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Effect of dietary microminerals in early weaning diets on growth, survival, mineral contents and gene expression in gilthead sea bream (*Sparus aurata,* L*)* larvae

Running title: Microminerals in early weaning diets for gilthead sea bream larvae

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Data Availability Statement

The data that support the findings of this study are openly available in [Orcid] at [https://orcid.org], reference number [0000-0002-8336-957X].

Abstract

Importance of a premix inclusion of Zn, Mn and Se in early weaning diets have shown for larval growth, and suggested a potential toxicity by one of these elements. The objective of the present study was to determine the effect of the single dietary inclusion of Zn, Mn, Se and Cu, their combination (Control+) or their absence (Control-). At the end of the trial, survival was significantly (P<0.05) lowest in fish fed C+ diet (17.16±7% mean±SD), followed by that of larvae fed Mn diet (21.91±7%). The highest survival was obtained by Cu diet (35.27±15%), followed by C- diet (34.58±9%). Cu and Se supplementation significantly improved total length and body weight, in comparison to the C- fish. On the contrary, fish fed Mn and C+ showed the lowest growth. Supplementation with Zn or Cu significantly increased *CuZnsod* whereas *gpx* was significantly up-regulated in fish fed Se and C+ diets.

ARA/EPA level was significantly highest and lowest DHA/EPA in larvae fed Cu diet and the opposite in fish fed C+ diet. The results pointed out the importance of supplementation with Cu, as well as Se and Zn, on early weaning diets for gilthead sea bream, and the potential toxic effect of Mn.

Keywords: gilthead seabream, copper, zinc, manganese, selenium, gene expression.

Abbreviations: dph: days post hatched, Zn: Zinc, Cu: Copper, Mn: Manganese, Se: Selenium, I: Iodine, P: Phosphorus, Ca: Calcium, *Mnsod*: Manganese superoxide dismutase, *cu/znsod*: Copper, zinc superoxide dismutase, *gpx*: glutathione peroxidase, *oc*: octeocalcin

1. Introduction

Improvement of diets for marine fish larvae requires the complete understanding of nutritional requirements of these early life stages in the different species (Kolkovski, Lazo, Leclercq, & Izquierdo, 2009; Hamre, Yufera, Ronnestad, Boglione, Conceição, & Izquierdo, 2013). In spite of the abundance of research regarding lipids and essential fatty acids (Izquierdo & Koven, 2011), proteins and aminoacids (Conceição, Aragão, & Rønnestad, 2010) or vitamins A, C and E (Moren, Kvalheim, Harboe, Nordgreen, & Lie, 2011), studies aiming to determine the essentiality of other micronutrients are more scarce (Hamre, 2011). Regarding microminerals, essentiality of iodine (I) and selenium (Se) has been established for marine fish larvae (Ribeiro, Ribeiro, Sæle, Hamre, Dinis, & Moren, 2009; Hamre, Mollan, Sæle, & Erstad, 2008b; Moren, Opstad, Van der Meeren, & Hamre, K., 2006; Saleh, Betancor, Roo, Montero, Zamorano, & Izquierdo, 2014), whereas the effects of other dietary microminerals on such as zinc (Zn), copper (Cu) or manganese (Mn) growth performance of fish larvae have been much less studied (Hamre et al., 2013; Izquierdo, Ghrab, Roo, Hamre, Hernández-Cruz, Bernardini, Terova, & Saleh, 2017). Despite these minerals form part of key metalloenzymes that play important roles in fish physiology, it is not yet known if their requirements in larvae are covered by the dietary inputs obtained by the live preys used in hatcheries, such as rotifers and Artemia. Moreover, previous studies have demonstrated that the contents of these minerals in these live preys are lower than those of copepods, the natural prey for marine fish larvae (Hamre et al., 2013). Therefore, there is a lack of information on the effect of the inclusion of these minerals in early weaning diets for marine fish.

Zn is included in 20 metalloenyzmes that catalyze different metabolic pathways related to growth, reproduction, tissue development, vision or immunity (Watanabe, Kiron, & Satoh, 1997). Moreover, Zn plays important roles in oxidative stress and reducing cellular damage through antioxidant enzymes like alcohol dehydrogenases or cytosolic superoxidase dismutase (Watanabe et al., 1997; Lall, 2002). Besides, Zn intervenes on bone formation and mineralization by activating osteoblastic cells and inhibiting osteoclastic bone resorption, hence promoting ossification, bone development and subsequently growth (Yamaguchi, 1998). Studies have demonstrated that Zn can partially substitute calcium (Ca) in the bone due to

their similar physicochemical properties (Do Carmo e Sá, Pezzato, Lima, & de Magalhães Padilha, 2004). Uptake of Zn during larval development seems to be determinant to control mineral status in the larvae of amberjack (*Seriola dumerilii*) (Yamamoto, Matsunari, Iwasaki, Hashimoto, Kai, Hokazono, Hamada, Teruya, Hara, Furuita, & Mushiake, 2013) and improves growth and survival in red sea bream (*Pagrus major*) (Nguyen, Satoh, Haga, Fushimi, Kotani, 2008).

