



Effect of Dietary Manganese and Zinc Levels on Growth and Bone Status of Senegalese Sole (*Solea senegalensis*) Post-Larvae

Michael N. Viegas^{1,2,3} · Maria Antonia Salgado² · Carla Aguiar³ · Agostinho Almeida⁴ · Paulo Gavaia⁵ · Jorge Dias¹

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Abstract

Essential dietary trace elements, such as zinc (Zn) and manganese (Mn), critically influence a wide range of physiological, metabolic, and hormonal processes in fish larvae and post-larvae. Despite their importance for normal fish growth and skeletal development, trace mineral nutrition has not been extensively studied in the early stages of development of fish. Post-larvae of an emergent aquaculture species, Senegalese sole (*Solea senegalensis*), were the subject of this study in order to better understand the effects of diet supplementation of trace minerals upon fish larval development and performance. Sole post-larvae were fed a combination of organic Mn (45 and 90 mg kg⁻¹ feed) and organic Zn (100 and 130 mg kg⁻¹ feed) and survival, growth, mineral deposition rates, and vertebral bone status were assessed. Our results showed that although no significant effect was found on the growth performance of Senegalese sole post-larvae, Mn and Zn supplementation to a commercial microdiet for marine fish larvae at higher dietary levels (Mn at 90 mg kg⁻¹ and Zn at 130 mg kg⁻¹) improved larval survival, decreased the severity of vertebral malformations, and increased the deposition of Mn in bone.

Keywords Manganese · Zinc · *Solea senegalensis* · Post larvae · Growth · Malformations

Introduction

The aquaculture industry must solve a series of challenges throughout the entire production cycle of commercially raised fish in order to achieve its expected growth. One of the major bottlenecks faced by the marine aquaculture industry is the intensive culture of healthy, fast-growing fish larvae and juveniles, with high survival rates and a low incidence of skeletal deformities. The incidence of moderate to severe skeletal anomalies in fish can be high, not only between different

hatcheries but also among different lots within the same hatchery or even within the same batch of eggs [1]. In marine hatcheries, skeletal deformities can affect 7–20% on average of the produced juveniles, whereas occasionally this incidence has been shown to rise to 45–100% [2]. It has been estimated that a 50% reduction of skeletal anomalies could increase the production and profitability of marine hatcheries and save up to €25 million per year [1]. A majority of skeletal anomalies have their onset during bone development in early larval stages [1], although some vertebral curvatures like lordosis and kyphosis and some vertebrae fusion and related anomalies can also develop later in life [3–6]. Skeletal malformations can affect swimming ability and therefore the capability to compete for food, reduce growth rates, increase mortality, and significantly affect animal welfare. The development and severity of skeletal disorders in larval and juvenile fish are linked to inadequate nutrition, unfavorable environmental conditions, and genetic factors which are poorly understood.

Over the last decade, both academia and the industry have devoted a continuous research effort toward the optimization of fish larval nutrition and significant progress has been achieved on the quality of commercial microdiets for marine fish larvae. These advances are reflected not only by a significant enhancement of larval growth performance and survival during weaning but also later in the production of high-quality

✉ Michael N. Viegas
michaelviegas@sparos.pt

¹ SPAROS Lda, Área Empresarial de Marim, Lote C, 8700-221 Olhão, Portugal

² CIIMAR, ICBAS, R. Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

³ ADM Portugal S. A., Zona Industrial de Murte, 3060-372 Cantanhede, Portugal

⁴ LAQV/REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, R. Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

⁵ CCMAR, University of Algarve, Campus Gambelas, 8005-139 Faro, Portugal

juveniles. Nevertheless, there is still room for substantial improvements in microdiets for marine fish larvae. Available literature data shows that virtually all microdiet components have an impact on several aspects of bone development and bone remodeling processes. An inadequate quantitative and qualitative supply of peptide profile in hydrolysates [7], lipid classes (triglycerides, phospholipids) [8, 9], fatty acids ratios [10, 11], vitamins [12, 13], and minerals and trace elements [14] have all been associated to a modulation of bone formation and mineralization.

