21.3 mg Cu/kg (Fig.5). Increase in dietary Cu up to 18 mg/kg (diet 18 Cu) significantly ($P<0.05$) up-regulated *oc* expression at later stage (47 dph) (Table 8). Positive regressions were found between *oc* and *bmp2* expression at the three sampling points (Fig. 6).

(Insert Figures 3 and Table 8, Figure 4, 5 and 6)

3.5. Bone mineralization and incidence of skeletal anomalies

Bone mineralization was determined by counting the number of vertebrae completely mineralized found in larvae of each size class and fed each of the 4 Cu dietary levels. As expected, regardless the dietary Cu level fed, the number of mineralized vertebrae was increased by size class (Supplementary Figure 1), following highly correlated ($R^2>0.90$, $P<0.05$) linear regressions (Cu 17: $y=1.60x+3.46$, $R^2 = 0.92$; Cu 18: $y = 2.15x +1.83$, $R^2 = 0.96$; Cu 19: $y = 2.22x - 1.03$, $R^2 = 0.97$; Cu 25: $y = 2.64x - 2.11$, $R^2 = 0.97$). Besides, the slope of the regressions was increased by the dietary Cu level, suggesting a highest number of mineralized vertebrae at 22.6 mg/kg dietary Cu (Fig. 7). Consequently, in larger size classes the number of mineralized vertebrae was lower in larvae fed 17 mg Cu/kg (17 mineralized vertebrae) than in those fed 18, 19 and 25 mg Cu/kg (20.3, 21.3 and 23 mineralized vertebrae, respectively) (Supplementary Figure 1).

(Insert Figure 7)

In present study, cephalic vertebrae kyphosis (A1), abdominal vertebrae kyphosis (B1), lordosis (B2), partial vertebral fusion (B3), total vertebral fusion (B3*), vertebral anomaly (B4), calculi (13), anomalous maxillary or pre-maxillary (14), anomalous dentary (15), anomalous branchiostegal ray (17*) were considered as severe anomalies. However, the most common skeletal anomalies found in the larvae in order of prevalence were: abdominal vertebrae kyphosis, fused branchiostegal ray, anomalous maxillary/premaxillary and abdominal vertebrae fusion (Table 9, Figure 8). The incidences of total severe anomalies (*A1, B1, B2, B3, B3*, B4, 13, 14, 15, 17**), abdominal vertebrae kyphosis and fused branchiostegal rays were highest in larvae fed the Cu17 diet and were reduced with the increase in dietary Cu. In particular, the percentage of fused branchiostegal rays was significantly ($P<0.05$) higher in larvae fed the Cu17 diet than in those fed the other 3 diets supplemented with Cu (Table 9). Polynomial regression models suggested the lowest incidence of severe anomalies at dietary Cu levels of 22.5 mg Cu/kg ($y = 2.03x^2 - 88.53x + 975.03$, $R^2 = 0.97$, Fig. 9), abdominal vertebrae kyphosis at 22 mg Cu/kg ($y = 1.33x^2 - 58.39x + 652.99$ $R^2 = 0.90$) and fused branchiostegal rays at 21.5 mg Cu/kg ($y = 0.47x^2 - 20.29x + 215.35$ $R^2 = 0.90$). Considering the different body regions, anomalies were significantly ($P<0.05$) higher in cranium and abdominal vertebrae of larvae fed the Cu 17 diet than in those fed the other 3 diets supplemented with Cu (Table 9). Polynomial regression models suggested the lowest incidence of cranium

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at dietary Cu levels of 22.5 mg Cu/kg ($y = 0.72x^2 - 30.80x + 325.57$, $R^2 = 0.95$) and of anomalies at the abdominal vertebrae at 22.2 mg Cu/kg ($y = 1.33x^2 - 58.40x + 652.99$, $R^2 = 0.90$, Fig. 10).

(Insert Table 9, Figure 8, 9 and 10).

4. Discussion

Copper is one of the important elements required for the normal growth of fish (NRC, 2011). In the present study, increase in dietary Cu from 17 to 25 mg/kg did not affect gilthead seabream larvae growth, in terms of total length (mm) or body weight (dry/mg). However, in a previous study, gilthead seabream larvae fed a diet containing 20 mg Cu/kg showed significantly higher total length and body weight than larvae fed a non-supplemented diet (17 mg Cu/kg) (Eryalçın et al., 2020). The low growth of fish fed 17 mg Cu/kg in the previous study may be related to the low dietary contents in other antioxidant minerals such as Mn (4 mg/kg) or Se (1.9 mg/kg), which seem to be required in higher amounts in marine fish larval diets (Izquierdo et al., 2017; Saleh et al., 2014). Besides, the non-supplemented diet (17 mg Cu/kg) of such previous study showed also a relatively low Zn content (Zn: 83 mg /kg) (Eryalçın et al., 2020). On the contrary, in the present study, dietary contents of these minerals (Zn: 89 mg/kg; Mn: 7.03 mg/kg; Se: 7.5 mg/kg) were higher than in the previous one and could be adequate levels for gilthead seabream larvae (Izquierdo et al., 2017; Saleh et al., 2014). Larval survival tended to increase with the elevation of the dietary Cu levels, following a lineal regression with whole-body contents in DHA. DHA is one of the most effective promoters of larval survival (Izquierdo et al., 1992) and therefore, the increased survival tendency could be partly related with the higher whole body DHA contents.

Increase in dietary Cu up to 19 mg/kg significantly raised the whole-body contents in DHA, EPA and n-3 PUFA, suggesting Cu deficiency induced lipid peroxidation in gilthead seabream larvae. Indeed, the values of TBARS, one of the most used methods to determine lipid peroxidation in animal tissues (Rosmini et al., 1996), were reduced by the increase in dietary Cu. A polynomial regression model suggested the lowest TBARS value at dietary Cu levels of 22 mg Cu/kg, whereas the highest dietary Cu level tested (25 mg/kg) were not associated to significantly lower TBARS values. Several studies with different dietary forms of Cu ($CuSO_4.5H_2O$, Cu peptide, tribasic copper chloride) have demonstrated that both deficiency or excess in dietary Cu induces lipid peroxidation in fish, damaging polyunsaturated membrane lipids or even proteins, and harming cells and tissues. For instance, among marine fish species, feeding grouper (*Epinephelus malabaricus*) juveniles with diets either low (0.19-1.81 mg Cu/kg) or excessively high (8.99-20.05 mg Cu/kg) in Cu markedly increased lipid peroxidation, regardless the inorganic or organic Cu source used (Lin et al., 2010, 2008). Excessive dietary Cu also caused peroxidation signs in intestine, kidney and liver as well as oxidative stress in Atlantic salmon (*Salmo salar*) (Berntssen et al., 2000). Similar results have been reported for freshwater species such as blunt snout bream (*Megalobrama amblycephala*) (Shao et al., 2012), beluga (*Huso huso*) (Mohseni et al., 2014), Russian

sturgeon (*Acipenser gueldenstaedtii*) (Wang et al., 2016), spotted snakehead (*Channa punctatus*) (Abdel-Hameid et al., 2017) or stinging catfish (*Heteropneustes fossilis*) (Zafar and Khan, 2020). Copper is required to prevent oxidative damage, by the action of the Cu-containing enzyme superoxide dismutase (Cuznsod), which removes the reactive oxygen species by catalyzing the dismutation of two superoxide radicals ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2). In turn, H_2O_2 can be neutralized by two antioxidant enzymes: the Fe-dependent catalase (Cat) and the Se-dependent glutathione peroxidase (Gpx) (Fattman et al., 2003). In the present study, expression of *cuznsod* gene in seabream larvae tended to increase with the elevation of dietary Cu following a polynomial regression model that suggested the highest expression at 21.3 mg Cu/kg diet. Thus, *cuznsod* expression tended to be down-regulated at the highest dietary Cu levels tested (25 mg/kg) in comparison to larvae fed 19 mg Cu/kg diet. Moreover, in larvae fed these highest dietary Cu levels the *cat* expression was significantly up-regulated, what could denote an increase in the oxidative risk, in agreement with previous studies (Domínguez et al., 2019; Lin et al., 2010; Shao et al., 2012; Tang et al., 2013). Therefore, the antioxidant properties of Cu could be responsible for the elevation of PUFA in larval whole-body when dietary Cu levels increased up to 22.5 mg/kg, this suggesting Cu could protect these polyunsaturated lipids.