Cu forms part of several enzymes, including superoxide dismutase, counteracts oxidative stress and regulates lipid and blood metabolism. Therefore, Cu has important functions in vital organs such as heart, eye, brain, liver or bone (Watanabe et al., 1997; Lall & Lewis-McCrea, 2007), whereas excessive levels lead to reduced growth (Lin, Shih, Kent, & Shiau, 2010) or toxicity that may induce necrosis in gills or liver (Murai, Andrews, & Smith, 1981; Woody & O'Neal, 2012). Deficient Cu levels in diets for juveniles may lead to Cu reduction in tissues and low growth (Tang, Feng, Jiang, Liu, Jiang, Li, Kuang, Tang & Zhou, 2013), however, its importance in larval diets has not been sufficiently addressed. For instance, Cu and Zn contents in rotifers are markedly reduced when they are fed to fish larvae, even if microalgae is supplied to the larval rearing tanks, whereas availability of both elements increase in rotifers with digestive replete in microalgae (Wang, Shu, & Wang, 2019).

Mn is known to be an essential mineral for growth, reproduction and prevention of skeletal anomalies in terrestrial animals and fish. Mn is important for mitochondria functioning, affecting lipid, protein and carbohydrate metabolism (Lall, 2002). Thus, the lack of dietary Mn leads to low growth in rainbow trout or carp (Ogino & Yang, 1980), whereas its presence promotes protein synthesis and growth (Romanenko, 1984). Despite fish can directly absorb Mn from the water, Ca and phosphorus (P) presence reduces Mn absorption, that therefore must be included in the diet (Watanabe et al., 1997). Mn deficiency in fish reduces growth (Satoh, Takeuchi, & Watanabe, 1987; Tan, Xie, Luo, Lin, Zhao, & Xi, 2012) and affects manganese superoxide dismutase (*Mnsod*) and copper, zinc superoxide dismutase (*Cu/Znsod*) activities (Lin, Shie, & Shiau, 2008b), as well as tissue mineral composition (Liu, Ai, Mai, Zhang, Zhang, & Zheng, 2013). Artemia seems to be deficient in Mn and enrichment with this mineral improves growth and reduces skeletal anomalies in red sea bream larvae (Nguyen et al., 2008).

Se forms part of a considerable number of selenoproteins including glutathione peroxidase (*gpx*) (Penglase, Hamre, & Ellingsen, 2014) and plays an important antioxidant role (Pacitti, Wang, Page, Martin, Sweetman, Feldmann, & Secombes, 2013), being essential for correct larval development (Hamre, Srivastava, Rønnestad, Mangor-jensen, & Stoss, 2008a). Se deficiency may reduce growth, *gpx* activity and survival (Hardy, Oram, & Möller, 2010; Moren et al., 2011; Le & Fotedar, 2013; Saleh et al., 2014). Absorption of organic Se has been found to be higher than that of mineral salts (Schrauzer, 2003). Se content in the different live preys used in larviculture markedly differs and have been related to growth improvements (Penglase, Nordgreen, Van der Meeren, Olsvik, Sæle, Sweetman, Bæverfjord, Helland, & Hamre, 2010; Ribeiro, Ribeiro, Sæle, Hamre, Dinis, & Moren, 2012). Increase of organic Se (SeMet) in larval diets for European seabass (*Dicentrarchus labrax*) reduces oxidative damage (Betancor, Caballero, Terova, Saleh, Atalah, Benítez-Santana, Bell, & Izquierdo, 2012) and for gilthead sea bream improved survival and stress resistance (Saleh et al., 2014).

Supplementation of these minerals seems to be necessary in microdiets for larval gilthead sea bream, since non-supplementation reduces growth and bone mineralization, leading to a high incidence of bone anomalies (Izquierdo et al., 2017). However, join supplementation with three of these minerals (Zn, Mn and Se) at the same time in inorganic forms in levels similar to those found in copepods had negative effects in larval performance, suggesting the deleterious effect of one or more of these minerals (Izquierdo et al., 2017). In that previous study it was not possible to demonstrate which of those minerals had a negative effect since all of them were supplemented together in a premix. Thus, the aim of the present study was to determine the effect of the single inclusion of Zn, Cu, Mn or Se in microdiets for gilthead sea bream on larval performance, whole body composition, and expression of selected genes.

2. Materials and methods

2.1. Feeding trial and larval performance

Larvae were obtained from natural spawns from the gilthead sea bream broodstock of Grupo de Investigación en Acuicultura (GIA) (EcoAqua Institute, Las Palmas de

Gran Canaria, Spain). Larvae (initial total length 6.79±0.42 mm, dry body weight 0.22±0.02 mg, mean ± *SD*) previously fed rotifers (*Brachionus plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until 20 days post hatching (dph) were randomly distributed in 18 experimental tanks at a density of 2100 larvae each tank (10.5 larvae/l) and were fed one of the experimental diets tested in triplicate for 22 days, at a water temperature of 19.2-21.1ºC. All tanks (200 L light grey color cylinder fiberglass tanks) were supplied with filtered seawater (37 g/L salinity) at an increasing rate of 0.4-1 L/min along the feeding trials. Water entered the tank from the bottom and was let out from the top; water quality was tested daily, and no deterioration was observed. Water was continuously aerated (125 mL/ min), attaining 6.0-6.2 g/L dissolved O_2, saturation ranging between 90% and 95%.