Trace minerals are often presented as key nutrients for skeletal development, growth, and bone remodeling; however, knowledge on the optimal dietary supply of these micronutrients in marine fish larvae is extremely scarce [1, 15–19]. Difficulties associated to the quantification of dietary mineral requirements in fish, specially at larval stages, include the potential contribution of minerals from the water itself, leaching of minerals from the diet prior to consumption, the limited data on mineral bioavailability, and availability of suitable test diets that have a low concentration of the targeted mineral [1, 20].

Manganese (Mn) and zinc (Zn) are essential trace minerals with important roles in several biological processes, functioning either as cofactors activating many enzymes that form metal–enzyme complexes or as integral parts of certain metalloenzymes in carbohydrate, lipid, and protein metabolism. Mn and Zn also have an antioxidant role as they are part of either Mn- or Cu/Zn-superoxide dismutase [16]. Data on the trace mineral requirements of fish are often generated at the juvenile stage. According to NRC (2011), dietary requirements for Mn in fishes range from 2 to 14 mg kg⁻¹ feed, while that of Zn ranges from 20 to 30 mg kg⁻¹. Fish larval nutrition and feeding features can differ significantly from that of juveniles, that is fish larvae show significantly higher growth rates, lower size feed pellets, and consequently a much higher surface/volume ratio, which causes longer floatability in water before ingestion that in turn makes them more prone to leaching and oxidation. These aspects explain why recommended dietary levels of micronutrients in larval feeds are in general significantly higher. In red seabream larvae, supplementation of enriched *Artemia* nauplii has been shown to enhance growth (from 12 to 43 mg Mn kg⁻¹ dry weight) and to promote normal skeletal development with a reduction of anomalies in neural spines and arches with both 12 to 43 mg Mn kg⁻¹ and 119 to 423 mg Zn kg⁻¹ dry weight [21]. In gilthead seabream larvae (from 20 to 44 days after hatching (DAH)), a simultaneous dietary supplementation with Mn (from 3 to 15 mg kg⁻¹), Zn (from 86 to 110 mg kg⁻¹), and selenium (from 2 to 5 mg kg⁻¹), incorporated in both organic and inorganic chemical forms, led to higher growth, enhancement of early mineralization, and reduction of anomalies in branchial arches [22, 23]. Moreover, in this same study, the survival rate of gilthead seabream larvae was negatively

correlated with the dietary Zn content [22]. Our aim was to further study the influence of Mn and Zn in fish larval nutrition the effect of combined dietary levels of organic Mn (45 and 90 mg kg⁻¹ feed) and organic Zn (at 100 and 130 mg kg⁻¹ feed) on survival, growth, mineral deposition rates, and bone status of an emergent aquaculture species, Senegalese sole (*Solea senegalensis*).

Materials and Methods

Experimental Diets

The trial comprised four dietary treatments (Table 1). All experimental diets were based on a single commercial feed formulation (WINFlat®, SPAROS LDA, Portugal), which was supplemented at the manufacturing stage with both manganese (Mn) and zinc (Zn) in the glycine chelated organic form (B-TRAXIM® 2C, Pancosma, Switzerland). Dietary target levels were as follows: Mn at 45 and Zn at 100 mg kg⁻¹ (diet M45Z100); Mn at 45 and Zn at 130 mg kg⁻¹ (diet M45Z130); Mn at 90 and Zn at 100 mg kg⁻¹ (diet M90Z100); and Mn at 90 and Zn at 130 mg kg⁻¹ (diet M90Z130). A detailed list of raw materials and additives cannot be disclosed due to industrial trade secret. Nevertheless, the formula contains high levels of premium marine and plant protein sources (> 75% of combined crustacean meal, squid meal, marine zooplankton meal, fish hydrolysates, fishmeal, wheat gluten, pea protein concentrate, and fish gelatin), while lipids are provided by marine oils and a vegetable lecithin source.

Diets were produced at SPAROS LDA (Olhão, Portugal). Powder ingredients were initially mixed according to each target formulation in a double-helix mixer, being thereafter ground in a micropulverizer hammer mill (SH1, Hosokawa-Alpine, Germany). The oils were subsequently added and after a moisturizing step, diets were agglomerated through low-shear extrusion (Dominioni Group, Italy). Upon extrusion, diets were dried in a vibrating fluid bed dryer. Dry microparticles were subsequently sieved to the desired size ranges (400–600 and 600–800 μm). Diets were kept at 4 °C until use. Samples of each diet were taken for relevant analytical characterization (Table 1).