Indeed, Cu has been also associated with lipid metabolism in both fish and animal. For instance, in fish studies showed that dietary Cu levels induced alterations on whole body lipid content (Berntssen et al., 1999) and lipid metabolism related gene expression (Meng et al., 2016). In mammal studies, dietary Cu promotes PPAR-α gene expression (Lei et al., 2017), enhancing fatty acid oxidation and the subsequent ATP synthesis (Espinosa et al., 2020). Moreover, Cu increased PUFA deposition in body tissues, together with a reduction in SFA in broiler chickens (Skřivan et al., 2000). In agreement with previous studies, the elevation of dietary Cu not only enhanced PUFA contents in whole body of larval seabream, but also reduced MUFA, particularly oleic acid (18:1n-9), a main source of energy in marine fish larvae, and tended to reduce SFA. Therefore, the PUFA increase in relative contents (%) in whole body of larval seabream could be also related to the reduction in saturated and monounsaturated fatty acids, which are preferred substrates for fatty acid oxidation. Moreover, interestingly, the increase in dietary Cu significantly raised the ratio 18:4n-3/18:3n-3, an indicator of fatty acid desaturase activity, suggesting the positive effect of Cu on PUFA synthesis. Several key enzymes related to lipogenesis and fatty acid synthesis have been found to be affected by Cu in other fish species, such as 6-phosphogluconate dehydrogenase (6PGDH), glucose 6-phosphate dehydrogenase (G6PDH), malic enzyme (ME) or isocitrate dehydrogenase (Meng et al., 2016). However, to our knowledge, the potential effect of dietary Cu levels on PUFA synthesis has not been yet investigated and deserves further studies.

In agreement with a previous study, where dietary Cu supplementation raised Fe contents in seabream larvae (Eryalçın et al., 2020), whole body contents in Fe in the present study followed a positive linear regression with the elevation of dietary Cu, even though the Fe content in the diet with highest Cu was the lowest. Cu has an important role in Fe metabolism by being part of the four ferroxidases that oxidize ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), which is loaded onto transferrin, the protein that regulates iron flux

in the body (Thackeray et al., 2011). Thus, iron mobilization from storage sites is impaired in copper deficient animals, supporting the role for ferroxidases (also known as multicopper oxidases) in iron metabolism (Thackeray et al., 2011). Moreover, in turbot (*Psetta maxima*) exposure of hepatocytes to Cu up-regulates the gene expression of M-ferritin, a protein for conserved iron storage (Zheng et al., 2010), which suggests a role for Cu in the protection of Fe, in agreement with the increased Fe contents found in this and previous studies in seabream larvae (Eryalçın et al., 2020).

Additionally, a solute carrier family (SLC11A2β) also known as dmt1, (divalent metal transporter), which can transport Cu^{2+} and Zn^{2+}, maintaining the homeostatic regulation. However, this suggests that the increased trend of Zn content in larval whole body by increasing dietary Cu, could be possibly due to the upregulated SLC11A2βexpression. Although SLC11A2βexpression did not determine in present study, a similar result of increased larval whole body Zn content by the Cu supplementation in diet was found in seabream larvae (Eryalçın et al., 2020). In the contrary, increasing dietary Cu levels shown no changes of Zn content in tissues, such as whole body, liver of gilthead seabream juvenile (dietary Cu level: 5.5-32 mg/kg) (Dominguez et al., 2019) and whole body of tilapia (dietary Cu level: 6.9 – 17.31 mg/kg) (Lin and Shin 2016). Although elevated dietary Cu shown to have an antagonistic effect on Zn uptake by competing for the transporter, this was not observed in the previous studies and the present study.

A Se dependent enzyme, glutathione peroxidase, protects cell membrane against oxidative damage and its activities related to the oxidation level in fish caused by the excess or deficient antioxidant nutrients. Whole body Se content increased with elevation of dietary Cu followed by a positive linear regression, whereas the lowest Se content found in larvae fed with Cu17 diet. This low Se level could be related to the use of Se for GPX synthesis to counteract the oxidative stress caused by deficient dietary Cu, in agreement with the highest TBARS contents found in these larvae. Similar results were found in previous studies were deficient dietary Cu increased TBARS value (Domínguez et al., 2019; Lin and Shiau, 2008).

Chromium (Cr) content in larvae increased with increasing dietary Cu level and showed a significant (R^2=0.96, P=0.02) positive linear correlation with larval EFAs. Chromium is involved in the regulation of the carbohydrate metabolism, and influences the lipid metabolism by up-regulating sterol regulatory element-binding protein (SREBP) (NRC, 2011; Shi et al., 2021) through actives *akt* (AKT serine/threonine kinase 1) expression. High oxidative stress (high hydrogen peroxide, MDA) occurred in chromium excess/deficient animal, whereas adequate Cr in animals improve the antioxidant capacity (Ahmad et al., 2006; Kim and Kang, 2016; Shi et al., 2021). Moreover, the reduction of glutathione (GSH) due to the elevation of oxidative stress is also caused by excess/deficient Cr levels resulting in high TBARS value (Shi et al., 2021; Zhang et al., 2022). These results agree well with the interaction between Cu and Cr found in Angus and Simmental cows (Stahlhut et al., 2006). Therefore, in the present study, increase in dietary Cu seemed to spare three other minerals with antioxidant properties such as Se, Cr and Zn whose levels tend to be increased in the larvae, together with the significant reduction in the TBARS values, and increase in the larval EFA contents.

At the end of the present study (47 dph), the number of mineralized vertebrae was increased by the

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elevation of dietary Cu up to 22.6 mg/kg. This faster mineralization process was not directly related to the whole-body contents in Ca, P or the ratio Ca:P, which were not affected by dietary Cu levels. However, the expression of *oc*, a molecular marker of bone mineralization and osteoblastic bone formation, tended to be up-regulated at early stage (40 dph) of larvae that fed higher dietary Cu levels, resulting in higher mineralization rate at later stage (47dph), deposition of Ca and P in bone cells and osteoblast maturation. Besides, the expression of *bmp2*, which plays important roles in modulating proliferation and differentiation of osteoblast and induces bone and cartilage formation, was directly related to the *oc* expression at early stage (40 dph).

These results well agree with the poor mineralization state described in rat affected by Cu deficiency (Smith et al., 2002). Also, in zebrafish embryo, Cu deficiency cause notochord distortion which could affect the later mineralization (Bagwell et al., 2020; Gansner et al., 2007). The slower mineralization of vertebral bodies in seabream larvae fed the lowest Cu level went along with the significantly higher incidence of anomalies in abdominal vertebrae, which followed a regression with dietary Cu levels with the lowest incidence at 22.2 mg Cu/kg. Indeed, poor mineralization during early bone development increase the prevalence of skeletal anomalies in previous studies (Izquierdo et al., 2013). Overall, these results suggested the beneficial effect of adequate dietary Cu levels on osteoblast maturation, mineralization of vertebral bodies and prevention of abdominal vertebrae anomalies. These results also well agree with the Cu modulation of calcium phosphate crystals deposition and alkaline phosphatase activity in mesenchymal stem cells differentiating into osteocytes observed in mammals (Rodríguez et al., 2002).