Six isonitrogenous and isolipidic experimental microdiets (pellet size <250 ⌈m) based on squid meal and krill oil with gelatin as a binder, attractants (Kanazawa *et al*., 1989), and vitamin and mineral premixes (Teshima, Kanazawa, & Sakamoto, 1982) lacking Zn, Cu, Mn and Se were formulated and produced according to Saleh *et al*. (2014). The negative control diet (Diet C-) was not supplemented with the target minerals and their levels were low (83 mg/kg Zn, 17 mg/kg Cu, 4 mg/kg Mn, and 1.9 mg/kg Se), being lower than their levels in copepods (120 mg/kg Zn (Fujita, 1972); 3 mg/kg Se and 8 mg/kg Mn (Hamre et al., 2008a,b). Another four diets were supplemented with either $ZnSO_4$, $CuSO_4$, $MnSO_4$ or organic Se (SeMet, Alltech, Lexington, KY, USA) at similar levels to those found in copepods and, finally, a fifth positive control diet (Diet C+) was supplemented with the four minerals (Table 1). Sulphate forms were preferred over oxide forms for the better absorption (Lorentzen & Maage, 1999; Maage, Lygren, & El-Mowafic, 2000), whereas organic Se was very effectively used by gilthead sea bream larvae in our previous studies (Betancor et al., 2012; Izquierdo et al., 2017). Diets were supplied manually every 45 min from 9:00 to 19:00 at a rate of 2.5-3.5 g/tank. Larvae were observed under the binocular microscope to determine feed acceptance. If apparent feed intake differences were observed along different experimental diets, diet acceptance was determined calculating the percentage of gut occupation of the microdiet by image analysis. For such studies, pictures were taken of the abdominal cavity of 30 larvae per tank (Leica Wild M3Z, Optotek, California, USA).

Growth was determined by measuring dry body weight (105ºC until constant weight) and total length (Profile Projector V-12A; Nikon, Tokyo, Japan) of 30 fish/tank at the beginning, in the middle, and at the end of the trial. Final survival was calculated by individually counting all the live larvae at the beginning and at the end of the experiment. Before the end of the experiment, an activity test was conducted by handling 20 larvae/tank out of the water in a scoop net for 1 min and, subsequently allocating them to another tank supplied with clean seawater and aeration to determine survival after 24 h. The remaining larvae in each tank were starved for 16 hours, washed with distilled water, sampled, and kept at 80ºC to analyze biochemical composition.

2.2. Proximate composition and fatty acids and mineral profiles

Moisture (AOAC, 1995), protein (AOAC., 1995), and crude lipid (Folch, Lees, & Sloane-Stanley, 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982), separated by gas-liquid chromatography (GLC), quantified by FID (GC-14A; Shimadzu, Tokyo, Japan) under the conditions described by Izquierdo, Watanabe, Takeuchi, Arakawa, & Kitajima C. (1990), and identified by comparison with previously characterized standards and GLC-MS. Mineral analysis was conducted according to the method of Julshamn, Malde, Bjorvatn, & Krogedal, (2004). Samples were acidified in a microwave digester (MarsXpress, CEM, Kamp-Lintfort, Germany) with 5 mL of 69% pure nitric acid, then poured into a 10-mL volumetric flask, and made up to volume with distilled water. A total of 0.4 mL of this solution was then added to a 10-mL sample tube; 10 µL of the internal standard (Ga and Sc, 10 ppm) was included and 0.3 mL of methanol added. The tubes were made up to volume with distilled water and total selenium, zinc, copper, manganese and iron were measured by collision/reaction ICP-MS (Thermo Scientific, Cheshire, UK) using argon and hydrogen as carrier gasses.

2.3. Gene expression

Molecular biology analysis was carried out at GIA laboratories. Total RNA from larvae samples (average weight per sample 60 mg) was extracted using the RNeasy Mini Kit (Qiagen). Total body tissue was homogenized using the Tissue Lyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were

centrifuged with chloroform for phase separation (12000 g, 15 min, 4°C). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column where total RNA bonded to a membrane and contaminants were washed away by RW1 and RPE buffers (Qiagen). Purified RNA was eluted with 30 lL of RNase-free water. The quality and quantity of RNA were analyzed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of cDNA was conducted using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:5, 1:10, 1:15, 1:20 and 1:25). The product size of the real-time qPCR amplification was checked by electrophoresis analyses using PB322 cut with HAEIII as a standard. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) using β-actin as the house-keeping gene in a final volume of 20 μl per reaction well, and 100 ng of total RNA reverse-transcribed to complementary cDNA. Each gene sample was analyzed once per gene. The PCR conditions were the following: 95 °C for 3 min 30s followed by 40 cycles of 95°C for15s, 61°C for 30s, and 72°C for 30s; 95°C for1min; and a final denaturing step from 61 °C to 95 °C for 10 s. Data obtained were normalized and the Livak method (2-ΔΔCt) used to determine relative mRNA expression levels, using β-actin as internal control and total samples average as ΔCt calibrator. Gilthead sea bream specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis Software (Molecular Biology Insights, Cascade, CO, USA). Detailed information on primer sequences and accession numbers is presented in Table 2.

