Aquaculture Research

Aquaculture Research, 2016, 1–16 doi: 10.1111/are.13119

Organic, inorganic and nanoparticles of Se, Zn and Mn in early weaning diets for gilthead seabream (*Sparus aurata;* Linnaeus, 1758)

Marisol S Izquierdo[1], Wafa Ghrab[1], Javier Roo[1], Kristin Hamre[2], Carmen M Hernández-Cruz[1], Giovanni Bernardini[3], Genciana Terova[3] & Reda Saleh[1,4]

[1]Grupo de Investigación en Acuicultura (GIA), Universidad de Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, Spain

[2]National Institute of Nutrition and Seafood Research (NIFES), Bergen, Norway

[3]University of Insubria, Varese, Italy

[4]Oceanography Department, Faculty of Science, Alexandria University, Alexandria, Egypt

Correspondence: M Izquierdo, Grupo de Investigación en Acuicultura (GIA), Universidad de Las Palmas de Gran Canaria, Transmontaña s/n, 35416 Arucas, Las Palmas de Gran Canaria, Spain. E-mail: reda-saleh@hotmail.com

Abstract

Levels of the oxidative stress-related minerals selenium (Se), zinc (Zn) and manganese (Mn) that should be supplied in microdiets for marine fish larvae depend on the availability of the molecular form of these minerals. The objective of this study was to determine how effectively Se, Zn and Mn in organic, inorganic and nanoparticle forms promote larval performance and bone development. Microdiets supplemented with Se, Zn and Mn were fed for 24 days to 20 dah seabream larvae. Microdiets without Se, Zn and Mn supplementation were associated with poor growth, low bone mineralization and a high incidence of anomalies in the branchial arches. Including Zn, Mn and Se in an amino acid chelate organic form promoted maximum larval growth, increased body lipid reserves, enhanced early mineralization and prevented branchial arches anomalies. In contrast, feeding with inorganic forms of these minerals was less effective than organic minerals in improving larval weight or bone mineralization in comparison to the non-supplemented diet. Moreover, the larvae were less resistant to stress, and fish showed higher bone anomalies in the pre-hemal region. Adding Zn, Mn and Se in the form of nanometals did not enhance growth, but improved stress resistance and bone mineralization. The study showed the need to supplement seabream with early weaning diets based on squid meal and krill oil with one or more of the antioxidant minerals, to promote larval growth, bone mineralization and prevention of skeleton anomalies, organic minerals being more effective than inorganic forms and nanometals in promoting mineralization and stress resistance.

Keywords: larval nutrition, selenium, manganese, zinc, skeletal anomalies, bone mineralization

Introduction

Despite the important roles of minerals for organ functioning and development, information about mineral nutrition in marine fish larvae is very scarce. Mineral content in copepods, natural live prey for these larvae, can vary greatly from species to species (Fujita 1972; Watanabe, Arakawa, Kitajima, Fukusho & Fujita 1978) and is probably affected by the abundance and variety of microalgae that they ingest. For instance, in copepods the selenium (Se), zinc (Zn) and manganese (Mn) levels are in the range of 3–5, 110–700 and 8–25 mg kg^{-1} respectively (Fujita 1972; Watanabe *et al.* 1978; Hamre, Mollan, Saele & Erstad 2008). Unfortunately, mineral content in hatchery live prey greatly differs from that in copepods, being in rotifers 0–20 mg kg^{-1} Se, 75–600 mg kg^{-1} Zn, and 6–11 Mn mg kg^{-1} (Hamre, Yúfera, Rønnestad, Boglione, Conceição & Izquierdo 2013;

Nordgreen, Penglase & Hamre 2013; Yamamoto, Matsunari, Iwasaki, Hashimoto, Kai, Hokazono & Mushiake 2013; Kim, Sakakur, Maruyama, Nakamura, Takiyama, Fujiki & Hagiwara 2014) and in Artemia 104–423 Zn mg kg^{-1} and 8–43 mg kg^{-1} Mn (Nguyen, Satoh, Haga, Fushimi & Kotani 2008). Moreover, the mineral content in rotifers may vary with the type of enrichment, the kind of microalgae used as green water in the rearing tanks and the period where they remain unfed in the rearing tanks (Yamamoto *et al.* 2013). Thus, mineral content in the live prey used in commercial hatcheries is frequently lower than in copepods (Hamre, Mollan *et al.* 2008) and even lower than the requirements estimated for juveniles or adults. Several authors have developed effective methods for enriching minerals in live prey (Nguyen *et al.* 2008; Srivastava, Stoss & Hamre 2011; Srivastava, Hamre, Stoss & Nordgreen 2012; Nordgreen *et al.* 2013; Penglase, Harboe, Sæle, Helland, Nordgreen & Hamre 2013). Nevertheless, the mineral and, overall, nutritional compositions of live prey are variable and difficult to control, and they change while they remain uneaten in the larval rearing tanks. Moreover, live prey production requires manpower and energy expenditure, increasing production costs. Therefore, a great research effort has been made during the last few decades to develop compound diets that can completely replace live prey (Kolkovski, Lazo Corvera & Leclercq 2009).

However, in order to develop effective early weaning diets for marine fish larvae, the nutritional requirements of the different marine fish species must be defined (Hamre *et al.* 2013). Studies that aimed to determine optimum levels of nutrients in compound diets for larvae have focused mainly on lipids and essential fatty acids (Izquierdo, Tandler, Salhi & Kolkovski 2001; Villeneuve, Gisbert, Zambonino-Infante, Quazuguel & Cahu 2005), protein and amino acids (Carvalho, Sá, Oliva-Teles & Bergot 2004; Kvåle, Harboe, Mangor-Jensen & Hamre 2009; Pinto, Figueira, Ribeiro, Yúfera, Dinis & Aragão 2010), and vitamins (Mazurais, Glynatsi, Darias, Christodoulopoulou, Cahu, Zambonino-Infante & Koumoundouros 2009; Betancor, Atalah, Caballero, Benítez-Santana, Roo, Montero & Izquierdo 2011), whereas research on dietary mineral content only started very recently (Betancor, Caballero, Terova, Saleh, Atalah, Benítez-Santana, Bell & Izquierdo 2012; Saleh, Betancor, Roo, Montero, Zamorano & Izquierdo 2014). The high surface volume of the small particle size of compound feeds for larvae and the long duration in the water until the larvae ingest them, greatly increase the risk of leaching and oxidation in this type of diet. Moreover, the high polyunsaturated fatty acid contents of these diets to fulfil requirements of the fast-growing marine larvae and, consequently, the further increase in the oxidative risk (Betancor *et al.* 2011) suggest that adequate levels of antioxidant minerals, such as Se, Zn or Mn, must be of high relevance.