Anomalies in branchiostegal rays and cranium were also significantly highest in larvae fed the Cu17 diet and were reduced by elevation of dietary Cu levels. Both types of bones are considered endochondral bones in gilthead seabream (Izquierdo et al., 2013), previously formed by cartilaginous molds with scattered chondrocytes that maintain a collagen rich matrix that is later mineralized. *Runx2* is a key transcription factor associated with osteoblast, as well as with chondroblast differentiation. Whereas at the end of the trial (47 dph) there were no differences in the expression of *runx2*, after 2 weeks of feeding (40 dph), when the formation of cartilage precursors for endochondral bones is still very high in seabream, the expression of this gene was directly related to the dietary Cu levels. The importance of copper for collagen synthesis in the chondrocytes denotes its relevant role in cartilage formation, and the present results denote its significance for prevention of anomalies in endochondral bones.

Nevertheless, the increased lipid peroxidation observed in larvae fed only 17 mg Cu/kg diet could be also responsible for the high levels of bone anomalies found, since free radicals and oxidation products have been found to destroy cartilaginous tissue (Beer and Wegener, 2011) and reduces bone cortex and bone strength (Qu et al., 2018). In fish, an increased oxidative risk has been associated with anomalies in the cranium and in the haemal and neural spines, also considered endochondral bones (Izquierdo et al., 2013). Besides, feeding Atlantic halibut (*Hippoglossus hippoglossus*) with a highly oxidized oil reduced vertebral ash content and increased anomalies (Lewis-McCrea and Lall, 2007), in agreement with the low vertebral mineralization, high abdominal vertebrae anomalies and high TBARs found in seabream fed the

non-supplemented diet in the present study. Also feeding diets deficient in the antioxidant vit C increases skeletal anomalies in Atlantic halibut (Lewis-McCrea and Lall, 2010) and hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*E. lanceolatus*) (Ebi et al., 2021). On the contrary, dietary supplementation with adequate amounts of antioxidants such as vitamin C, E or taurine reduces both the oxidative risk and the incidence of bone anomalies (Izquierdo et al., 2019).

Copper is an essential element with many important roles in fish metabolism (Watanabe et al., 1997). Despite the objectives of the present study did not include defining the Cu requirements for gilthead seabream larvae, the significant improvements of increasing dietary Cu supplementation on PUFA, TBARS and prevention of skeletal anomalies denote that the non-supplemented diet (17 mg Cu/kg diet) was deficient in this mineral. Moreover, polynomial regression models applied to whole body DHA, EPA, PUFA, 18:4n-3/18:3n-3 and TBARS contents, *cuznsod* expression and severe anomalies, abdominal vertebrae kyphosis, fused branchiostegal rays, cranial and abdominal vertebrae anomalies, all suggested optimum dietary Cu levels around 21.5-22.6 mg/kg. Dietary Cu requirements for juvenile marine fish have been reported in several species such as red drum (*Sciaenops ocellatus* L.,) (3 mg Cu/kg, Chen et al., 2020), yellow croaker (*Larimichthys croceus*) (3.41-7.05 mg Cu/kg, Cao et al., 2014), or grouper (*Epinephelus malabaricus*) (4-6 mg Cu/kg, Lin et al., 2008). Whereas studies in fish fed with plant based diet, the non-Cu supplemented basal diet (5.5 mg Cu/kg) and a nutrient package (100-150 NP) containing 13-14 mg Cu/kg were enough to cover requirements for gilthead seabream juvenile and Atlantic salmon post-smolt, respectively (Antony Jesu Prabhu et al., 2019; Domínguez et al., 2019). Thus, this suggests that the dietary Cu requirement level for juvenile marine fish around 6.44 mg/kg was based on growth, liver Cuznsod activity, body Cu retention, whole body, liver and vertebrae Cu content.

However, optimum dietary nutrient levels for fish larvae are higher than for juveniles or adults, as the larvae are poorly developed at first feeding, undergo dramatic morphological and physiological changes and have a very high metabolic rate (Hamre et al., 2013; Izquierdo, 1996). For instance, Atlantic salmon fry have a large requirement for Cu (35 mg/kg diet) to maintain growth (Berntssen et al., 1999), whereas at the parr stage Cu requirements are 3-4 times lower (8.5-13.7 mg Cu/kg diet) (Lorentzen et al., 1998). As well as gilthead seabream larvae require higher dietary Se levels (11.51 mg/kg; Saleh et al., 2014) than the juvenile stage (0.94 mg/kg, Dominguez et al., 2019). In agreement, in the present study, seabream larvae seemed to have high Cu requirements (21.5-22.6 mg/kg), whereas those estimated for juveniles (5.5 mg Cu/kg) are 4 times lower (Domínguez et al., 2019). Indeed, these suggested dietary Cu levels for gilthead sea bream larvae are in the range of the contents of Cu in copepods (12-38 mg/kg), a common wild prey for marine fish larvae in the wild (Hamre et al., 2008).

5. Conclusion

In summary, dietary supplementation with $CuSO_4 \cdot 5H_2O$ from 17 to 25 mg Cu/kg for 21 days did not significantly affect seabream larvae growth performance. However, the larvae fed the diet non-supplemented with Cu (17mg Cu/kg) showed the highest lipid peroxidation, the lowest content of EFA

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and the highest incidence of bone anomalies. On the contrary, dietary Cu supplementation reduced MUFA in relation to the role of Cu on beta-oxidation; raised the whole-body contents in DHA, EPA and n-3 PUFA and reduced TBARS, denoting the protective effect of Cu against lipid peroxidation; and reduced the incidence of severe, cranial, branchiostegal rays and abdominal vertebral anomalies. Polynomial regression models applied to all these parameters suggested optimum dietary Cu levels to be 21.5-22.6 mg/kg in gilthead seabream larvae microdiets supplemented with $CuSO_4.5H_2O$. These levels are on the verge of the maximum dietary levels authorized by EU Commission in feeds for cultured fish species (25 mg Cu/kg diet).

Acknowledgements

This project Biomedaqu has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No.766347. And part of the research was funded by Aquaexcel2020 (AQUAculture infrastructures for EXCELlence in European fish research towards 2020) under H2020 programme, in the frame of Transnational Access –TNA No.AE100014. The mineral analyses were supported by the Norwegian Institute of Marine Research (IMR). The authors want to acknowledge the assistance of C.M. Hernandez-Cruz in the production of larvae.

"This output reflects the views only of the authors, and the European Union cannot be held responsible for any use which may be made of the information contained therein".

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Table 1. Ingredients and proximate composition of the experimental diets containing different Cu levels

	Diet
Ingredients (g/kg)	
Squid powder[1]	
Gelatin[2]	
Sel-Plex® 2000[3]	
Krill oil[4]	
$CuSO_4 \cdot 5H_2O$[5]	
Taurine[6]	
Mineral Premix (free-Cu)[7]	
Vitamin Premix[8]	
Attractants[9]	
Proximate composition (%)	
Moisture	
Ash	
Total lipid	
Crude protein	
Mineral contents	
Cu (mg/kg)	
Fe (mg/kg)	
Se (mg/kg)	
Mn (mg/kg)	
Cr (mg/kg)	
Zn (mg/kg)	
Ca (mg/g)	
P (mg/g)	
Ca:P	

[1] Bacarel.

[2] Panreac.

[3] Sel-Plex® 2000, 2000mg Se/kg, yeast dervied selenium; Alltech.

[4] High phospholipids, Aker BioMarine, Fjordalléen, Norway.

[5&6] Sigma-Aldrich

[7] Mineral premix (mg/100g) supplied for 100g diet: NaCl, 215.133 mg; $MgSO4.7H_2O$, 677.545 mg; $NaH_2PO_4.H_2O$, 381.453 mg; K_2HPO_4, 758.949 mg; $Ca(H_2PO_4).2H_2O$, 671.610 mg; $FeC_6H_5O_7$, 146.884; $C_3H_5O_3.1/2Ca$, 1617.210 mg; $Al_2(SO_4)3.6H_2O$, 0.693 mg; $ZnSO_4.7H_2O$, 14.837; KI, 0.742 mg; $CoSO_4.7H_2O$, 10.706 mg.