2.4. Statistical analysis

All data were tested for normality and homogeneity of variances with Levene´s test, and treated using one-way ANOVA. Means were compared by Duncan's test (p <.05) using SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA). All values presented as percentage were arc cosine transformed before performing any statistical test.

3. Results

3.1. Feeding trial and larval performance

Microdiets were well accepted by the larvae, neither feed rejection nor differences in feed gut occupation were observed. After 21 days of feeding, at 42 dph, sea bream larvae had increased their initial body weight 4 times (Table 3). The highest survival was found in fish fed the Cu supplemented diets, followed by those fed non-supplemented target minerals (C-), both being significantly higher than in larvae fed the combination of the 4 minerals (C+) ($p < .05$, Table 3), which showed the lowest survival rate. Survival of larvae fed diets supplemented with Zn, Se or Mn was intermediate and did not differed from that of larvae fed Cu, C- or C+ diets (Table 3). Cu supplementation also significantly improved total length at 42 dph, as well as Se supplementation, in comparison to larvae fed a non-supplemented diet (C-) or Zn supplemented diet ($p < .01$, Table 3), whereas the lowest length was obtained in larvae fed Mn-supplemented and C+ diets. Similarly, the highest larval body weight at 42 dph was found in larvae fed Cu supplementation, followed by those fed Se supplementation, whereas the lowest body weight was obtained in C+ larvae, followed by C- ($p < .01$, Table 3). Larvae fed Zn and Mn supplementation showed intermediate larval weight values (Table 3). Regarding condition factor, Cu supplementation produced heavier larvae, whereas larvae fed with either C- or C+ diets were 33% thinner (Table 3). No significant differences were found in survival after air exposure stress test (92-98%).

3.2. Proximate composition and fatty acids and mineral profiles

At the end of the feeding trial there were no significant differences in lipid or protein contents in larval whole body (Table 4). There were neither significant differences in the fatty acid profiles of whole body among larvae fed the different microdiets (Table 4). However, EPA/ARA ratios were significantly ($p < .05$) highest in larvae fed C- or Cu or Zn supplemented diets (Table 4) and lowest in fish fed C+. Besides, there was a significant negative relation between DHA/EPA contents in larvae and survival rate ($R^2 = 0.70165$).

Increase in dietary Zn levels in diets Zn and C+ did not significantly increase whole body Zn content in gilthead sea bream larvae (Table 5, $p > .05$). However, whole body Zn contents increased in larvae fed diets supplemented with Cu (Table 5, $p < .05$). Cu whole body contents were significantly highest in larvae fed Cu

supplementation followed by those fed C+ (Table 5, p >.05). Elevation of dietary Mn levels (diets Mn and C+) significantly raised larval Mn contents (p < .05). Whole body Se contents were not affected by dietary Se contents. Finally, Fe body contents were significantly (p > .05) higher in larvae fed Mn, followed by those fed Zn or Cu.

3.3. Gene expression

Molecular studies for antioxidant enzymes genes showed that *cat* expression was significantly highest in larvae fed C- diet (p < .05, Table 6) and was down-regulated by dietary mineral supplementation. Expression of *CuZnsod* was highest in larvae fed Zn supplemented diets, being significantly (p < .05) higher than in larvae fed diets C-, C+ or Se supplemented. *CuZnsod* expressions also were higher in larvae fed the Cu and Mn supplemented diets, without significant differences with larvae fed Zn supplementation. No significant differences were found in expression of *Mnsod* (Table 6). Expression of *gpx* was significantly (p < .05) highest in larvae fed C+ diet, followed by those fed Se supplementation, and lowest in larvae fed any of the other diets (Table 6). The *oc* expression was significantly highest in larvae fed C+ diet (p < .05, Table 6).

4. Discussion

Even though microminerals are present in rotifers and artemia in lower amounts than in copepods (Hamre, Holen, & Moren, 2007; Hamre et al., 2008b), a natural prey for marine fish larvae, studies on the importance of supplementation of these minerals in larval diets are scarce (Hamre et al., 2013). In the present study, combined supplementation of Zn, Cu, Mn and Se (112, 20, 11 and 1.8 mg/kg, respectively) at dietary levels similar to those found in copepods (Fujita, 1972; Hamre et al., 2008a), negatively affected survival. The results are in agreement with the poor survival found in previous studies (Izquierdo et al., 2017) and suggest the harmful effect of at least one of those minerals. Despite these previous studies found a negative effect of these minerals in larval survival after handling stress (Izquierdo et al., 2017), in the present study there was no differences in stress resistance among larvae fed the different diets. Indeed all larvae showed a very high survival to handling stress, suggesting that the test was too mild.