Fish Rearing and Sampling

Senegalese sole larvae 20 DAH originating from SAFIESTELA/SEA8 (Estela, Portugal) were reared at the Instituto Português do Mar e da Atmosfera (IPMA) facilities (Olhão, Portugal). Larvae were initially acclimatized to 20 white plastic tanks (0.1 m², 8 L) and maintained in a semi-closed seawater recirculating system with four water renewals per hour. Larval density in each tank was 4000 larvae m⁻² (400 larvae per replicate treatment). Light intensity was

Table 1 Proximate and mineral composition of experimental diets

	M45Z100	M45Z130	M90Z100	M90Z130
Dry matter (DM) (%)	94.2 ± 1.3	94.2 ± 1.5	94.4 ± 1.2	94.0 ± 1.3
Ash (% DM)	8.9 ± 0.4	9.0 ± 0.1	8.8 ± 0.2	8.9 ± 0.1
Crude protein (% DM)	62.8 ± 0.2	62.7 ± 0.2	62.4 ± 0.2	62.9 ± 0.2
Crude fat (% DM)	21.4 ± 0.1	21.4 ± 0.3	20.9 ± 0.0	20.7 ± 0.1
Phosphorus (g kg ⁻¹ DM)	18.6 ± 0.4	18.5 ± 0.4	18.7 ± 0.2	18.7 ± 0.3
Calcium (g kg ⁻¹ DM)	9.9 ± 0.1	9.6 ± 0.1	9.7 ± 0.2	9.9 ± 0.1
Sodium (g kg ⁻¹ DM)	5.9 ± 0.1	5.8 ± 0.1	5.8 ± 0.1	5.9 ± 0.1
Potassium (g kg ⁻¹ DM)	6.9 ± 0.4	6.8 ± 0.6	7.8 ± 0.7	7.3 ± 0.7
Magnesium (g kg ⁻¹ DM)	2.3 ± 0.1	2.2 ± 0.1	2.4 ± 0.1	2.4 ± 0.2
Copper (mg kg ⁻¹ DM)	19.5 ± 0.5	18.8 ± 0.9	18.8 ± 0.2	16.9 ± 0.8
Iron (mg kg ⁻¹ DM)	177 ± 3.0	171 ± 5.9	175 ± 9.2	174 ± 3.2
Manganese (mg kg ⁻¹ DM)	47.9 ± 2.4	46.4 ± 5.2	91.2 ± 11.7	88.4 ± 2.0
Zinc (mg kg ⁻¹ DM)	104 ± 5.7	130 ± 3.3	108 ± 3.7	133 ± 7.5

Values are presented as means ± SD ($n = 2$)

40 lx and light:dark cycles of 3:21 h were used to maximize feed ingestion, since Senegalese sole exhibits a pronounced nocturnal feeding behavior. Fish larvae were initially fed with frozen *Artemia* previously enriched with commercial products. At 32 DAH, the benthic post-larvae were suddenly weaned onto a commercial control diet (WINFlat, SPAROS LDA). At 42 DAH, the fish were randomly distributed in the rearing tanks to establish four dietary treatments in quadruplicate ($n = 4$). Feeding was performed using automatic feeders, where the experimental diets were distributed to the fish in eight meals per day, each meal lasting a period of 2 h following a 1-h break. Water quality parameters were measured daily and adjusted to maintain the temperature at $20 \text{ }^\circ\text{C} \pm 1$, oxygen saturation level above 90%, and salinity at 35 g dm^{-3} . At 60 DAH, the post-larvae were submitted to a live calcein staining (described below) and returned to the tanks.

At the end of the trial (81 DAH), the survival rate (SR) in each replicate treatment was determined and fish for sampling were sacrificed with an overdose of tricaine methanesulfonate (MS222; Sigma). Growth performance parameters, dry weight (DW), standard length (SL), relative growth rate (RGR), and feed conversion ratio (FCR) were also determined ($n = 4$, total per treatment = 160). A total of 60 fish were sampled per replicate treatment for subsequent analysis.