[8] Vitamin premix (mg/100g) supplied for 100g diet: Cyanocobalamin, 0.03 mg; Astaxanthin, 5.0 mg; Folic acid, 5.44 mg; Pyridoxine-HCL, 17.28 mg; Thiamine-HCL, 21.77 mg; Riboflavin, 72.53 mg; Calcium Pantothenate, 101.59 mg; 4-Aminobenzoic acid, 145 mg; Nicotinic acid, 290.16 mg; myo-inositol, 1450.90 mg; L-Ascorbic acid, 180.00 mg; Choline chloride, 2965.80 mg; Retinoic acid, 0.24 mg; Menadione, 17.28 mg; α-Tocopherol acetate, 150 mg.

[9] Attractants premix (mg/100g) supplied for 100g diet: Inosine 5-monophosphate, 500 mg; Betaine, 660 mg; L-Serine, 170 mg; L- Tyrosine, 170 mg; Phenylalanine, 250 mg; DL- Alanine, 500 mg; L-Aspartic acid sodium, 330 mg; L- Valine, 250 mg; Glycine, 170 mg.

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Table 2. Fatty acid composition of experimental diets (% of total identified fatty acids)

Fatty acid	Diet			
	Cu 17	Cu 18	Cu 19	Cu 25
14:0	4.89	2.67	4.13	4.46
14:1n-7	0.08	0.04	0.07	0.07
14:1n-5	0.16	0.09	0.15	0.14
15:0	0.28	0.20	0.25	0.26
15:1n-5	0.06	0.03	0.03	0.03
16:OISO	0.10	0.05	0.07	0.07
16:0	20.64	15.90	19.15	19.48
16:1n-7	4.92	3.57	4.41	4.55
16:1n-5	0.21	0.17	0.20	0.19
16:2n-6	0.02	0.02	0.02	0.01
16:2n-4	0.45	0.29	0.42	0.44
17:0	0.15	0.10	0.12	0.12
16:3n-4	0.19	0.17	0.20	0.19
16:3n-3	0.33	0.28	0.32	0.31
16:3n-1	0.25	0.24	0.26	0.24
16:4n-3	0.47	0.40	0.44	0.48
16:4n-1	0.04	0.03	0.03	0.04
18:0	4.11	3.86	4.04	3.96
18:1n-9	12.55	11.72	12.46	12.30
18:1n-7	7.05	6.59	7.07	6.92
18.1n-5	0.23	0.24	0.25	0.25
18:2n-9	0.09	0.11	0.12	0.10
18.2n-6	1.88	1.83	1.87	1.85
18:2n-4	0.11	0.07	0.08	0.07
18:3n-6	0.27	0.23	0.22	0.24
18:3n-4	0.05	0.05	0.04	0.05
18:3n-3	0.91	0.90	0.87	0.90
18.3n-1	0.01	0.02	0.02	0.01
18:4n-3	2.14	2.10	2.08	2.21
18:4n-1	0.07	0.04	0.04	0.03
20:0	0.19	0.25	0.25	0.25
20:1n-9	0.21	0.25	0.24	0.24
20:1n-7	4.01	4.33	4.28	4.01
20.1n-5	0.53	0.58	0.57	0.53
20:2n-9	0.02	0.03	0.02	0.01
20:2n-6	0.24	0.28	0.28	0.25
20:3n-9	0.03	0.03	0.04	0.02
20:3n-6	0.08	0.08	0.09	0.07
20:4n-6	1.03	1.16	1.11	1.06
20:3n-3	0.38	0.39	0.37	0.34
20:4n-3	0.36	0.42	0.39	0.39
20:5n-3	14.87	18.11	15.64	15.95
22:1n-11	0.27	0.28	0.24	0.20
22:1n-9	1.69	2.04	1.86	1.63
22:4n-6	0.19	0.24	0.24	0.19

22:5n-6	0.24	0.27	0.30	0.26
22:5n-3	0.64	0.92	0.74	0.70
22:6n-3	12.32	18.26	13.92	13.94
Σ SAT*	30.36	23.03	28.00	28.60
Σ MUFA*	31.97	29.93	31.82	31.05
Σ PUFA*	37.68	47.04	40.18	40.36
Σ n-3	32.42	41.87	34.78	35.22
Σ n-6	3.95	4.10	4.14	3.92
Σ n-9	14.59	14.18	14.75	14.31
n-3/n-6	8.21	10.20	8.40	8.98
DHA/EPA*	0.83	1.01	0.89	0.87
EPA/ARA*	14.44	15.67	14.15	15.11

*SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ARA: arachidonic acid.

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Table 3. Primer pair, T_m (°C), and GenBank accession numbers of specific primers[1]

	5'–3' primer sequence			
Gene[1]	Forward	Reverse	T_m (°C)	GenBank accession no.
runx2	GCCTGTCGCCTTTAAGGTGGTTGC	TCGTCGTTGCCCGCCATAGCTG	61	AJ619023
bmp2	GTGGCTTCCATCGTATCAACATTTT	GCTCCCCGCCATGAGT	60	JF261172.1
oc	AGCCCAAAGCACGTAAGCAAGCTA	TTTCATCACGCTACTCTACGGGTT	58.1	AF048703
cuznsod	TTGGAGACCTGGGCAACGTGA	TCCTGCTTGCCTCCTTTTCCC	58.1	JQ308832.1
cat	ATGGTGTGGGACTTCTGGAG	AGTGGAACTTGCAGTAGAAAC	58.1	JQ308823
ctr1	CGGGTCTGCTCATCAACACCC	TGTGCGTCTCCATCAGCACCG	58.1	AJ630205.3
β-actin[2]	TCTGTCTGG ATC GGAGGCTC	AAGCATTTG CGGTGGACG	58.1	X89920

[1] *runx2*, runt-related transcription factor 2; *bmp2*, bone morphogenetic protein 2; *oc*, osteocalcin, *cuznsod*, Cu/Zn superoxide dismutase; *cat*, catalase; *crt1*, copper transporter 1

[2] *β-actin* cDNA was used as an internal control.

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Table 4. Growth performance of gilthead seabream larvae (47 dph) fed diets with different levels of dietary Cu for 21 days (mean ± SD)

	Diet			
	[illegible] 17	[illegible] 18	[illegible] 19	[illegible] 25
Final body weight (dry weight, mg)	± 0.30	± 0.22	±0.10	± 0.10
Final total length (mm)	± 0.39	± 0.53	[illegible] ± 0.12	± 0.22
Body weight gain (dry weight, mg)	± 0.30	± 0.22	± 0.09	± 0.08
Total length gain (mm)	± 0.39	± 0.53	± 0.12	± 0.22
Specific growth rate (%)	± 0.69	± 0.51	± 0.73	± 0.26
Survival (%)	± 6.47	± 9.80	[illegible] ± 4.48	± 4.45
Biomass (g)	± 0.44	± 0.56	± 0.28	± 0.20

Initial dry weight: 0.46 ± 0.09 mg

Initial total length: 7.9 ± 1.61 mm

Table 5. Final whole-body composition of gilthead seabream larvae (47 dph) fed diets with different levels of dietary Cu for 21 days (mean ± SD)

	Diet			
(Dry weight %)	Cu 17	Cu 18	Cu 19	Cu 25
Ash	10.51 ± 0.49	11.17 ± 0.58	10.97 ± 0.18	10.82 ± 0.29
Total lipid	21.[illegible]7 ± 0.77	21.89 ± 2.14	20.62 ± 1.39	19.83 ± 0.83
Crude protein	73.27 ± 1.78	72.71 ± 2.04	71.44 ± 0.89	72.01 ± 0.78