Analysis of the mineral contents in larvae fed the combined supplementation of these target minerals (diet C+) showed that Mn contents were significantly raised in

comparison to larvae fed the non-supplemented diets. Moreover, Mn contents in larvae fed the Mn supplemented diet (Mn diet) were also increased, and these larvae showed also a low survival rate, not significantly different from that of larvae fed Mn in combination with the other minerals (diet C+). In agreement, in juveniles of other fish species Mn contents are also increased in several tissues, particularly in bone, by the elevation of dietary Mn levels (Ogino & Yang, 1980; Gatlin, Poe, & Wilson, 1986; Knox, Cowey, & Adron, 1981; Lorentzen & Maage, 1999; Lorentzen, Maage, & Julshamn, 1996.; Maage et al., 2000; Pan, Zhu, Xie, Lei, Han, & Yang, 2008). These results suggest that increase in dietary Mn supplementation from 4 to 11-13 mg/kg as $MnSO_4$ in microdiets for gilthead sea bream larvae was harmful and reduced survival by 40-50%, compared to larvae fed the non-supplemented diet (C-). This is in agreement with the negative correlation previously found between larval survival and dietary Mn levels (Izquierdo et al., 2017) when Mn was supplemented together with Zn and Se. Thus, the present study confirms the negative effect of excess dietary $MnSO_4$ levels on sea bream larvae growth. However, increase in dietary Mn (10-43 mg/kg) improves growth in larvae of another sparid of similar age (Nguyen et al., 2008) and the Mn levels tested in the present study (11-13 mg/kg) were in the range of those found in live preys for marine fish larvae. Similarly, rotifers enriched with inorganic iodine (NaI) at levels similar to copepods (129 mg/kg DW) are toxic to cod larvae and the requirement is suggested to be 3.5 mg/I kg DW (Penglase et al., 2013). Therefore, it could be possible that in the present study, rather than the dietary Mn levels themselves, the supplementation with an inorganic form of Mn would be responsible for the reduced survival. In a recent study in gilthead sea bream juveniles, supplementation with an inorganic form of Mn (Mn oxide) had a pro-oxidant effect in comparison to amino acids chelated Mn, raising the levels of thiobarbituric acid reactive substances (Dominguez, Robaina, Zamorano, Karalazos, & Izquierdo, 2019). Besides, supplementation with Mn oxide did not affected *Mnsod* expression (Dominguez et al., 2019). In agreement, in the present study, *Mnsod* expression was not affected by dietary Mn levels. In mammals, prolonged or excess exposure to Mn has been associated to several behavioral and neuronal pathologies including atypical parkinsonian syndrome (manganism) and causes neuronal apoptosis and mitochondrial dysfunction, in relation to increased mitochondrial H_2O_2 production and *sod2* activity (Zolkipli-Cunningham & Falk, 2017). In fish juveniles, *Mnsod* activity in liver is reduced either by insufficient or excess dietary levels of Mn (Satoh,

Takeuchi, & Watanabe, 1991; Lin et al., 2008a; Tan et al., 2012; Prabhu, Schrama, & Kaushik, 2016). In the present study, elevation of dietary and body Mn contents did not up-regulate *Mnsod* or *cat* gene expression in whole body. This fact suggests that even though the Mn content in whole body doubled that of the fish fed diets non-supplemented with minerals, their levels were not causing a pro-oxidant effect of excess Mn as described in zebrafish (Arndt, Borella, & Espósito, 2014). Indeed, larval fatty acid composition did not reflect a potential pro-oxidant effect with increased dietary Mn levels; i.e. a reduction in polyunsaturated fatty acids. Further studies are being conducted to determine the mechanisms implied on the negative effect of dietary Mn levels on gilthead sea bream survival and possible consequences for larval behavior or neural development.

On the contrary, Cu supplementation markedly improved growth in terms of total length and body weight, raised Zn contents in whole body and led to the highest Cu and Fe contents. Cu^{2+} shares the same metal ion transporter protein with Zn^{2+} and Fe^{2+} and other elements, the solute carrier transporter SLC11A2β, whose gene expression may be regulated by dietary minerals, maintaining their homeostasis as we have seen in previous studies in the same species (Terova, Rimoldi, Izquierdo, Pirrone, Ghrab, & Bernardini, 2018). Therefore, it is possible that in the present study the increase in dietary Cu up-regulated also the solute carrier transporter SLC11A2β, leading to increased Cu, Zn and Fe transport and, hence, to the higher deposition of this minerals in larval tissues as shown by the hole larvae mineral analysis. Besides, dietary Cu up-regulated *CuZnsod* expression in whole body in comparison to non-Cu supplemented diet (C-), increasing the protection against peroxidation. In agreement, in juveniles of different fish species, the low growth and superoxide dismutase activity in fish fed deficient dietary Cu levels is increased by the elevation of dietary Cu (Shiau & Ning, 2003; Tan et al., 2011; Tang et al., 2013; Wang, Zhang, Bai, Chen, Guo, & Xing, 2015a; Wang, Zhu, Wang, Li, Du, Qin, & Chen, 2018b; Abdel-Hameid, Zehra, & Khan 2017). The lower values in *cat* expression, agree well with the increased protection against oxidation by the up-regulation of *CuZnsod*. Apart from its important role in defense against oxidation, Cu is an essential element involved in energy production through the C cytochrome oxidase, neurotransmission, collagen synthesis and melanin production (Lall, 2002). The larvae of the present study were previously fed on rotifers, which in comparison to copepods have a low content in Cu (around 6 mg/kg dry matter) (Watanabe et al., 1997) that could be insufficient to

cover the requirements of the fast growing gilthead sea bream larvae. This would explain the elevation of Cu content in larvae fed increased dietary Cu levels. Whereas dietary Cu requirements in juveniles range between 3-13 mg/kg, these requirements may be increased during fast growing larval development (Prabhu, P. A. J., Schrama, J. W., Mariojouls, C., Godin, S., Fontagne-Dicharry, S., Geurden, I., Surget, A., Bouyssiere, B., & Kaushik, S. J., 2014).