Biochemical Analysis

Analysis of the proximate composition of feeds ($n = 2$) was performed by following the methodologies described by AOAC (2006). The dry matter content was determined after drying samples at $105 \text{ }^\circ\text{C}$ for 24 h. Total ash was analyzed by combustion ($550 \text{ }^\circ\text{C}$ for 6 h) in a muffle furnace (Nabertherm L9/11/B170, Germany). Crude protein ($\text{N} \times 6.25$) was analyzed by a flash combustion technique followed by a gas

chromatographic separation and thermal conductivity detection with a Leco N analyzer (Model FP-528, Leco Corporation, USA). Following an acid hydrolysis step, crude lipid was determined by dichloromethane extraction ($40\text{--}60 \text{ }^\circ\text{C}$) using a Soxtec™ 2055 Fat Extraction System (Foss, Denmark). Gross energy was measured in an adiabatic bomb calorimeter (Werke C2000 basic, IKA, Germany).

Mineral quantification was performed on feed ($n = 2$), whole fish ($n = 4$, total per treatment = 160), and vertebral bone ($n = 4$, total per treatment = 40) samples. For macromineral analysis, dry samples were weighed (50 mg) and digested in 3 mL of nitric acid (HNO_3 , 70%) for 2 h at $95 \text{ }^\circ\text{C}$ in a dry bath. After 2 h, 1 mL of hydrogen peroxide (H_2O_2 , 30%) was slowly added and the samples were kept at $95 \text{ }^\circ\text{C}$ for an additional 30 min. The samples were then cooled to room temperature and made up to 10 mL with ultrapure water. Before analysis, the solutions were diluted 16-fold in ultrapure water and standard curves were prepared in ultrapure water. Mineral quantification was performed by Microwave Plasma-Atomic Emission Spectrometer (MP-AES; Agilent, model 4200). Blank samples, containing only the decomposition reagents, were included to control contamination, and results were subtracted from the samples.

For micromineral analysis, dry samples were accurately weighed (90–380 mg) in a Teflon digestion vessel and then 3 mL of nitric acid 69% (v/v) + 1 mL of 30% (v/v) H_2O_2 (both TraceSELECT™, from Fluka) were added. The digestion vessel was tightly closed and placed inside a high-performance microwave digestion unit (Milestone MLS 1200 Mega, Sorisole, Italy) and the decomposition was carried out according to the following program: step 1—250 W, 1 min; step 2—0 W, 1 min; step 3—250 W, 5 min; step 4—400 W, 5 min; step 5—650 W, 5 min. Afterwards, the digests were left to cool and the volume was made up to 25 mL with ultrapure,

deionized water. The samples were then diluted 10-fold and scandium (Sc), yttrium (Y), indium (In), and terbium (Tb) were added as internal standards to a final concentration of $10 \mu\text{g L}^{-1}$. The samples were analyzed for trace elements using inductively coupled plasma mass spectrometry (ICP-MS; using a iCAP Q instrument, Thermo Fisher Scientific). Blank samples, containing only the digestion reagents, were included to control contamination, and analytical values were subtracted from the samples.

Certified reference materials (Fish muscle ERM@-BB422 and Fish Protein DORM-3) were tested to assess the accuracy of the analytical procedures and the results were within 5% of certified content for all elements.

Quantitative X-Ray Microradiograph Imaging

The relative bone mineral content (RMC, bone density) of 81 DAH Senegalese sole vertebrae ($n = 4$, total per treatment = 20) was determined using quantitative X-ray imaging (qXRI). Digital X-ray images of whole fish were recorded at a $30 \mu\text{m pixel}^{-1}$ resolution using a Kodak DXS 4000 X-ray imaging system (Carestream Molecular Imaging; www.carestream.pt) with the following settings: X-ray energy 35 kV; exposure 3×45 s; FOV 120; focal plane 20. The RMC of vertebrae was determined as previously reported [24] with the following modifications: a 1.67-mm-thick copper-covered steel plate and a 0.5-mm-thick polyester plate were used on each microradiograph and served as standards. Microradiographs were saved as 16-bit DICOM images and the histograms were stretched from the polyester (gray level 0) to the copper-covered steel (gray level 255) standards using ImageJ 1.51j (Wayne Rasband, National Institutes of Health, USA). Subsequently, images were saved into 8-bit Tiff files. After selecting the first three caudal vertebrae (free of malformations) of 5 similar-sized fish from each replicate tank, the frequency of occurrence of an i gray level (F_i) was calculated as previously reported [25]:

$$F_i = 100 \times \frac{N_i}{N_t},$$

where N_i represents the number of pixels with the i gray level and N_t the total number of pixels. The frequency distribution as a function of gray level was plotted, and the mean gray level (GL_{mean}) of each set of vertebrae was deduced from this distribution using the following formula:

$$GL_{\text{mean}} = \frac{\sum(F_i \times GL_i)}{100},$$

where GL_i represents the value of the i gray level. RMC units are gray level mean.

Vertebral Malformation Scoring

An evaluation of the degree of severity of observed vertebral column malformations ($n = 4$, total per treatment = 160) was performed on the previously obtained digital radiographs (described above). Fish vertebral columns were divided into three anatomical regions, abdominal, caudal, and caudal complex (according to Gavaia et al. [26]), and the observed malformations were classified with a score ranging from 0 to 3, according to the criteria presented by Losada et al. [27]. Minor or slight alterations usually involved a small number of vertebrae and consisted of a slight loss of structure or shape of vertebrae, mild deviations of the axis, or minor changes in the intervertebral spaces. Major alterations comprised vertebral fusions and changes in the vertebral bodies and intervertebral spaces that may result in moderate or severe axis deviations. Severe skeletal malformations consisted of any kind of malformation that altered the external morphology. A weighted average of scores (MS) was used to compare the effect of the different dietary treatments on the severity of the observed vertebral malformations and was calculated as follows:

$$MS = \frac{\sum(MF \times S)}{\sum MF},$$

where MF is the percentage of fish with a malformation score of 0, 1, 2, or 3, and S is the score from 0 to 3. MS units are a qualitative score from 0 to 3.

Evaluation of the Mineral Apposition Rate (MAR) of Vertebrae

At 60 DAH, Senegalese sole post-larvae were immersed in a calcein staining solution as previously reported [28], with some modifications. The staining solution was made in $0.45 \mu\text{m}$ filtered seawater with calcein powder (Sigma) at 0.1%, immediately before use. pH was adjusted with NaOH to 7.6 and any residual calcein powder was removed by filtering. For staining, live fish larvae were immersed in the staining solution for 5 min. Stained fish were then placed in calcein-free seawater for more than 10 min to remove any unbound calcein. At 81 DAH, fish were sacrificed with an overdose of tricaine methanesulfonate (MS222; Sigma) and placed in paraformaldehyde (PFA) 4% in phosphate-buffered saline (PBS), 4°C for 20 h. Vertebral columns ($n = 4$, total per treatment = 40) were removed by dissection and placed in KOH 2% overnight with gentle agitation. Muscle tissue was then removed mechanically by light scraping and placed in KOH 2% to help remove the remaining muscle. Samples were then stained with an Alizarin red-S (ARS) solution 0.1% in KOH 0.1% and subsequently submitted to graded concentrations of glycerol (25–75%) in KOH 0.1%, for 3 h, for subsequent observation on an Olympus IX81 motorized

fluorescence microscope equipped with an F-View camera (Olympus). Imaging was performed under green (510–550 nm) and blue (450–480 nm) fluorescent light to image ARS and calcein staining, respectively. To determine the mineral apposition rate (MAR), the same three caudal vertebrae (free of malformations) were selected. The interlabel thickness in the vertebrae (distance between calcein staining mark and Alizarin red-S staining mark) was measured using the software ImageJ 1.51j (Wayne Rasband, National Institutes of Health, USA) (Fig. 1). MAR was calculated as follows:

$$\text{MAR} = \frac{\overline{IrL.th}}{IrL.t}$$

where $\overline{IrL.th}$ represents the average interlabel thickness and $IrL.t$ represents the chosen period (number of days between the first and second staining). MAR unit is in micrometers per day.