Selenium is an essential micronutrient with several physiological functions. It is an integral part of the enzyme glutathione peroxidise, which prevents oxidative damage of cytoplasmic structures, and it promotes bone formation and mineralization. Positive effects of the Se content in live prey have been described for larvae of Atlantic cod (*Gadus morhua*) (Hamre, Srivastava, Rønnestad, Mangor-Jensen & Stoss 2008; Penglase, Nordgreen, Van der Meeren, Olsvik, Sæle, Sweetman, Baeverfjord, Helland & Hamre 2010) and Senegalese sole (*Solea senegalensis*) (Ribeiro, Ribeiro, Saele, Dinis & Moren 2012). Our previous studies of feeding larvae with early weaning diets showed that an increase in Se levels from 1.3 to 6.27 mg selenomethionine (Se-Met) kg^{-1} promotes growth and reduces muscle dystrophy in larval European sea bass (*Dicentrarchus labrax*) (Betancor *et al.* 2012), whereas an increase from 1.7 to 11.65 mg Se-Met kg^{-1} improves larval survival, stress resistance and bone mineralization in larval gilthead sea bream (*Sparus aurata*) (Saleh *et al.* 2014). In juveniles, the bioavailability of aa-chelated (organic) Se seems to be higher than that of mineral salts (inorganic) of Se since absorption of Se-Met occurs through Na^+-dependent neutral amino acid transport and seems more effective than selenite absorption by passive diffusion (Daniels 1996; Schrauzer 2003).

Zinc is a very important element in fish nutrition (Lall 1989), not only as part of the enzyme superoxide dismutase that plays an important role in preventing peroxidation but also as an integral part of 20 other metalloenzymes, such as alkaline phosphatase (Watanabe, Kiron & Satoh 1997), that are required for bone mineralization. By enriching *Artemia nauplii* with Zn and Mn, growth performance improved significantly and fewer skeletal anomalies developed (Nguyen *et al.* 2008; Matsumoto, Satoh, Kotani & Fushimi 2009). However, no studies have been conducted to test Zn supplementation to early weaning diets. In

juveniles, like Se, organic Zn in the form of amino acid-chelated Zn seems to be more bioavailable than inorganic forms of zinc, such as zinc carbonate, zinc sulphate or zinc oxide, promoting growth (Apines, Satoh, Kiron, Watanabe, Nasu & Fujita 2001) and fish health (Zn-proteinates) (Paripatananont & Lovell 1997). However, in other studies organic Zn did not improve growth in comparison to inorganic Zn (Ma, Hou, Mai, Bharadwaj, Ji & Zhang 2014).

In addition to its antioxidant role in forming part of the Mn-superoxide dismutase, Mn is a cofactor for a large number of enzymes and forms metalloenzyme complexes essential for carbohydrate, lipid and protein metabolism (Watanabe *et al.* 1997; Lall 2002). Thus, Mn activates glycosyltransferase, kinases, transferases, hydrolases and decarboxylases (Watanabe *et al.* 1997). Only very few studies have aimed to study Mn nutrition in fish larvae. The content of Mn in cultured live prey is lower than in wild zooplankton and enriching them with Mn promotes larval growth in terms of total length and body weight and, together with Zn, reduces bone malformations (Nguyen *et al.* 2008; Matsumoto *et al.* 2009). In rainbow trout (*Onchorhynchus mykiss*) juveniles, a greater amount of organic amino acid-chelated Mn accumulated in the whole body than inorganic Mn sulphate (Apines, Satoh, Caipang, Kiron, Watanabe & Aoki 2004), and a lower dietary content is required to fulfil the Mn requirements of this species with organic Mn (7 mg amino acid-chelated Mn per kg) than inorganic Mn (14 mg Manganese sulphate per kg) (Satoh, Apines, Tsukioka, Kiron, Watanabe & Fujita 2001).

These previous studies on juveniles suggest that the optimum dietary levels of Se, Zn and Mn may depend on the molecular form supplemented since the form may affect their absorption and retention. This may be particularly important for marine fish larvae in relation to the limited digestion and absorption capacity in comparison to juveniles. Moreover, in larval microdiets the high risk of leaching and oxidation of these micronutrients needs to be minimized. However, up to now no studies have compared the effect of different delivery forms for minerals in early weaning diets for marine fish larvae. Apart from organic and inorganic forms, nanotechnology is now being studied in animal nutrition since the nanometre dimension offers a great specific surface area, a high surface activity with many active centres, and extreme catalytic efficiency (Gao & Hiroshi 2005). Thus, nanoparticles differ from bulk material in their chemical and physical properties and present novel and interesting behaviours in their interaction with biological matter. They are capable to cross biological barriers (Castellini, Ruggeri, Mattioli, Bernardini, Macchioni, Moretti & Collodel 2014; Coccini, Gornati, Rossi, Signoretto & Vanetti 2014), are rapidly taken up by the cells and present a higher bioavailability than mineral salts (Hilty, Arnold, Hilbe, Teleki, Knijnenburg, Ehrensperger, Hurrell, Pratsinis, Langhans & Zimmermann 2010). In terrestrial animals, nanoparticles are transported rapidly and have higher absorption efficiencies than mineral salts (Liao, Hung, Jan, Yeh, Ho & Hwang 2010).

The objective of this study was to assess early weaning diets for gilthead seabream larvae supplemented with Se, Zn and Mn in organic, inorganic and nanoparticle forms to determine how effectively they are in promoting larval performance and bone development. For that purpose, basal early weaning formulations previously developed for seabream (Saleh *et al.* 2014) were supplemented with Se, Zn and Mn in levels and ratios found in copepods in those different delivery forms.

Materials and methods

Experimental animals and trial conditions

Larvae were obtained from natural spawns from the gilthead sea bream (*Sparus aurata*) broodstock of Grupo de Investigación en Acuicultura (GIA) (Las Palmas de Gran Canaria, Spain). Larvae (initial total length 5.10 ± 0.43 mm, dry body weight 0.12 ± 0.03 mg, mean $\pm$ SD) previously fed rotifers (*Brachionus plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until 20 dah were randomly distributed in 12 experimental tanks at a density of 2100 larvae in each tank (10.5 larvae L^{-1}) and were fed one of the experimental diets tested in triplicate for 24 days, at a water temperature of 19.5–20°C. All tanks (200-L, light grey colour cylinder fibreglass tanks) were supplied with filtered seawater (37 g L^{-1} salinity) at an increasing rate of 0.4–1 L min^{-1} 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^{-1}), attaining 5–8 g L^{-1}

dissolved O_2, saturation ranging between 60% and 80%.