Supplementation with Zn raised dietary Zn levels from 86 to 110 mg/kg, increasing the whole body Zn contents with 6% and and up-regulating *CuZnsod.* These results agree well with the increase in superoxide dismutase activity found in Atlantic cod (*Gadus morhua*) when rotifer Zn contents where increased up to 90 mg kg^{-1} (Penglase et al., 2011). However, the present levels of Zn supplementation did not affect sea bream growth, in agreement with the results found in red sea bream larvae fed rotifers with increased Zn supplementation (119–306 mg/kg) (Nguyen et al., 2008). Moreover, increased dietary Zn did not negatively affected gilthead sea bream larval survival as occurred in larvae of other species fed enriched rotifers (33–245 mg kg^{-1}), *Artemia* (119–306 mg/kg) or copepods (340–570 mg/kg) (Nguyen et al., 2008; Yamamoto et al., 2013).

Supplementation with organic Se raised dietary Se contents, increased final larval body weight and condition index, and up-regulated *gpx.* These results agree well with the increased GPX activity found in other species fed increased dietary Se levels (Hamre et al., 2008b; Pacitti et al., 2013; Penglase et al., 2014; Ramesh, Sankaran, Veera-Gowtham, & Poopal, 2014; Kim, Kim, & Song, 2014). Optimum organic Se (SeMet) contents in microdiets for gilthead sea bream have been recently determined (Saleh et al., 2014) and show that dietary Se increase from 1.73 up to 11.65 mg/kg reduced oxidative risk and improved survival and stress resistance, but did not affect larval growth in terms of total length in agreement with the present study.

In summary, these results pointed out the importance of supplementation with Cu, as well as Se and Zn on early weaning diets for gilthead sea bream larvae, and the potential toxic effect of Mn. Therefore, copepod-levels of some of the micro-minerals may be toxic when supplemented in inorganic forms in early weaning diets. Further studies are being conducted to determine optimum levels of these nutrients in diets for marine fish larvae.

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Table 1. Ingredients analyzed proximate composition and mineral contents in the experimental microdiets supplemented with minerals to feed gilthead sea bream larvae

Experimental diets (g/kg)						
	C-	**Zn**	**Cu**	**Mn**	**Se**	**C+**
Squid Powder†	710	710	710	710	710	710
Gelatine‡	30	30	30	30	30	30
Krill Phospholipids§	125	125	125	125	125	125
Mineral Premix (no Zn, Mn, Cu or Se)	44.8	44.8	44.8	44.8	44.8	44.8
$ZnSO_4.7H_2O$¶	-	0.15	-	-	-	0.15
$CuSO_4.5H_2O$**	-	-	0.013	-	-	0.013
$MnSO_4.H_2O$††	-	-	-	0.03	-	0.03
Organic Se‡‡	-	-	-	-	0.00003	0.00003
Vitamins§§	60	60	60	60	60	60
Attractants***	30	30	30	30	30	30
Proximate composition (g/kg)						
Dry matter	902.2	900	902.7	912.8	905.5	909
Protein	654	652	650	656	654	675
Lipid	168	177	175	175	175	156
Ash	67.8	65.2	67.5	67.2	68.9	63.4
Zn (mg/kg)	83	119	88	85	83	112
Cu (mg/kg)	17	17	21	17	17	20
Mn (mg/kg)	4	4	4	13	4	11
Se (mg/kg)	1.9	1.8	1.9	1.7	2	1.8

† Rieber & Son, Bergen, Norway.
‡ Panreac, Barcelona, Spain.
§ Qrill (high in PL) Aker BioMarine, Fjordalleen, Norway.
¶ $ZnSO_4.7H_2O$
** $CuSO_4.5H_2O$
†† $MnSO_4.H_2O$
‡‡ Organic Se (SeMet, Sel-Plex, Alltech, Lexington, KY, USA).

§§Eryalçın, Roo, Saleh, Atalah, Benitez, Betancor, Hernandez-Cruz & Izquierdo (2013).

***Betancor, Nordrum, Atalah, Caballero, Benitez-Santana, Roo, Robaina & Izquierdo (2012a) and Betancor, Caballero, Terova, Cora, Saleh, Benitez-Santana, Bell, Hern andez-Cruz & Izquierdo (2012b).