Data Analysis

Results were expressed as means \pm standard deviation (SD) from treatment replicates ($n = 4$). Group mean differences were tested by two-way ANOVA, where the effects of dietary Mn and Zn levels and its interaction were examined. The significance level was $p < 0.05$. Data were previously checked for normal distribution and homogeneity of variances, and the results (expressed as a percentage) were based on arcsine transformed data [29]. All statistical tests were performed using the GraphPad Prism software (Version 5.0; GraphPad Software, San Diego, CA).

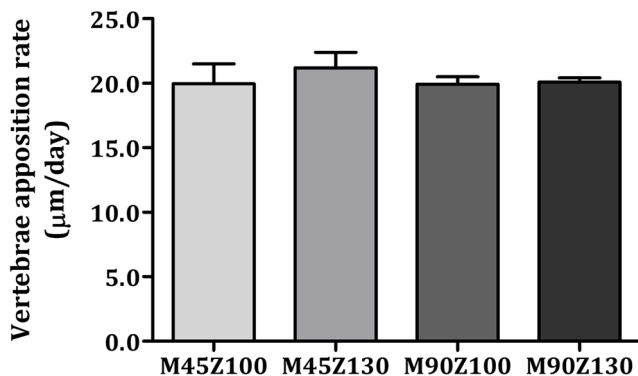


Fig. 1 Graphical representation of vertebral mineral apposition rates of Senegalese sole fed basal diets supplemented with Mn and Zn. Values are mean \pm SD ($n = 4$)

Results

Growth Performance

The growth performance parameters of 81 DAH Senegalese sole are shown in Table 2. In all treatments, survival was high ($> 84\%$). An increase of dietary Zn level from 100 to 130 mg kg^{-1} resulted in a significantly higher survival ($p < 0.05$), while changes on dietary Mn levels had no effect on survival ($p > 0.05$) or standard length (SL) ($p > 0.05$). For all treatments, fish increased their dry weight (DW) by at least fiftyfold (initial dry weight 0.008 g). Fish fed with the highest Zn levels (130 mg kg^{-1} feed) presented a significantly lower DW ($p < 0.05$) than those fed with the lower level of Zn (100 mg kg^{-1}). However, the relative growth rate (RGR) was not significantly affected by dietary treatments ($p > 0.05$). Similarly, the feed conversion rate (FCR) was not significantly affected by changes in dietary Mn and Zn levels ($p > 0.05$).

Whole-Body Mineral Composition and Deposition Rate

The whole-body mineral composition of 81 DAH Senegalese sole fed the different dietary treatments is presented in Table 3. A rise in dietary Mn level, from 45 to 90 mg kg^{-1} , resulted in a significant increase of whole-body Mn content and daily Mn deposition rate ($p < 0.05$). The whole-body content and deposition rate of all other minerals were unaffected ($p > 0.05$) by changes on dietary Mn and Zn levels.

Bone Mineralization and Skeletal Deformities

The vertebral bone mineral content of sole larvae fed the various diets is presented in Table 4. An increase in dietary Mn level, from 45 to 90 mg kg^{-1} , resulted in a significantly higher bone Mn content. An increase in dietary Zn level, from 100 to 130 mg kg^{-1} , resulted in a significant increase in bone Na content ($p < 0.05$). Vertebral bone content of all other minerals was unaffected ($p > 0.05$) by dietary changes. Similarly, X-ray microradiograph imaging showed that vertebral bone density was also not affected by dietary treatments ($p > 0.05$; Table 4). Fish fed the different dietary treatments did not present a high level of severe malformations ($< 10\%$), that is, any type of malformations that alter the gross morphology of fish. Fish juveniles fed the different dietary treatments presented no statistical differences ($p > 0.05$) in severe malformation scoring in the abdominal or caudal region of the vertebral column (Table 4). However, fish fed with the highest Zn levels (130 mg kg^{-1} feed) presented a significantly lower malformation rating in the caudal complex region of the vertebral column ($p < 0.05$) than those fed with the lower level of Zn (100 mg kg^{-1}). Changes in dietary Mn levels had no effect ($p > 0.05$) on malformation scoring in any anatomical region

Table 2 Growth performance of sole post-larvae fed the experimental diets for 39 days