Diets and feeding

Four isonitrogenous and isolipidic experimental microdiets (Pellet size <250 μm) based on squid meal and krill oil with gelatin as a binder, attractants (Kanazawa, Koshio & Teshima 1989), and vitamin and mineral premixes (Teshima, Kanzawa & Sakamoto 1982) lacking Se, Zn and Mn were formulated and produced according to Saleh *et al.* (2014). The control diet (Diet C) was not supplemented with the target minerals and their levels (86 mg kg^{-1} Zn, 1.9 mg kg^{-1} Se and 3.3 mg kg^{-1} Mn) were lower than the minimum content of copepods found in the literature (120 mg kg^{-1} Zn (Fujita 1972); 3 mg kg^{-1} Se and 8 mg kg^{-1} Mn (Hamre, Mollan *et al.* 2008)). The other three diets were supplemented with Zn, Se and Mn to obtain similar levels in the diets, reaching copepod levels: Diet O (organic minerals) was supplemented with peptide-chelated Mn and Zn (Mn or Zn-Bioplex, Alltech, Lexington, KY, USA) and Se-yeast (Alltech); Diet I was supplemented with MnSO4, ZnSO4 and NaSe; and Diet N was supplemented with Mn, Zn and Se in nanoparticle form. Nanoparticles were kindly supplied by Colorobbia SpA (Montelupo Fiorentino, Firenze, Italy) and were produced with a 'bottom-up' synthesis by co-precipitation in water of metal-organic precursors. Diets were formulated according to the information provided by the mineral suppliers.

Proximate and mineral composition of the diets is shown in Table 1. Diets were supplied manually every 45 min from 9:00 to 19:00 at a rate of 2.5–3.5 g $tank^{-1}$. 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, CA, USA).

Table 1 Ingredients, analysed proximate composition and mineral contents in the experimental microdiets for 20 dah gilthead seabream larvae. Diet C: control without supplementation of Mn, Se and Zn; Diet O: organic Mn-Bioplex, Se-yeast and Zn-Bioplex (Alltech, Lexington, KY, USA); Diet I: inorganic MnSO4, ZnSO4 and NaSe; Diet N: Supplementation of Mn, Se and Zn as nanometals

Ingredients %	Diet C	Diet O	Diet I	Diet N
Squid powder*	71.1	71.1	71.1	71.1
Krill oil†	10	10	10	10
Gelatine	3	3	3	3
Taurine	1	1	1	1
Attractants‡	3	3	3	3
Vitamin Premix§	6.15	6.15	6.15	6.15
Basal Mineral Premix¶	4.5	4.5	4.5	4.5
Manganese**	0	0.0065	0.0030	0.0030
Zinc††	0	0.0224	0.0148	0.0840
Selenium‡‡	0	0.1304	0.0004	0.0003
Analysed composition				
Lipid (%dw)	16.16	17.14	15.98	16.57
Moisture (%)	8.71	8.58	8.66	8.21
Ash (%dw)	6.04	6.2	5.76	5.31
Protein (%dw)	68.52	69.10	71.78	71.12
Selenium (mg kg^{-1})	1.9	4.9	3.6	5.4
Zinc (mg kg^{-1})	86	110	110	96
Manganese (mg kg^{-1})	3.3	15	9.4	9.8

*Rieber & Son, Bergen, Norway.

†Qrill, high phospholipids, Aker BioMarine, Fjordalléen, Norway.

‡Attractants supplied per 100 g diet: inosine-5-monophosphate, 500 mg; betaine, 660 mg; L-serine, 170 mg; L-phenylalanine, 250 mg; DL-alanine,500 mg; L-sodium aspartate, 330 mg; L-valine, 250 mg; glycine, 170 mg; Tyropsina, 170 mg.

§Vitamins supplied per 100 g diet: cyanocobalamin 0.03 mg; astaxanthin 5.0 mg; folic acid 5.4 mg; pyridoxine-HCl 17.2 mg; thiamin 21.7 mg; riboflavin 72.5 mg; calcium-pantothenate 101.5 mg; p-aminobenzoic acid 145.0 mg; nicotinic acid 290.1 mg; myo-inositol 1450.9 mg; retinol acetate 0.2 mg; ergocalcipherol 3.6 mg; menadione 17.3 mg; alfa-tocopheryl acetate 150.0 mg.

¶Minerals supplied per 100 g diet: NaCl, 215.133 mg; MgSO4.7H2O, 677.545 mg; NaH2PO4.H2O, 381.453 mg; K2HPO4, 758.949 mg; Ca(H2PO4).2H2O, 671.610 mg; C3H5 O3.0·5Ca, 1617.210 mg; Al2(SO4)3.6H2O, 0.693 mg; KI, 0.74 mg; CoSO4.7H2O, 10.706 mg.

**Manganese sources O: peptide chelated Mn-Bioplex (Alltech, Lexington, KY, USA); I: MnSO4.H2O; N: Nannoparticulated Mn.

††Zinc sources O: peptide chelated Zn-Bioplex (Alltech, Lexington, KY, USA), I: ZnSO4.7H2O, N:Nanoparticulated Zn.

‡‡Selenium sources O: Se-Yeast (Alltech, Lexington, KY, USA), I: NaSe, N: nanoparticulated Se.

Larval performance

Growth was determined by measuring dry body weight (105°C until constant weight) and total length (Profile Projector V-12A; Nikon, Tokyo, Japan) of 30 fish per tank at the beginning, in the middle (30 dah) and at the end of the trial. Final survival was calculated by individually counting all the alive 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 per 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 h, washed with distilled water, sampled and kept at 80°C to analyse biochemical composition.

Chemical analysis

Moisture (AOAC 1995), protein (AOAC 1995) and crude lipid (Folch, Lees & Stanley1957) contents of larvae and diets were analysed. 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, Arakawa, Takeuchi, Haroun and Watanabe (1992) and identified by comparison with previously characterized standards and GLC-MS. Mineral analysis was conducted in NIFES (Bergen, Norway) according to the method of Julshamn, Lundebye, Heggstad, Berntssen and Boe (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 was measured by collision/reaction ICP-MS (Thermo Scientific, Cheshire, UK) using argon and hydrogen as carrier gases.

Whole mount staining for skeleton studies

To determine the presence of skeletal anomalies and mineralization, 150 larvae per treatment were sampled at 34 dah (earliest moment to determine complete mineralization in seabream larvae), fixed and stored in buffered (10% phosphate) formaldehyde after a light sedation with 10% clove oil solution. Staining procedures with alizarin red were conducted to evaluate the skeletal anomalies and vertebral mineralization following methods (Izquierdo, Scolamacchia, Betancor, Roo, Caballero, Terova & Witten 2013) modified from previous studies (Vandewalle, Gluckmann & Wagemans 1998). The effects of the different treatments on skeletal mineralization were analysed considering the average number of mineralized vertebrae in each size class and the ossification degree expressed as the average percentage of individuals with a complete mineralization of the vertebral column (Izquierdo *et al.* 2013).

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 ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS, Chicago, IL, USA). All values presented as percentage (skeletal anomalies, mineralization of the column, total survival and activity test survival) were arc cosine transformed before performing any statistical test.