Table 2. Sequences of forward and reverse primers (5'-3') for real-time quantitative PCR of gilthead sea bream

	5'-3' primer sequence		
Gene	**Forward**	**Reverse**	**GenBank Accession no.**
cat	ATGGTGTGGGACTTCTGGAG	AGTGGAACTTGCAGTAGAAAC	FJ860003
CuZnsod	AAGAATCATGGCGGTCCTACTGA	TGAGCATCTTGTCCGTGATGTCT	AJ937872
Mnsod	AGTGCCTCCTGATATTTCTCCTCTG	CCTGACCTGACCTACGACTATGG	JQ3088331
gpx	TCCATTCCCCAGCGATGATGCC	TCGCCATCAGGACCAACAAGGA	DQ524992
oc	AGCCCAAAGCACGTAAGCAAGCTA	TTTCATCACGCTACTCTACGGGTT	AF048703
[a]β-actin	TCTGTCTGGATCGGAGGCTC	AAGCATTTGCGGTGGACG	X89920

Abbreviations: Cat, catalase; CuZnsod, superoxide dismutase; Mnsod, manganese superoxide dismutase; gpx, glutathione peroxidase; oc, osteocalcin.

[a]β-Actin cDNA was used as an internal control.

Table 3. Survival and growth in gilthead sea bream larvae fed for 21 days with diets containing different mineral supplementation

	Age (dph)	C-	Zn	Cu	Mn	Se	C+	*p*-values
Survival* (%)	42	34.58±9*	26.31±5	35.27±15*	21.91±7	22.29±4	17.16±7	.04
Total length** (mm)	31	7.63±0.68	7.66±0.68	7.83±0.76	7.68±0.77	7.87±0.81	7.83±0.82	.12
	42	9.42±0.02	9.51±0.02	9.76±0.09**	9.30±0.05	9.65±0.06**	9.30±0.08	**.01**
Dry body weight*** (mg)	31	0.39±0.04	0.43±0.02	0.58±0.17	0.43±0.08	0.44±0.03	0.40±0.08	.17
	42	0.89±0.07	1.05±0.19	1.34±0.07***	1.00±0.18	1.20±0.07	0.79±0.08	**.002**
CF[a]	31	0.03	0.03	0.05*	0.03	0.03	0.03	**.04**
	42	0.08	0.10	0.13*	0.09	0.12*	0.07	**.03**

*n = 3, $p < .05$. **n = 90, $p < .01$. ***n = 90, $p < .01$. Values are mean of three tanks ± SEM. Asterisks indicate significant difference among mineral contents fed groups, *, $p < .05$; Duncan's multiple range test.
[a]Condition factor (CF) = 100*(DW/TL^3). TL stands for total length (cm).

Table 4. Lipid and protein contents (% dry weight) and main fatty acids profiles (% total identified fatty acids) of gilthead sea bream larvae fed microdiets with different minerals from 20 to 42 dph

	Initial	C-	Zn	Cu	Mn	Se	C+	*p*-values
Proximate composition (g/kg)								
Crude lipid	16.46	18.44±2.33	19.29±1.28	17.32 ± 1.71	19.90 ± 1.54	19.82 ± 1.22	17.88 ± 4.43	.32
Crude protein	69.81	66.06±1.32	65.23±1.08	66.53 ± 0.45	65.62 ± 1.25	66.52 ± 2.74	67.75 ± 4.70	.41
Fatty acid profile (g/kg total lipid)								
C14:0	0.02	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.02	0.08 ± 0.0	0.08 ± 0.04	.97
C15:0	0.01	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	.87
C16:0	0.33	0.69 ± 0.19	0.77 ± 0.05	0.61 ± 0.04	0.69 ± 0.08	0.82 ± 0.19	0.81 ± 0.23	.59
C16:1n-7	0.11	0.08 ± 0.02	0.09 ± 0.00	0.07 ± 0.00	0.08 ± 0.01	0.09 ± 0.02	0.09 ± 0.03	.62
C16:3n-4	0.01	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	.89
C18:0	0.22	0.22 ± 0.06	0.25 ± 0.02	0.20 ± 0.01	0.23 ± 0.03	0.27 ± 0.05	0.28 ± 0.07	.34
C18:1n-9	0.33	0.27 ± 0.07	0.29 ± 0.02	0.24 ± 0.02	0.27 ± 0.03	0.32 ± 0.07	0.31 ± 0.08	.39
C18:1n-7	0.11	0.13 ± 0.04	0.15 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.16 ± 0.04	0.15 ± 0.04	.43
C18:2n-6	0.21	0.04 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	.23
C18:3n-3	0.09	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	.95
C18:4n-3	0.01	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.02	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	.77
C20:0	0.00	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	.78
C20:1n-9	0.03	0.05 ± 0.01	0.05 ± 0.00	0.06 ± 0.03	0.05 ± 0.00	0.05 ± 0.01	0.06 ± 0.02	.55
C20:4n-6	0.07	0.04 ± 0.01	0.05 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	.20
C20:4n-3	0.02	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	.
C20:5n-3	0.11	0.34 ± 0.04	0.37 ± 0.08	0.31 ± 0.07	0.32 ± 0.08	0.35 ± 0.02	0.30 ± 0.02	.75