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Table 6. Final whole-body fatty acid profile of gilthead seabream larvae (47 dph) fed diets with different levels of dietary Cu for 21 days (% of total identified fatty acids; mean ± SD)

Fatty acid	Diet			
	Cu 17	Cu 18	Cu 19	Cu 25
14:0	2.08 ± 0.59	2.34 ± 1.04	1.08 ± 0.66	1.51 ± 0.66
14:1n-7	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.04	0.01 ± 0.01
14:1n-5	0.02 ± 0.01	0.04 ±0.04	0.05 ± 0.04	0.02 ± 0.01
15:0	0.27 ± 0.03	0.29 ± 0.08	0.17 ± 0.10	0.20 ± 0.06
15:1n-5	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.01
16_OISO	0.06 ± 0.01	0.04 ± 0.03	0.06 ± 0.01	0.06 ± 0.02
16:0	23.85 ± 2.11	24.15 ± 4.34	15.51 ± 10.09	18.47 ± 3.28
16:1n-7	3.38 ± 0.46	3.21 ± 0.60	2.20 ± 1.49	2.55 ± 0.53
16:1n-5	0.20 ± 0.02	0.22 ± 0.01	0.15 ± 0.04	0.16 ± 0.06
16:2n-4	0.16 ± 0.01	0.17 ± 0.01	0.14 0.04	0.12 ± 0.05
17:0	0.08 ± 0.02	0.08 ± 0.00	0.08 ± 0.03	0.07 ± 0.01
16:3n-4	0.22 ± 0.01	0.23 ± 0.03	0.19 ± 0.04	0.18 ± 0.02
16:3n-3	0.26 ± 0.03	0.29 ± 0.01	0.28 ± 0.03	0.24 ± 0.01
16:3n-1	0.48 ± 0.06	0.52 ± 0.16	0.62 ± 0.15	0.50 ± 0.04
16:4n-3	0.27 ± 0.04[b]	0.19 ± 0.02[a]	0.27 ± 0.03	0.19 ± 0.04[a]
16:4n-1	0.13 ± 0.04	0.17 ± 0.08	0.15 ± 0.04	0.14 ± 0.03
18:0	8.08 ± 0.45	8.52 ± 0.13	8.16 ± 1.55	6.63 ± 0.11
18:1n-9	16.04 ± 3.75	12.04 ± 0.27	12.11 ± 1.90	10.23 ± 0.48
18:1n-7	5.52 ± 0.42	5.79 ± 0.13	5.78 ± 0.77	5.11 ± 0.39
18:1n-5	0.23 ± 0.02	0.23 ± 0.01	0.24 ± 0.02	0.20 ± 0.01
18:2n-9	0.16 ± 0.11	0.06 ± 0.02	0.07 ± 0.04	0.05 ± 0.01
18:2n-6	2.31 ± 0.33	1.65 ± 0.10	1.96 ± 0.71	1.51 ± 0.10
18:2n-4	0.10 ± 0.01	0.09 ± 0.02	0.12 ± 0.02	0.10 ± 0.01
18:3n-6	0.32 ± 0.01	0.32 ± 0.06	0.34 ± 0.05	0.32 ± 0.03
18:3n-4	0.05 ± 0.01	0.06 ± 0.03	0.07 ± 0.03	0.10 ± 0.01
18:3n-3	0.74 ± 0.22	0.45 ± 0.05	0.55 ± 0.14	0.46 ± 0.03
18:3n-1	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.03	0.01 ± 0.01
18:4n-3	0.86 ± 0.10	0.89 ± 0.06	1.17 ± 0.13	1.11 ± 0.06
18:4n-1	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.03	0.04 ± 0.01
20:0	0.27 ± 0.04	0.29 ± 0.02	0.38 ± 0.23	0.25 ± 0.02
20:1n-9	0.15 ± 0.03	0.16 ± 0.03	0.18 ± 0.09	0.13 ± 0.01
20:1n-7	2.06 ± 0.24	2.08 ± 0.14	2.41 ± 0.99	1.81 ± 0.13
20:1n-5	0.33 ± 0.05	0.35 ± 0.04	0.45 ± 0.22	0.38 ± 0.09
20:2n-9	0.03 ± 0.02	0.02 ± 0.01	0.07 ± 0.08	0.03 ± 0.01
20:2n-6	0.23 ± 0.02	0.21 ± 0.04	0.26 ± 0.15	0.19 ± 0.03
20:3n-9	0.02 ± 0.00	0.01 ± 0.01	0.05 ± 0.06	0.02 ± 0.02
20:3n-6	0.13 ± 0.03	0.13 ± 0.02	0.18 ± 0.09	0.15 ± 0.02
20:4n-6	1.50 ± 0.27	1.70 ± 0.23	2.23 ± 0.73	1.83 ± 0.20
20:3n-3	0.22 ± 0.06	0.21 ± 0.03	0.28 ± 0.11	0.22 ± 0.03
20:4n-3	0.27 ± 0.06	0.30 ± 0.05	0.39 ± 0.12	0.34 ± 0.04
20:5n-3	10.38 ± 1.82[a]	11.50 ± 1.86[a]	14.97 ± 2.14[b]	15.66 ± 1.19[b]
22:1n-11+ 22:1n-9	1.06 ± 0.18	1.24 ± 0.12	1.45 ± 0.62	1.07 ± 0.15
22:4n-6	0.18 ± 0.09	0.19 ± 0.06	0.27 ± 0.23	0.19 ± 0.06
22:5n-6	0.32 ± 0.09	0.34 ± 0.06	0.49 ± 0.23	0.40 ± 0.06

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22:5n-3	1.05 ± 0.23	1.16 ± 0.22	1.46 ± 0.26	1.52 ± 0.23
22:6n-3	15.87 ± 3.27[a]	18.01 ± 3.27[ab]	22.87 ± 3.08[bc]	25.50 ± 3.49[c]
Σ SFA*	34.68 ± 2.33	35.71 ± 5.53	25.43 ± 9.52	27.20 ± 4.02
Σ MUFA*	29.03 ± 3.49[b]	25.39 ± 0.73[ab]	25.06 ± 3.30[ab]	21.68 ± 0.94[a]
Σ PUFA*	36.29 ± 5.82[a]	38.90 ± 6.22[ab]	49.50 ± 7.52[bc]	51.12 ± 4.97[c]
Σ n-3 PUFA	29.93 ± 5.65[a]	33.01 ± 5.50[ab]	42.23 ± 5.76[bc]	45.24 ± 4.79[c]
Σ n-6 PUFA	4.98 ± 0.32	4.53 ± 0.46	5.74 ± 2.11	4.58 ± 0.21
Σ n-9 PUFA	17.36 ± 3.63[b]	12.46 ± 0.20 [a]	12.72 ± 2.32[a]	10.63 ± 0.43[a]
n-3/n-6	6.01 ± 0.98[a]	7.25 ± 0.52[a]	7.81 ± 2.13[ab]	9.85 ± 0.58[b]
DHA/EPA*	1.53 ± 0.08	1.56 ± 0.04	1.53 ± 0.07	1.62 ± 0.10
EPA/ARA*	6.94 ± 0.34	6.76 ± 0.24	7.01 ± 1.53	8.60 ± 0.61
18:3n-6/18:2n-6	0.14 ± 0.02	0.19 ± 0.04	0.18 ± 0.04	0.21 ± 0.00
18:4n-3/18:3n-3	1.22 ± 0.33[a]	1.97 ± 0.13[b]	2.19 ± 0.43[b]	2.40 ± 0.05[b]
EFAs*	27.74 ± 5.31[a]	31.21 ± 5.36[ab]	40.07 ± 5.61[bc]	42.[illegible]8 ± 4.84[c]

*SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ARA: arachidonic acid; EFAs: essential fatty acids (ARA, EPA, DHA). Mean values in the same line with different superscript letters are significantly different (*P*<0.05).