Results

All experimental microdiets were well accepted by the larvae and no significant differences were found by image analysis of the guts. After 24 days of feeding, larval weight had increased over sixfold and growth of larvae fed Diet C without Se, Zn and Mn supplementation was the lowest ($P < 0.05$) in terms of total length (Fig. 1). Adding organic (Diet O), inorganic (Diet I) and nanometals

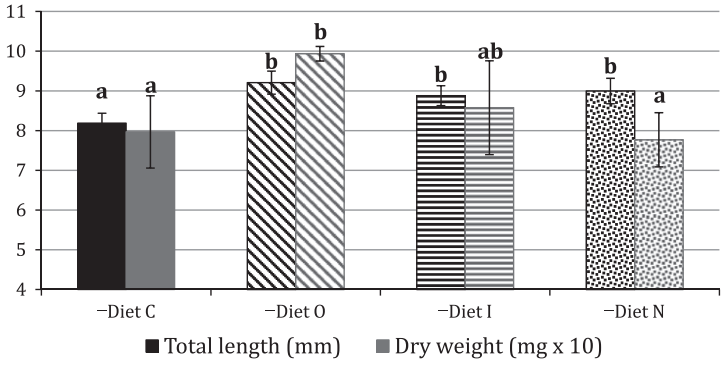

Figure 1 Total length (mm) and dry body weight (mg) of 44 dah gilthead seabream larvae fed the experimental microdiets supplemented with different sources of Se, Zn and Mn for 24 days. Mean and standard deviation, different letters among columns indicate significant differences ($n = 3$, $P < 0.05$).

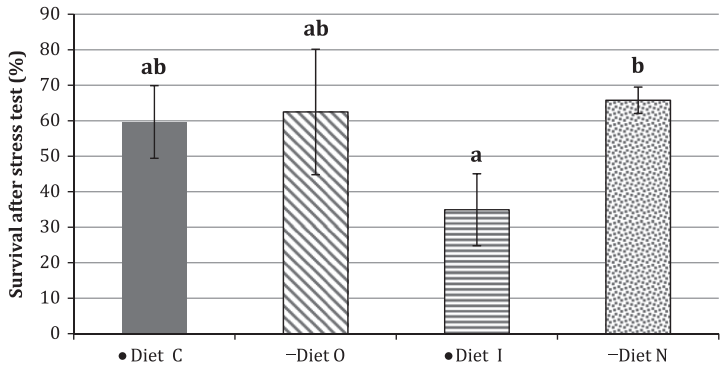

Figure 2 Survival after air exposure of 44 dah gilthead seabream larvae fed for 24 days the experimental microdiets supplemented with different sources of Se, Zn and Mn. Mean and standard deviation, different letters among columns indicate significant differences ($n = 3$, $P < 0.05$).

(Diet N) significantly ($P < 0.05$) improved total larval length, whereas body weight was only significantly ($P < 0.05$) improved by supplementing the diet with organic Se, Zn and Mn (Fig. 1). Feeding the larvae with inorganic minerals was associated with the lowest survival after air exposure, whereas feeding nanometals significantly ($P < 0.05$) increased larval survival in comparison to those fed inorganic minerals (Fig. 2). At the end of the trial, survival of 44-dah larvae was significantly ($P < 0.05$) higher in fish fed Diet C without supplementation of Se, Zn or Mn (22.19%) than fish fed organic (Diet O, 7.22%), inorganic (Diet I, 7.46%) or nanoparticulate minerals (Diet N, 8.84%). Moreover, the survival rate at the end of the trial correlated negatively to the dietary Mn and, particularly, Zn contents (Fig. 3).

Bone mineralization was markedly affected by the diets as illustrated in Plate 1. The effects of the different treatments on bone mineralization were analysed considering the average number of mineralized vertebrae in each given size class (Fig. 4), and the average percentage of individuals with a complete mineralization of the vertebral column at 44 dah (Fig. 5). Considering the number of mineralized vertebrae from all the larvae studied (150 larvae of 6–14 mm), the highest mineralization was found in fish fed organic minerals (Diet O), followed by those fed nanometals (Diet N) (Fig. 4). Further analysis of each given size class showed that in the smallest size class (6–8 mm) larvae fed diet O showed a significantly ($P < 0.05$) higher number of mineralized vertebrae than those fed diet I. Despite significant differences were found only at a 6–8-mm fish size, a similar tendency was found for larvae of size class 8–10 mm, whereas in the larger size classes (10–12 and 12–14 mm) larvae fed diet N showed the highest mineralization (Fig. 4). In most size classes, the lowest number of mineralized vertebrae was found in fish fed either inorganic minerals (Diet I) or a non-supplemented diet (Diet C) (Fig. 4). In line with these findings, the ossification degree was statistically significant ($P < 0.05$) and highest in fish fed diet O, followed by diet N (Fig. 5), whereas the lowest percentage of larvae with a complete vertebral mineralization was found in fish fed diets C and I.

A high incidence of minor anomalies, such as those in branchial arches, was found in 44 dah gilthead seabream larvae, particularly in fish fed

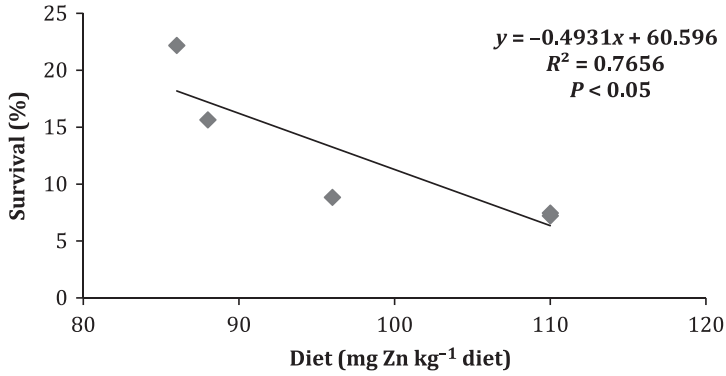

Figure 3 Correlation between dietary Zn content and survival rates of 44 dah gilthead seabream larvae fed the experimental microdiets supplemented with different sources of Se, Zn and Mn for 24 days.