C22:5n-6	0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	.53
C22:5n-3	0.04	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	.88
C22:6n-3	0.29	0.58 ± 0.05	0.62 ± 0.16	0.45 ± 0.12	0.54 ± 0.18	0.59 ± 0.10	0.58 ± 0.13	.38
ΣSFAs	0.59	0.93 ± 0.26	1.13 ± 0.07	0.92 ± 0.04	1.03 ± 0.10	1.19 ± 0.28	1.19 ± 0.34	.32
ΣMUFAs	0.64	0.57 ± 0.15	0.64 ± 0.04	0.56 ± 0.05	0.60 ± 0.05	0.70 ± 0.16	0.68 ± 0.18	.64
Σ n-3	0.59	1.03 ± 0.10	1.10 ± 0.25	0.93 ± 0.22	0.96 ± 0.28	1.06 ± 0.11	0.99 ± 0.15	.89
Σ n-6	0.33	0.11 ± 0.02	0.12 ± 0.01	0.10 ± 0.01	0.12 ± 0.02	0.13 ± 0.01	0.13 ± 0.01	.26
Σ n-9	0.36	0.28 ± 0.07	0.31 ± 0.01	0.26 ± 0.03	0.29 ± 0.03	0.34 ± 0.07	0.33 ± 0.08	.55
Σ n-3 HUFAs	0.48	0.98 ± 0.10	1.05 ± 0.25	0.86 ± 0.20	0.91 ± 0.27	1.00 ± 0.11	0.94 ± 0.16	.87
EPA/ARA	1.65	7.64 ± 0.69*	7.87 ± 1.09*	7.97 ± 0.71*	7.25 ± 0.32	6.01 ± 0.50	5.73 ± 0.36	**.04**
DHA/EPA	2.56	1.68 ± 0.09	1.67 ± 0.10	1.60 ± 0.14	1.69 ± 0.16	1.68 ± 0.21	1.90 ± 0.33	.59
DHA/ARA	4.21	12.84 ± 1.82	13.17 ± 2.55	12.77 ± 2.02	12.29 ± 1.63	11.76 ± 2.28	10.92 ± 2.55	.84
n-3/n-6	1.77	9.132 ± 1.13	9.057 ± 1.53	9.26 ± 1.21	8.32 ± 1.08	8.26 ± 1.52	7.56 ± 1.62	.70

All data are presented as mean values expressed in mean ± *SD*, *n=3* (represents one pool per tank). *, $p < .05$; Duncan's multiple range test.
Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFAs, monounsaturated fatty acids; HUFAs, highlyunsaturated fatty acids; SEM, standard error of the mean; SFAs, saturated fatty acids.

Table 5. Mineral content of gilthead sea bream larvae fed for 21 days with diets containing different mineral supplementation (Mean ± *SD*, n=3, one pool of larvae per tank)[a]

Diets	C-	Zn	Cu	Mn	Se	C+	*p*-values
Zn (mg/kg)	16.21 ± 2.42	15.94 ± 0.39	23.17 ± 6.3*	18.40 ± 4.10	14.56 ± 0.83	16.33 ± 2.07	**.005**
Cu (mg/kg)	0.61 ± 0.04	0.59 ± 0.03	0.75 ± 0.17*	0.64 ± 0.07	0.63 ± 0.03	0.70 ± 0.08	**.006**
Mn (mg/kg)	0.19 ± 0.04	0.19 ± 0.02	0.19 ± 0.07	0.38 ± 0.11*	0.18 ± 0.01	0.34 ± 0.07*	**.001**
Se (mg/kg)	0.27 ± 0.04	0.26 ± 0.01	0.29 ± 0.03	0.27 ± 0.01	0.24 ± 0.03	0.29 ± 0.03	.35
Fe (mg/kg)	5.4 ± 0.4	10.8 ± 4.6	14.9 ± 7.4*	7.8 ± 1.2	5.5 ± 1.1	8.7 ± 2.7	**.007**

[a] Values are means of three replicates per treatment. Asterisks indicate significant difference among mineral contents fed groups, *, $p < .05$; Duncan's multiple range test.

Table 6. Expression of antioxidant enzymes and osteocalcin genes in gilthead sea bream larvae fed for 21 days with diets containing different mineral supplementation

Diets	**C-**	**Zn**	**Cu**	**Mn**	**Se**	**C+**	***p*-values**
cat	1.24 ± 0.19*	1.15 ± 0.25	1.16 ± 0.24	0.94 ± 0.26	0.76 ± 0.37	0.62 ± 0.32	**0.04**
CuZnsod	0.81 ± 0.26	1.38 ± 0.19*	1.26 ± 0.26	1.23 ± 0.22	0.99 ± 0.14	1.01 ± 0.26	**0.03**
Mnsod	1.20 ± 0.38*	1.02 ± 0.05	1.01 ± 0.47	0.98 ± 0.16	1.04 ± 0.10	0.97 ± 0.51	**0.03**
gpx	0.78 ± 0.34	0.95 ± 0.23	0.91 ± 0.15	0.89 ± 0.21	1.25 ± 0.31	2.03 ± 0.73*	**0.01**
oc	0.84 ± 0.57	0.98 ± 0.24	1.05 ± 0.49	1.05 ± 0.36	1.11 ± 0.30	1.58 ± 0.79*	**0.04**

All data are presented as mean values expressed in mean ± *SD*, *n=3* (represents one pool per tank). Asterisks indicate significant difference among different mineral contents fed groups. *, $p < .05$; Duncan's multiple range test.