	Dietary treatments				Two-way ANOVA		
	M45Z100	M45Z130	M90Z100	M90Z130	Mn	Zn	MnxZn
Survival (%)	86.9 ± 4.9	91.3 ± 6.5	84.1 ± 2.9	91.3 ± 5.3	–	*	–
SL (cm)	5.27 ± 0.19	5.07 ± 0.25	5.28 ± 0.14	5.18 ± 0.12	–	–	–
DW (g)	0.43 ± 0.04	0.39 ± 0.04	0.45 ± 0.04	0.41 ± 0.04	–	*	–
RGR (% day ⁻¹)	10.7 ± 0.62	10.9 ± 0.77	10.8 ± 0.35	11.1 ± 1.1	–	–	–
FCR	0.75 ± 0.06	0.79 ± 0.09	0.75 ± 0.07	0.77 ± 0.06	–	–	–

Values are presented as means ± SD ($n = 4$)

*Denotes a statistical difference at a significance level of $p < 0.05$

of the vertebral column. The vertebral mineral apposition rate (MAR) of 81 DAH Senegalese sole, fed the different dietary treatments, are shown in Fig. 1. No statistical differences were observed in the MAR of selected vertebrae of juveniles fed diets with variable levels of Mn and Zn ($p > 0.05$).

Discussion and Conclusions

Over the last decade, significant advances have been achieved on fish larval nutrition, which resulted in faster growing

larvae, higher survival rates, and better quality juveniles. Optimal nutrition at the early developmental stages plays a pivotal role on fostering growth performance, skeletal development, and stress resistance [30, 31], not only at the larval stage but potentially also, through epigenetic changes, with life-long effects [32–34]. However, there are still areas related to the nutritional requirements of fish larvae where knowledge is lacking, leaving room for substantial improvements.

Although trace minerals are essential nutrients with a critical role in several physiological, metabolic, and hormonal pathways, and consequently on survival, growth, feed

Table 3 Whole-body content and daily deposition rate of minerals in sole post-larvae

	Dietary treatments				Two-way ANOVA		
	M45Z100	M45Z130	M90Z100	M90Z130	Mn	Zn	MnxZn
Whole-body							
P (g kg ⁻¹ DM)	9.19 ± 0.52	9.36 ± 0.39	9.42 ± 0.43	9.16 ± 0.26	–	–	–
Ca (g kg ⁻¹ DM)	27.7 ± 0.56	27.6 ± 1.4	27.7 ± 1.0	27.5 ± 0.97	–	–	–
Na (g kg ⁻¹ DM)	4.95 ± 0.22	5.00 ± 0.40	4.84 ± 0.32	5.21 ± 0.45	–	–	–
K (g kg ⁻¹ DM)	14.8 ± 0.38	14.9 ± 0.29	15.0 ± 0.91	14.5 ± 0.50	–	–	–
Mg (g kg ⁻¹ DM)	1.28 ± 0.13	1.26 ± 0.04	1.29 ± 0.07	1.23 ± 0.05	–	–	–
Cu (mg kg ⁻¹ DM)	6.96 ± 0.41	6.79 ± 0.49	6.73 ± 0.68	6.85 ± 0.74	–	–	–
Fe (mg kg ⁻¹ DM)	180 ± 1.9	183 ± 8.8	181 ± 6.0	176 ± 9.5	–	–	–
Mn (mg kg ⁻¹ DM)	17.0 ± 0.82	16.3 ± 1.4	22.2 ± 0.57	21.5 ± 1.7	*	–	–
Zn (mg kg ⁻¹ DM)	42.4 ± 1.0	42.0 ± 3.9	42.2 ± 1.4	43.6 ± 1.8	–	–	–
Daily mineral deposition (μg g ⁻¹ day ⁻¹)							
P	86.5 ± 6.3	88.8 ± 4.9	88.7 ± 3.3	87.3 ± 3.6	–	–	–
Ca	259 ± 9.0	262 ± 16	260 ± 8.4	261 ± 12	–	–	–
Na	46.2 ± 2.5	47.0 ± 4.5	45.1 ± 3.4	49.4 ± 5.4	–	–	–
K	138.2 ± 5.6	140 ± 3.8	140 ± 7.6	138 ± 7.3	–	–	–
Mg	11.9 ± 1.3	11.9 ± 0.50	12.0 ± 0.64	11.6 ± 0.62	–	–	–
Cu	0.07 ± 0.0	0.07 ± 0.00	0.06 ± 0.01	0.07 ± 0.01	–	–	–
Fe	1.68 ± 0.04	1.73 ± 0.08	1.70 ± 0.05	1.66 ± 0.07	–	–	–
Mn	0.17 ± 0.01	0.16 ± 0.01	0.22 ± 0.00	0.21 ± 0.01	*	–	–
Zn	0.39 ± 0.02	0.39 ± 0.03	0.39 ± 0.02	0.41 ± 0.03	–	–	–