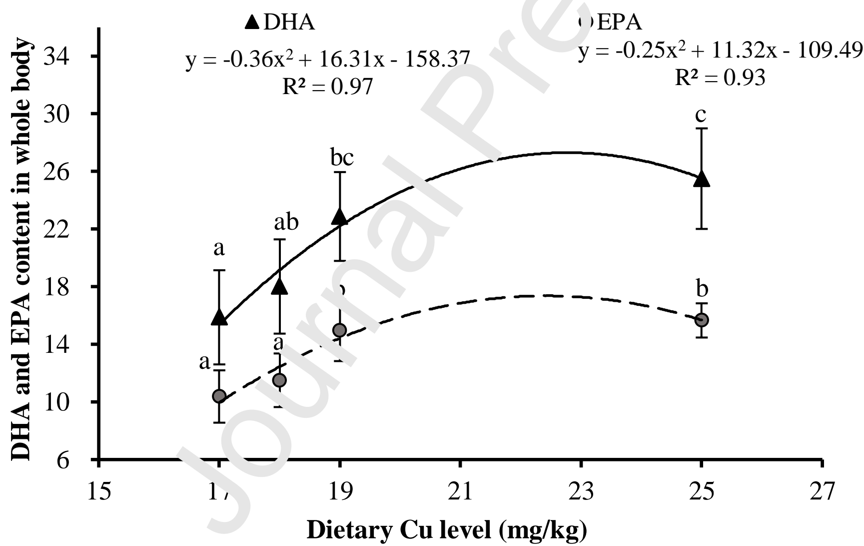

Figure 1. DHA and EPA contents in whole body of gilthead seabream larvae (47 dph) fed diets containing 17, 18, 19 and 25 Cu mg/kg. Means with different letters denote significant differences (*P*<0.05).

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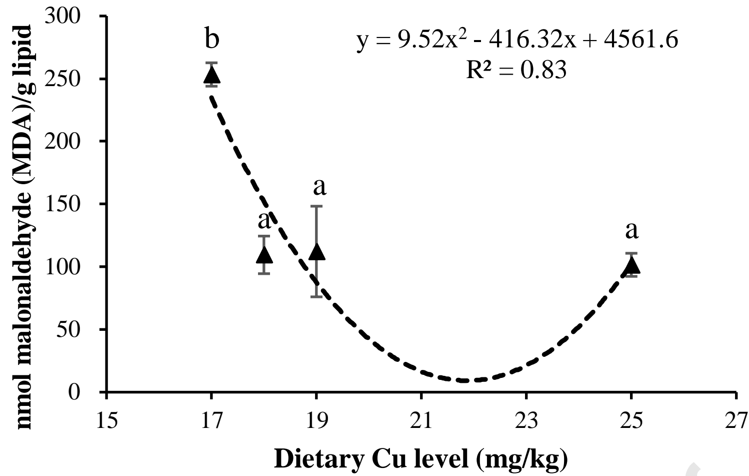

Figure 2. TBARs contents in gilthead seabream larvae (47 dph) fed diets containing 17, 18, 19 and 25 Cu mg/kg. Means with different letters denote significant differences (P<0.05).

Table 7. Whole-body mineral composition of gilthead seabream larvae (47 dph) fed diets with different levels of dietary Cu for 21 days (mean ± SD, n=3 tanks)

(Dry weight)	Diet			
	Cu 17	Cu 18	Cu 19	Cu 25
Cu (mg/kg)	2.83 ± 0.15	2.80 ± 0.10	[illegible].70 ± 0.20	2.87 ± 0.12
Fe (mg/kg)	61.0 ± 10.0	62.33 ± 21.94	[illegible]6.67 ± 19.04	76.33 ± 8.62
Se (mg/kg)	4.80 ± 0.10	4.90 ± 0.1[illegible]	4.97 ± 0.06	5.03 ± 0.06
Mn (mg/kg)	2.13 ± 0.06	2.30 ± 0.[illegible]6	2.17 ± 0.12	2.40 ± 0.10
Cr (mg/kg)	1.88 ± 1.25	1.9[illegible] ± [illegible]33	4.67 ± 2.71	5.27 ± 1.07
Zn (mg/kg)	90.67 ± 3.51	[illegible]2.0[illegible] ± 3.61	90.67 ± 3.21	94.00 ± 3.00
Ca (g/kg)	14.00 ± 1.00	[illegible]5.33 ± 0.58	15.00 ± 1.00	15.00 ± 1.00
P (g/kg)	17.67 ± 0.[illegible]	[illegible]8.67 ± 0.58	18.33 ± 0.58	18.67 ± 0.58
Ca: P	0.79 ± 0.[illegible]4	0.82 ± 0.03	0.82 ± 0.06	0.80 ± 0.03

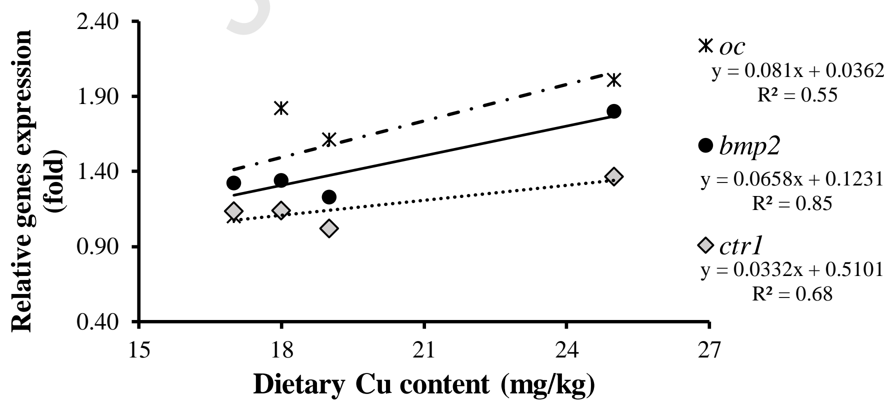

Figure 3. Correlation between *oc*, *bmp2* and *crt1* genes expression of gilthead seabream larvae (40 dph) and different dietary Cu levels after 15 days of feeding different diets. *oc*, osteocalcin; *bmp2*, bone morphogenetic protein; *crt1*, copper transporter 1

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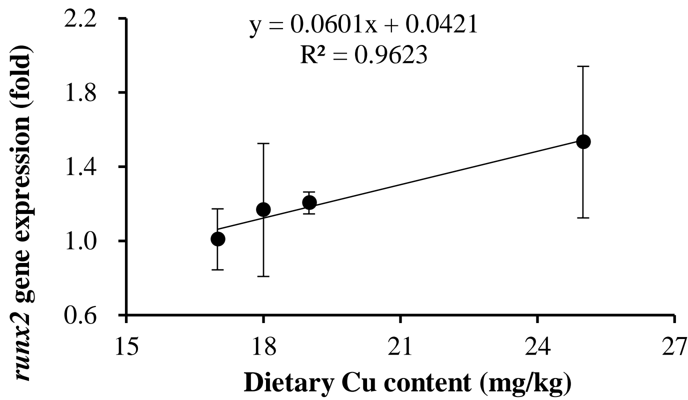

Figure 4. Expression of *runx2* gene in gilthead seabream larvae (40 dph) after 15 days of feeding diets containing 17, 18, 19 and 25 Cu mg/kg. *runx2*, runt-related transcription factor 2.