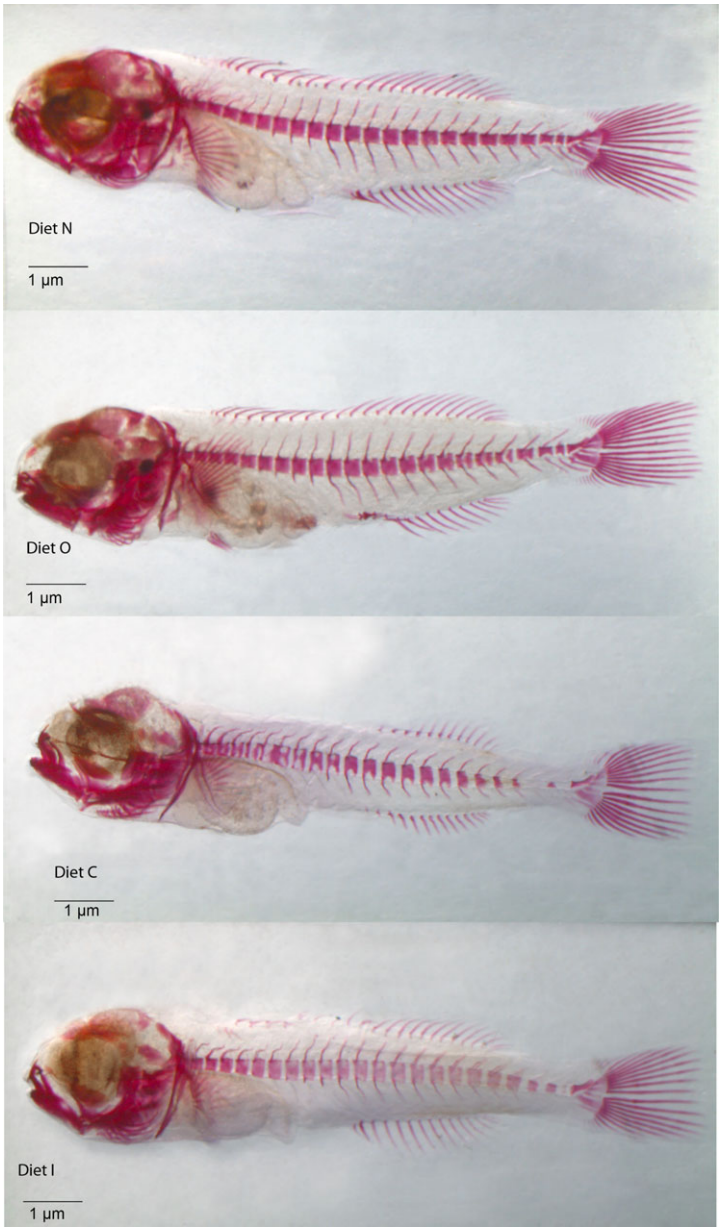

Plate 1 Images of alizarine red staining of 44 dah gilthead seabream larvae fed the experimental microdiets supplemented with different sources of Se, Zn and Mn for 24 days, showing the most mineralized larvae for each treatment. Diet C: control without supplementation of Mn, Se and Zn; Diet O: organic Se, Zn and Mn; Diet I: inorganic Se, Zn and Mn; Diet N: Mn, Se and Zn nanometals.

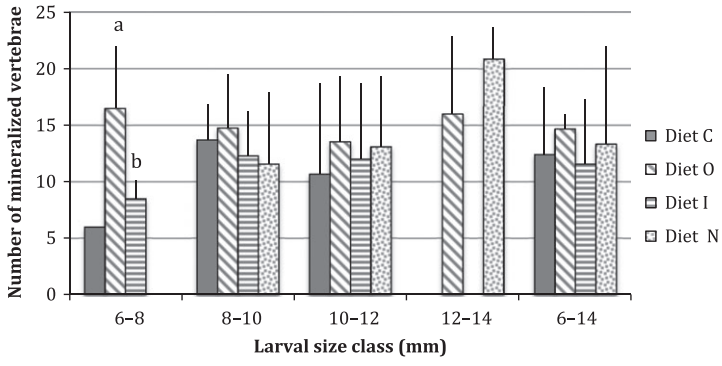

Figure 4 Average number of mineralized vertebrae for each size class of 44 dah gilthead seabream larvae fed the experimental microdiets supplemented with different sources of Se, Zn and Mn for 24 days (different letters for a given size class denote significant differences, $n = 150$).

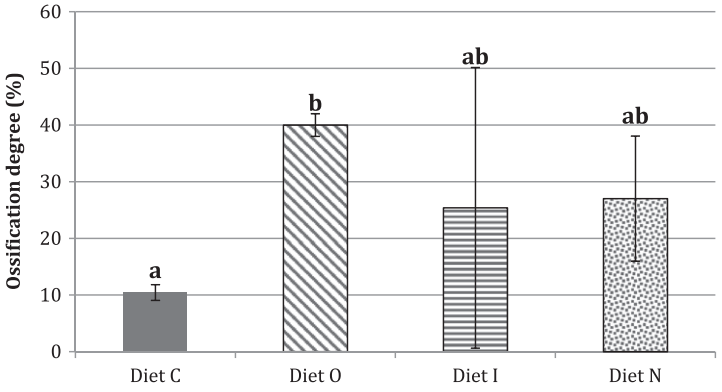

Figure 5 Bone mineralization frequency expressed as the average percentage of individuals with a completely mineralized vertebral column in 44 dah gilthead seabream larvae fed the experimental microdiets supplemented with different sources of Se, Zn and Mn for 24 days. Mean and standard deviation, different letters among columns indicate significant differences ($n = 3$, $P < 0.05$).

diet C (Fig. 6). Supplementation with Se, Zn and Mn in inorganic (Diet I) and nanometal (Diet N) forms tended to reduce these anomalies, but only by adding organic minerals could they be significantly reduced (Fig. 6). Moreover, feeding inorganic Se, Zn and Mn significantly increased vertebral body anomalies (Fig. 7) and, when distributed by different regions, a higher percentage of bone anomalies in the pre-hemal region was found in larvae fed inorganic minerals (Fig. 7). No significant differences were found in other types of anomalies.

Regarding fish biochemical composition, although dietary lipid content and fatty acid composition did not differ among the experimental diets (Table 2), feeding organic Se, Zn and Mn significantly increased larval lipid contents, mainly due to the increase in monounsaturated fatty acids, such as 18:1n-9, saturated fatty acids, such as 18:0, and n-6 fatty acids, such as 18:2n-6, 18:3n-6 and 20:2n-6 (Table 3). In contrast, feeding inorganic minerals reduced larval lipid contents in comparison to fish fed control diet, due to a reduction in 16:1n-7, 16:1n-5, 18:1n-7,

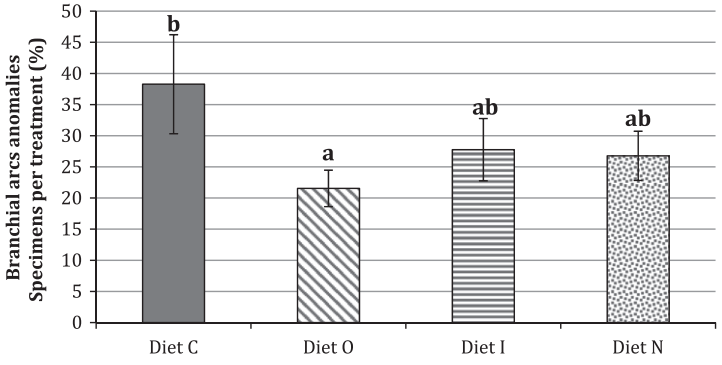

Figure 6 Branchial arch anomalies found in 44 dah gilthead seabream larvae fed the experimental microdiets supplemented with different sources of Se, Zn and Mn for 24 days. Mean and standard deviation, different letters among columns indicate significant differences ($n = 3$, $P < 0.05$).