Table 8. Expression of genes related to Cu or bone metabolism in whole body of gilthead seabream larvae (47 dph) fed different levels of dietary Cu for 21 days (relative expression; mean ± SD) [1]

	Diet			
	17	18	19	25
ctr1	1.01 ± 0.16	1.27 ± 0.35	1.2[illegible] ± 0.6[illegible]	1.05 ± 0.19
cuznsod	1.00 ± 0.07	0.95 ± 0.28	1.[illegible]3 ± 0.29	1.12 ± 0.40
cat	1.15 ± 0.69[a]	1.11 ± 0.48[a]	1.17 ± 0.78[a]	3.61 ± 0.73[b]
runx2	1.02 ± 0.28	1.37 ± 0.11	0.86 ± 0.15	1.14 ± 0.26
bmp2	1.24 ± 1.02	2.91 ± 0.59	1.[illegible]3 ± 1.83	2.43 ± 1.43
oc	1.55 ± 1.27[a]	4.25 ± 0.60[b]	1.31 ± 1.28[a]	2.21 ± 0.86[a]

Mean values in the same line with different superscript letters are significantly different (P<0.05).

[1] *crt1*, copper transporter 1; *cuznsod*, Cu/Zn superoxide dismutase; *cat*, catalase; *runx2*, runt-related transcription factor 2; *bmp2*, bone morphogenetic protein 2; *oc*, osteocalcin.

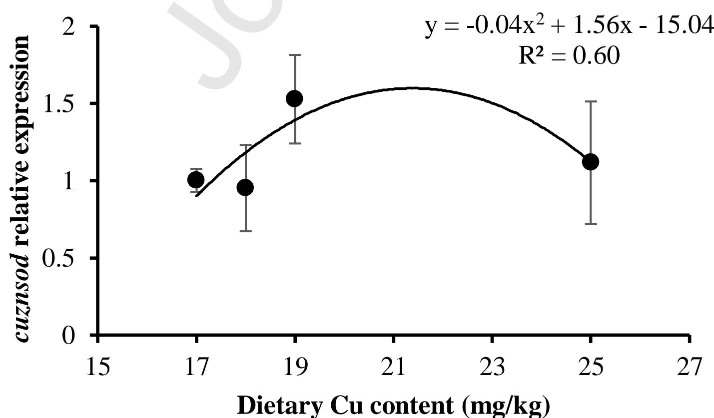

Figure 5. Expression of *cuznsod* gene in gilthead seabream larvae (47 dph) after 21 days of feeding diets containing 17, 18, 19 and 25 Cu mg/kg. *cuznsod*, Cu/Zn superoxide dismutase.

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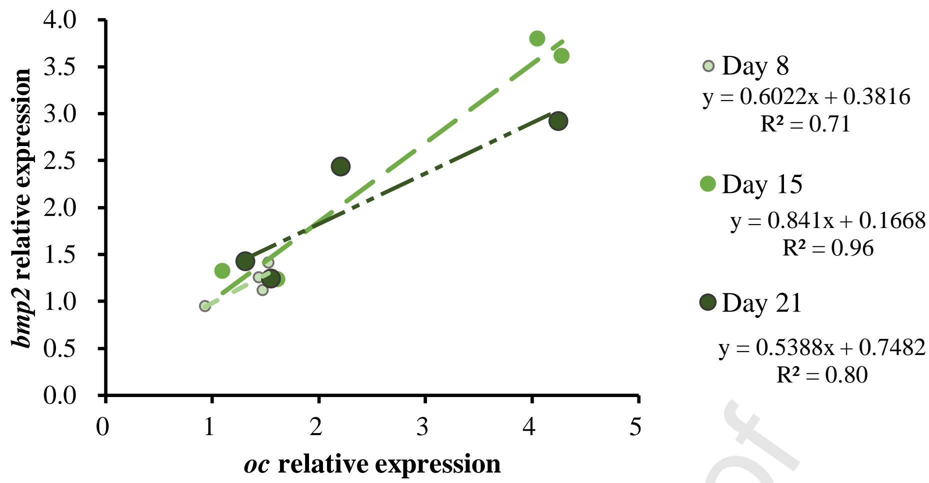

Figure 6. Correlation between *bmp2* and *oc* gene expression of gilthead seabream larvae fed diets with different levels of dietary Cu for after 8, 15 and 21 days of feeding. *bmp2*, bone morphogenetic protein 2; *oc*, osteocalcin

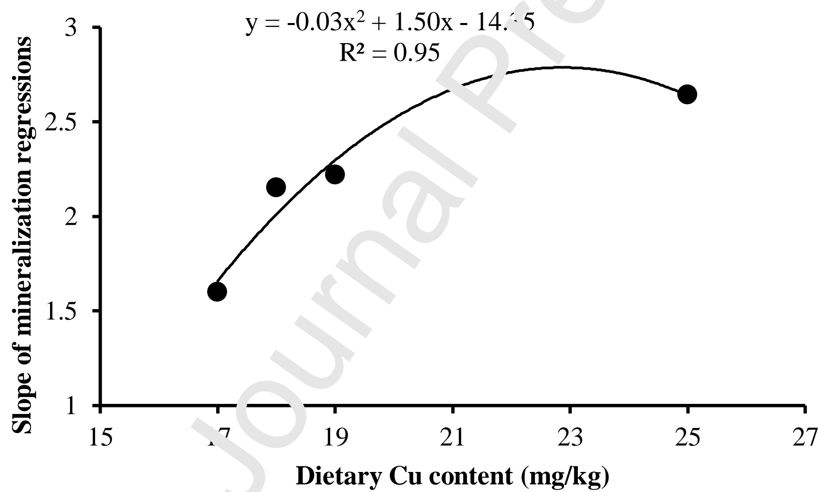

Figure 7. Relation between the slope of the mineralization lines (average number of completely mineralized vertebra in each size class) and the dietary Cu levels.

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Table 9. Frequency (%) of severe skeletal anomalies and regions of gilthead seabream larvae (47 dph) fed the experimental diets for 21 days (225 larvae per group were examined)

	Diet			
Anomalies	Cu 17	Cu 18	Cu 19	Cu 25
Severe anomalies	53.80 ± 14.46	41.46 ± 17.55	22.04 ± 11.31	27.82 ± 6.71
Kyphosis in abdominal vertebrae	38.81 ± 9.69	33.76 ± 11.93	17.99 ± 11.42	18.43 ± 3.06
Vertebral fusion in abdominal vertebrae	0.88 ± 1.52	0.43 ± 0.75	0.00 ± 0.00	0.90 ± 0.78
Anomalous maxillary/premaxillary	3.54 ± 2.79	0.42 ± 0.73	0.00 ± 0.00	2.21 ± 2.73
Anomalous fused branchiostegal ray	6.60 ± 4.73^{b}	0.86 ± 0.75^{a}	0.00 ± 0.00^{a}	1.37 ± 1.37^{a}
Anomalies by regions				
Cranium	10.14 ± 4.26^{b}	2.58 ± 1.29^{a}	0.45 ± 0.78^{a}	4.48 ± 1.95^{a}
Abdominal vertebrae	41.01 ± 9.55^{b}	33.92 ± 12.69^{b}	18.89 ± 11.94^{a}	21.14 ± 3.01^{a}
Haemal vertebrae	0.88 ± 0.76	0.44 ± 0.76	0.00 ± 0.00	0.088 ± 1.52

*Different letters in a row denote significant differences between groups fed different diets (mean ± SD, P <0.05).