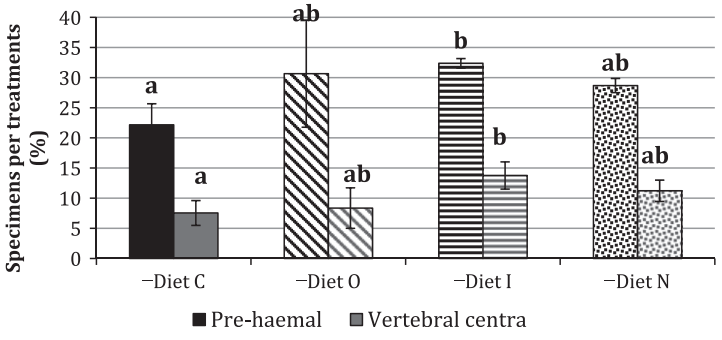

Figure 7 Total anomalies in the pre-hemal region and anomalies in vertebral centra found in 44 dah gilthead seabream larvae fed the experimental microdiets supplemented with different sources of Se, Zn and Mn for 24 days. Mean and standard deviation, different letters among columns indicate significant differences ($n = 3$, $P < 0.05$).

Table 2 Main fatty acid composition (% total identified fatty acids) of the experimental microdiets supplemented with different sources of Se, Zn and Mn fed to gilthead seabream larvae

Fatty acids	Diet C	Diet O	Diet I	Diet N
14:0	9.38	9.94	9.50	9.58
14:1n-5	0.18	0.22	0.19	0.19
14:1n-7	0.16	0.16	0.15	0.15
16:0	22.62	24.32	23.50	23.68
16:1 n-7	5.83	6.00	5.90	5.92
16:1n-5	0.23	0.24	0.24	0.24
16:2n-6	0.49	0.50	0.49	0.50
16:2n-4	1.24	1.13	1.16	1.22
18:0	2.71	3.05	3.16	3.10
18:1 n-9	10.18	10.76	10.19	10.28
18:1 n-7	5.85	5.99	5.86	5.91
18:1 n-5	0.28	0.28	0.27	0.28
18:2n-9	0.06	0.05	0.05	0.06
18:2 n-6	1.43	1.51	1.38	1.41
18: 3n-6	0.21	0.21	0.21	0.21
18:3 n-3	0.99	0.96	0.93	0.93
18:3n-1	0.01	0.01	0.01	0.01
18:4 n-3	2.80	2.43	2.52	2.49
18:4 n-1	0.04	0.04	0.04	0.04
20:0	0.11	0.12	0.12	0.12
20:1 n-9	0.12	0.13	0.13	0.13
20: 1n-7	2.68	2.91	3.02	3.04
20: 1n-5	0.32	0.33	0.32	0.33
20: 2n-9	0.01	0.01	0.01	0.01
20:2 n-6	0.12	0.13	0.14	0.13
20:3 n-6	0.04	0.04	0.04	0.04
20:4 n-6	0.66	0.67	0.71	0.70
20: 3n-3	0.24	0.25	0.28	0.27
20:4 n-3	0.30	0.27	0.28	0.27
20:5 n-3	14.19	12.40	13.06	12.95
22:1 n-11	0.87	0.88	0.85	0.85
22:1 n-9	0.16	0.17	0.16	0.16
22:4 n-6	0.10	0.10	0.10	0.10
22:5 n-6	0.14	0.14	0.16	0.15
22:5 n-3	0.41	0.37	0.39	0.38
22:6 n-3	12.65	11.14	12.33	12.02
Saturated	35.37	37.99	36.84	37.03
Monounsaturated	26.91	28.14	27.36	27.57
n-3	32.51	28.69	30.68	30.20
n-6	3.19	3.31	3.23	3.24
n-9	10.55	11.14	10.56	10.67

20:1n-7 and 20:1n-5 (Table 3). Feeding nanometals further reduced larval lipid contents without affecting the proportions of different fatty acids (Table 3). Larval lipid contents correlated significantly to the larval dry body weight (Fig. 8).

Discussion

Despite the importance of minerals in fish metabolism and organ development, information regarding mineral nutrition in marine fish larvae is very limited (Hamre *et al.* 2013). Feeding 20–44 dah gilthead sea bream larvae weaning diets without Se, Zn and Mn supplementation was associated with poor growth, low bone mineralization and a high incidence of anomalies in the branchial arches, denoting a deficiency in one or more of these minerals. Dietary Se promotes growth in juveniles of several fish species (Liu, Wang, Ai, Mai & Zhang 2010; Zhu, Chen, Liu, Yang, Liang & Tian 2012; Le & Fotedar 2013; Lin 2014). However, the low Se levels (2 mg kg^{-1}) in the diet without Se supplementation did not seem to be the cause for the low larval growth since in a previous study of feeding larval gilthead seabream under diets and conditions similar to those in the present trial, increasing Se levels from 1.7 to 6 mg kg^{-1} did not significantly affect larval growth (Saleh *et al.* 2014). In agreement with this result, an increase in Se from 0.73 to 8 mg kg^{-1} did not affect growth in rainbow trout juveniles (Rider, Davies, Jha, Fisher, Knight & Sweetman 2009). Low Zn levels (86 mg kg^{-1}) did not seem to be responsible for the low gilthead seabream growth since Zn supplementation through rotifers (119–306 mg kg^{-1}) for larval red seabream (*Pagrus major*) (Nguyen *et al.* 2008) or microdiets (85–100 mg kg^{-1}) for gilthead seabream (Izquierdo, unpublished data), respectively, did not improve growth. However, Mn supplementation (10–43 mg kg^{-1}) significantly improved growth in larvae of red sea bream (Nguyen *et al.* 2008) and juveniles of several species (Pan, Zhu, Xie, Lei, Han & Yang 2008; Tan, Xie, Luo, Lin, Zhao & Xi 2012; Liu, Ai, Mai, Zhang, Zhang & Zheng 2013), altering bone formation (Watanabe *et al.* 1997; Satoh *et al.* 2001), which is in line with the lower bone mineralization obtained in larval sea bream fed non-supplemented diets in the present study. Moreover, the gilthead seabream larvae used in the present study were previously reared on enriched rotifers, which were probably low in Mn since the absorption and retention of this mineral in rotifers is much less efficient than for Zn or Se (Nordgreen *et al.* 2013). Therefore, insufficient Mn could contribute for the low mineralization, and, particularly, increased anomalies obtained in the larvae fed the non-supplemented diet.