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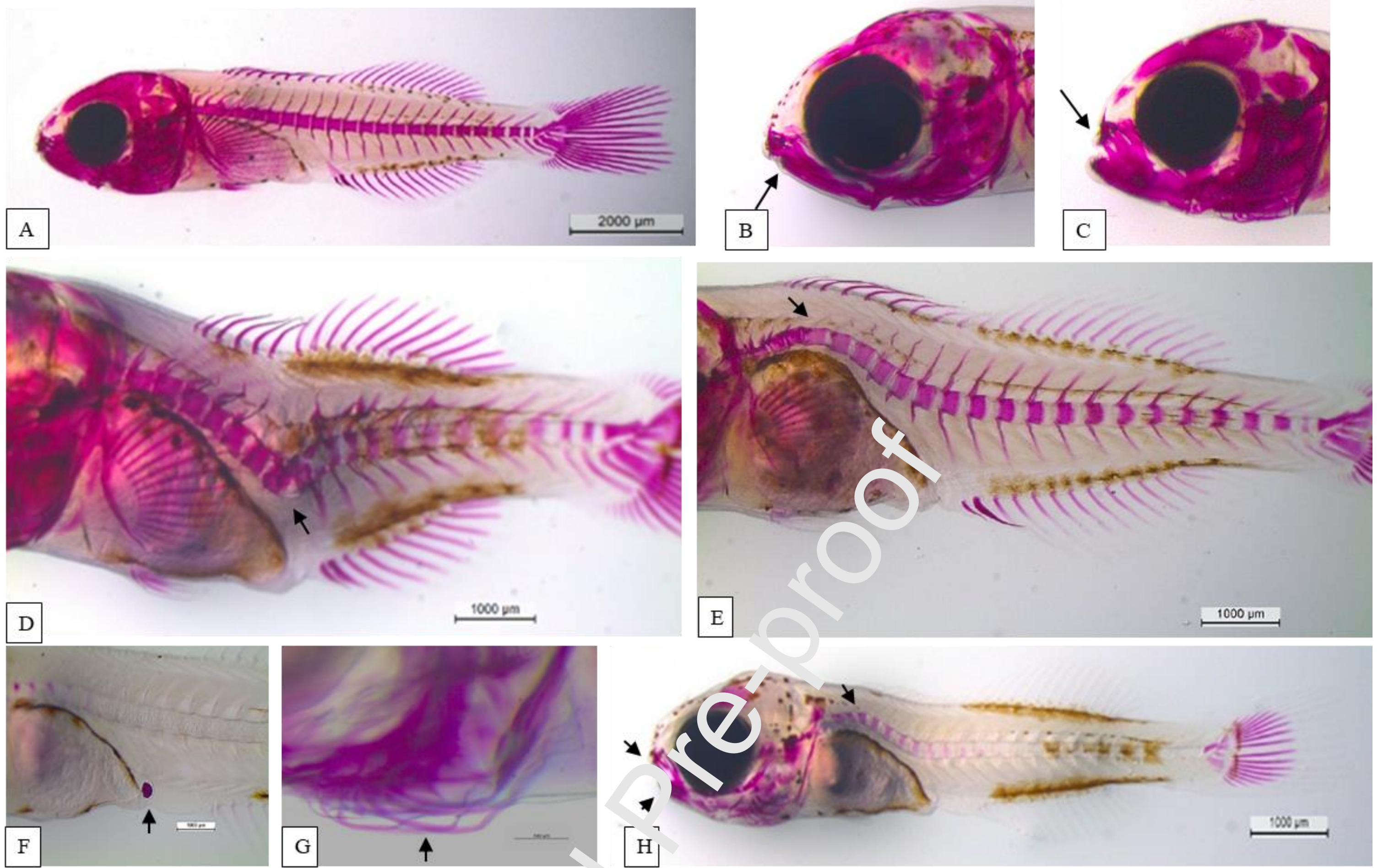

Figure 8. Different type of skeletal anomalies in gilthead seabream larvae (47 dph): A: Normal larvae with normal vertebrae column; B: Anomalous dentary; C: Abdominal premaxillary; D: Lordosis affecting abdominal and haemal vertebrae; E: Anomalous vertebrae kyphosis; F: Presence of calculi in the terminal tract of the urinary ducts; G: Anomalous branchiostegal rays; H: Anomalous jaw and abdominal vertebrae kyphosis. The black arrows point at the anomalies.

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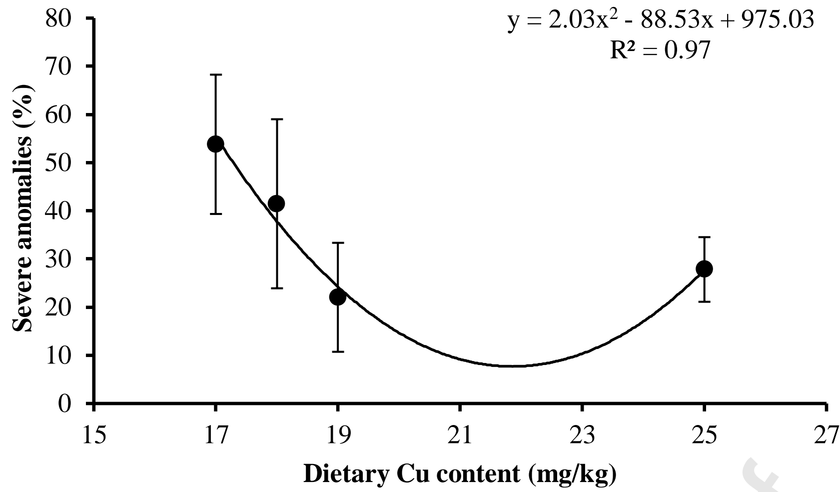

Figure 9. Relation between the dietary Cu levels and the incidence of severe anomalies in gilthead seabream (47 dph) fed different dietary Cu levels for 21 days.

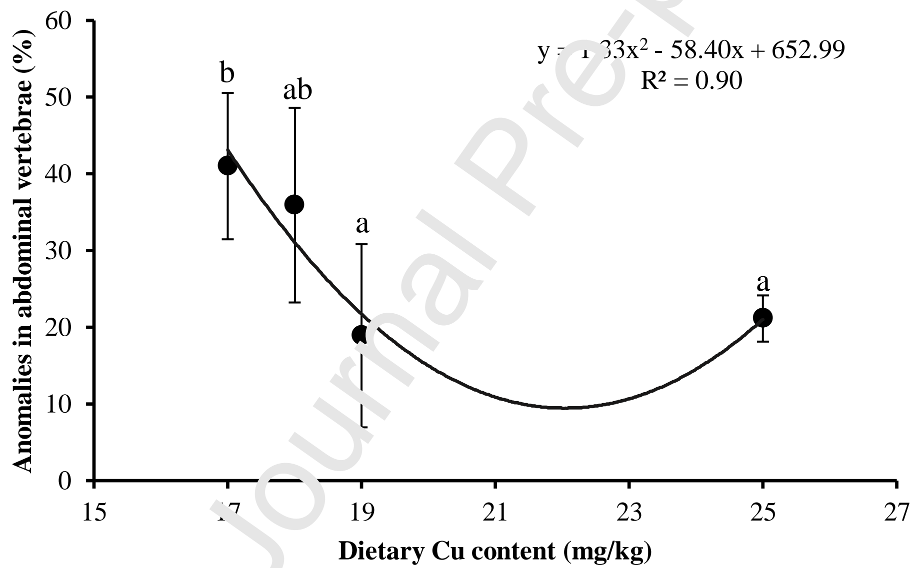

Figure 10. Relation between the dietary Cu levels and the incidence of total anomalies in abdominal vertebrae of gilthead seabream (47 dph) fed different dietary Cu levels 21 days. Means with different letters denote significant differences ($P<0.05$).

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Highlights:

- Dietary Cu levels from 17 to 25 mg Cu/kg did not significantly affect seabream larvae growth performance.
- The elevation of dietary Cu levels increased the whole-body contents in DHA, EPA and n-3 PUFA, and reduced lipid peroxidation and severe, cranial, branchiostegal rays and abdominal vertebral anomalies.
- Non-supplemented diet (17 mg Cu/kg) negatively affected the larvae by increasing lipid peroxidation, delaying mineralization and causing a high prevalence of skeletal anomalies.
- Polynomial regression models applied to all these parameters suggesting optimum dietary Cu levels to be 21.5-22.6 mg/kg in gilthead seabream larvae.

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