The inclusion of Zn, Mn and Se in an organic form (amino acid-chelate) as a source of trace elements for *Sparus aurata* larvae promoted maximum growth, increased larval lipid reserves, enhanced early mineralization and prevented branchial arch

Table 3 Total lipid level (%dw) and fatty acid composition (% total identified fatty acids) of gilthead seabream larvae fed the experimental microdiets supplemented with different sources of Se, Zn and Mn for 24 days. Different letters among values in the same line indicate significant differences ($n = 3$, $P < 0.05$)

	Initial larvae	Diet C	Diet O	Diet I	Diet N
Fatty acids/Total lipid	13.33 ± 0.37	16.93 ± 0.15^{c}	19.60 ± 0.26^{d}	15.69 ± 0.21^{b}	13.47 ± 0.48^{a}
20:4 n-6	4.53 ± 0.45	1.47 ± 0.31	1.57 ± 0.29	1.78 ± 0.24	1.57 ± 0.19
20:5 n-3	4.29 ± 0.36	10.41 ± 0.43^{a}	8.41 ± 0.64^{c}	9.61 ± 0.63^{b}	10.53 ± 0.74^{a}
22:6 n-3	16.80 ± 0.62	22.08 ± 0.95^{a}	17.55 ± 0.80^{b}	21.37 ± 0.85^{a}	20.52 ± 0.94^{a}
Saturated	31.95 ± 1.01	35.61 ± 1.13	37.11 ± 1.06	37.02 ± 1.19	36.33 ± 1.31
Monounsaturated	22.66 ± 0.89	21.49 ± 0.97^{b}	25.03 ± 0.91^{a}	21.38 ± 0.79^{b}	22.28 ± 0.90^{b}
n-3	25.13 ± 0.94	35.86 ± 1.19^{a}	28.99 ± 0.99^{b}	33.85 ± 1.08^{a}	34.20 ± 1.13^{a}
n-6	15.26 ± 0.53	3.35 ± 0.27^{b}	5.15 ± 0.40^{a}	3.80 ± 0.23^{b}	3.56 ± 0.28^{b}
n-9	14.38 ± 0.61	11.59 ± 0.45^{b}	15.35 ± 0.87^{a}	12.44 ± 0.59^{b}	12.39 ± 0.82^{b}

anomalies. In contrast, feeding larval sea bream with inorganic forms of these minerals was less effective than organic minerals in improving larval weight or bone mineralization in comparison to the non-supplemented diet. Moreover, in these larvae the lipid content was even lower than in the non-supplemented fish, as was resistance to stress, and the fish showed even higher bone anomalies in vertebral centra and in the pre-hemal region. Dietary organic aa-chelated minerals seem to be better utilized than inorganic forms (Wang & Lovell 1997; Satoh *et al.* 2001; Apines *et al.* 2004; Le & Fotedar 2014). For instance, organic Se (Met-Se and yeast Se) has been found to improve Se digestibility, absorption and retention in comparison to inorganic Se (Na-selenite) (Wang & Lovell 1997; Le & Fotedar 2014). Furthermore, bioavailability of organic Zn (Zn-methionine or Zn-propionate) has been found to be better (ZnO or ZnSO4·7H2O) (Spears 1989; Wedekind, Hortin & Baker 1992; Hahn & Baker 1993) and to be a better growth promoter (Paripatananont & Lovell 1995) than inorganic Zn. Similarly, chelated Mn is better absorbed and retained than inorganic Mn, denoting the higher bioavailability of the former (Apines *et al.* 2004), even in the presence of dietary inhibitors (Satoh *et al.* 2001) as there is a lower chance of forming insoluble precipitates. Moreover, Mn is closely associated with lipid metabolism (Watanabe *et al.* 1997), and dietary Mn promotes lipid deposition in the whole body (Liu *et al.* 2013), which is in agreement with the higher lipid larval content found here. Particularly the end products of lipid biosynthesis, 18:0 and 18:1n-9, were increased in seabream larvae fed organic minerals. Mn concentration in the skeleton also increases with dietary Mn (Gatlin & Wilson 1984; Wang & Zhao 1994; Lorentzen, Maage & Julshamn 1996; Maage, Lygren & El-Mowafi 2000; Pan *et al.* 2008). An increase in organic Mn also enhanced Zn deposition in the skeleton (Satoh *et al.* 2001) and Ca and P deposition in the

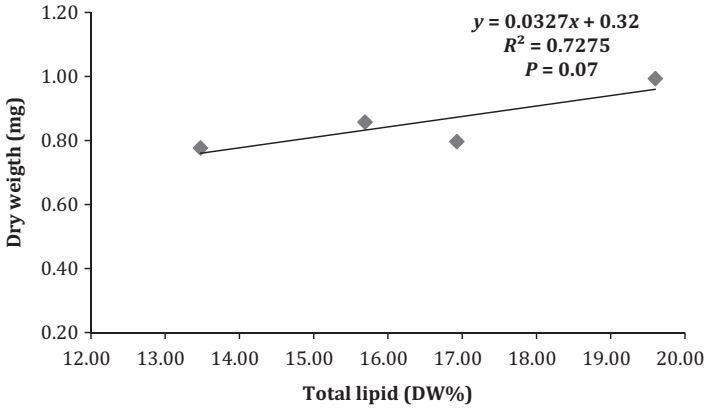

Figure 8 Correlation between larval lipid contents and dry body weight in 44 dah gilthead seabream larvae fed the experimental microdiets supplemented with different sources of Se, Zn and Mn for 24 days.

whole body (Ye, Tian, Yang, Liang, Niu & Liu 2009). This could explain the higher mineralization found in larval gilthead seabream fed organic minerals in the present study. Zn has a potent stimulatory effect on bone formation and mineralization (Yamaguchi, Oishi & Suketa 1987), activating aminoacyl-tRNA synthetase in osteoblastic cells, stimulating cellular protein synthesis (Yamaguchi 1998) and inhibiting osteoclastic bone resorption (Yamaguchi 1998). It can affect bone mineralization either directly, as a divalent cation acting on nucleation and mineral accumulation, or indirectly, as a cofactor of enzymes involved in this process, such as alkaline phosphatase (Gómez, Rizzo, Pozzi-Mucelli, Bonucci & Vittur 1999). Particularly, organic Zn markedly increases alkaline phosphatase activity in rainbow trout in comparison to inorganic Zn (Kucukbay, Yazlak, Sahin, Tuzcu, Cakmak, Gurdogan, Juturu & Sahin 2006). Therefore, Zn and Mn are essential for the development and mineralization of the skeleton of gilthead seabream during late metamorphosis and may have played an important role in preventing branchial arch anomalies found in larvae supplemented with organic minerals. These results are in line with the reduced branchial arch and spine anomalies found in larval red sea bream fed Artemia supplemented with both Zn and Mn (Nguyen *et al.* 2008). These skeletal elements are endochondral bones that develop from cartilaginous precursors, which are destroyed by reactive oxygen species when fish diets do not contain a sufficient amount of antioxidant nutrients (Izquierdo *et al.* 2013). Therefore, in the present study, even before bone mineralization, supplementation of organic minerals with antioxidant properties may have protected the cartilaginous anlagen of endochondral branchial arches and avoided malformations. Moreover, feeding inorganic minerals increased anomalies in vertebral centra, which are anosteocytic bones that mineralize directly without a cartilaginous precursor. The vertebral centra anomalies may be related to the lower mineralization found in this fish (Izquierdo *et al.* 2013) caused by an imbalance in mineral deposits in the bones or by reduced alkaline phosphatase activity (Kucukbay *et al.* 2006). The positive effects of organic minerals in the present study could not be related to the slightly higher Mn content in this diet (15 mg kg^{-1}) in comparison to diets containing inorganic and nanoparticulated minerals (9.4 and 9.8 mg kg^{-1}), since larvae fed the latter diet showed a very good mineralization pattern with a number of mineralized vertebrae, ossification degree and bone anomalies very similar to the larvae fed organic minerals. Indeed, Mn contents in copepods, the natural food of gilthead seabream larvae, are in the range of 8–29 mg kg^{-1} (Hamre, Mollan *et al.* 2008; Hamre, Srivastava *et al.* 2008) and requirements have been found to be around 6–13 mg $kg^{-1}$for other species (Pan *et al.* 2008; Tan *et al.* 2012).

Adding Zn, Mn and Se in the form of nanometals did not enhance growth or larval lipid contents and did not prevent branchial arch anomalies, in comparison to the control diet. However, it markedly improved stress resistance and bone mineralization as compared with inorganic minerals. Dietary minerals at the nanoscale level cross into cells more readily than larger counterparts and this accelerates their assimilation process in fish (Acosta 2009; Bouwmeester, Dekkers, Noordam, Hagens, Bulder, de Heer & Sips 2009). Previous studies have shown the good bioavailability of nanoselenium and its high effectiveness in preventing oxidative stress (Wang, Yan & Fu 2013). Other studies have demonstrated that dietary selenium nanoparticles improve growth and muscle Se concentration (Zhou, Wang, Gu & Li 2009). Indeed, Se seems to be essential for overcoming different types of stress situations in several species (Rider *et al.* 2009; Saleh *et al.*2014). Increase in dietary Se has been also found to upregulate the *osteocalcin* gene (Saleh *et al.* 2014), an effective molecular marker of bone mineralization in gilthead seabream (Saleh, Betancor, Roo, Benítez-Dorta, Zamorano & Izquierdo 2015a,b). The positive effect of nanominerals on stress resistance does not seem to be related to the slightly higher Se content (5.4 mg kg^{-1}) in comparison to the diet with inorganic minerals (3.6 mg kg^{-1}), since in previous studies with the same fish species and diet formulation, the increase from 4 to 6 mg kg^{-1} Se did not affect this parameter (Saleh *et al.* 2014). No information could be found in the literature regarding the use of nanoscale Zn or Mn.

In general, the addition of minerals in organic, inorganic or nanometal forms markedly reduced fish survival. These results suggest that one or more of these minerals have a negative effect. The low survival did not seem related to high Se, since the elevation of Se from 2 to 6 mg kg^{-1} did not affect larval sea bream survival in previous studies

with very similar conditions to the present trial (Saleh *et al.* 2014). Furthermore, growth reduction is the most sensitive indicator of Se excess (Lin & Shiau 2005; Jaramillo, Peng & Gatlin 2009), and seabream larvae growth in the present study was good despite the low survival. Excess Se levels are higher than 20 mg kg^{-1} in diets for juvenile yellowtail kingfish (*Seriola lalandi*) (Le & Fotedar 2014). Regarding Mn, the least toxic of the trace elements (McDowell 2003), only doses of 1000 mg kg^{-1} reduced survival in grouper juveniles (*Epinephelus coioides*) (Ye *et al.* 2009), whereas the dietary Mn levels in the present study were much lower (9.4–15 mg kg^{-1}). Several authors have discussed the potentially toxic effect of Zn (Clearwater, Farag & Mayer 2002; Luo, Tan, Zheng, Chen & Liu 2011). In gilthead sea bream juveniles, an increase in Zn dietary levels from 60.9 to 900 mg kg^{-1} significantly reduced weight (Serra, Isani, Cattani & Carpenè1996; Carpenè, Serra, Manera & Isani 1999). Moreover, feeding high levels of inorganic Zn seems to affect fish health as the alternative complement pathway activity (ACH50) in juvenile grouper, (*Epinephelus malabaricus*) (Houng-Yung, Yu-Chun, Li-Chi & Meng-Hsien 2014) and the occurrence of macrophage aggregates in gilthead seabream juveniles (Manera, Serra, Isani & Carpené 2000) were reduced by 50%. However, high Zn dietary levels did not affect survival in juveniles of yellow catfish (*Pelteobagrus fulvidraco*) (76.36 mg kg^{-1}, Luo *et al.* 2011), turbot (1000 mg kg^{-1}, Overnell, Fletcher & McIntosh 1988), or rainbow trout (88 mg, Kock & Bucher 1997). In the present study, although dietary Zn levels (86–110 mg kg^{-1}) correlated significantly to larval survival, these levels were in the range of those obtained in enriched rotifers (33–245 mg kg^{-1}), Artemia (119–306 mg kg^{-1}) or copepods (340–570 mg kg^{-1}) without negative consequences for larval red sea bream, cod or greater amberjack respectively (Nguyen *et al.* 2008; Yamamoto *et al.* 2013). Interestingly, increase in rotifers content in other minerals such as iodine (I) up to the levels found in copepods were found to be toxic for cod larvae, suggesting that I availability or nutrient interactions, rather than excess levels of this mineral, are responsible for the toxicity (Penglase *et al.* 2013). Since Zn has been found to reduce Fe and Cu retention in different tissues (Luo *et al.* 2011), the potential toxic effect of Zn could be related to the levels of other nutrients. Further studies are being conducted to elucidate the potentially negative effect of these minerals for marine fish larvae.

In conclusion, early weaning diets in gilthead seabream based on squid meal and krill oil must be supplemented with one or more of the antioxidant minerals Zn, Mn and Se, to promote larval growth and bone mineralization and prevent skeletal anomalies. However, further studies are needed to determine which minerals should be supplemented and the optimal inclusion levels. Organic minerals were more effective than inorganic minerals in promoting fish weight and mineralization and reducing malformations in endochondral bone. However, nanometals can be considered an interesting source of minerals since they promoted mineralization and stress resistance.

Acknowledgments

This work has been partly funded under the EU seventh Framework Programme by the ARRAINA project N288925: Advanced Research Initiatives for Nutrition & Aquaculture. Acknowledgement is also given to CIHEAM for a Master's Student grant to Wafa Ghrab.

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