Values are presented as means ± SD ($n = 4$)

*Denotes a statistical difference at a significance level of $p < 0.05$

Table 4 Vertebral bone mineral content, bone density, and skeletal malformations in sole post-larvae

	Dietary treatments				Two-way ANOVA		
	M45Z100	M45Z130	M90Z100	M90Z130	Mn	Zn	MnxZn
P (g kg ⁻¹)	44.8 ± 2.8	45.1 ± 2.3	43.9 ± 2.9	45.2 ± 0.46	–	–	–
Ca (g kg ⁻¹)	184 ± 13	170 ± 4.3	183 ± 17	178 ± 13	–	–	–
Na (g kg ⁻¹)	2.97 ± 0.10	3.11 ± 0.13	2.74 ± 0.18	3.25 ± 0.08	–	*	*
K (g kg ⁻¹)	18.4 ± 6.5	16.5 ± 4.4	21.4 ± 4.35	20.9 ± 5.3	–	–	–
Mg (g kg ⁻¹)	4.66 ± 0.20	5.33 ± 0.28	4.71 ± 0.26	5.03 ± 0.51	–	–	–
Cu (mg kg ⁻¹)	0.53 ± 0.13	0.42 ± 0.08	0.44 ± 0.19	0.41 ± 0.24	–	–	–
Mn (mg kg ⁻¹)	129 ± 3.6	124 ± 11	166 ± 13	167 ± 19	*	–	–
Zn (mg kg ⁻¹)	60.0 ± 2.0	60.0 ± 4.8	59.5 ± 1.4	62.3 ± 5.6	–	–	–
Bone density (gray level)	166.92 ± 9.56	164.82 ± 1.57	174.91 ± 8.78	165.01 ± 7.06	–	–	–
Skeletal malformations score**							
Abdominal	1.06 ± 0.05	1.02 ± 0.16	1.23 ± 0.17	0.89 ± 0.26	–	–	–
Caudal	1.01 ± 0.03	0.99 ± 0.25	1.21 ± 0.21	0.84 ± 0.27	–	–	–
Caudal complex	0.37 ± 0.13	0.34 ± 0.15	0.49 ± 0.11	0.25 ± 0.11	–	*	–

Values are presented as means ± SD ($n = 4$)

*Denotes a statistical difference at a significance level of $p < 0.05$

**Score range: 0 (no radiographic alterations) to 3 (presence of severe vertebral malformations that alter the gross morphology of fish)

utilization, skeletal formation, immune response, and susceptibility to pathologies, knowledge on the qualitative and quantitative requirements in fish larvae is extremely incipient [1, 17, 18].

At present, microdiets for marine fish larvae are generally formulated with extremely high levels of premium marine-derived ingredients, such as fish meal, krill meal, squid meal, and marine protein hydrolysates, which are generally rich in most essential macro and trace minerals. Although not based on scientific data, such larval microdiets are not expected to be nutritionally limiting in terms of trace minerals. However, as with almost all other nutritional components, at a highly sensitive life stage such as larvae, minor deviations from the optimal balance of the various nutrients may have significant effects on a vast array of biological processes.

In our study, average performance criteria of Senegalese sole at 81 DAH (survival 88%; DW 0.42 g; RGR 10.86% day⁻¹) can be considered as within the expected ranges according to a recent review [35] describing the historical progress in weaning strategies for Senegalese sole larvae. Our data shows that an increase in dietary Zn level from 100 to 130 mg kg⁻¹ enhanced survival of sole larvae. However, these findings are applicable only to the Senegalese sole, since they are supported by some studies but in conflict with others depending on the fish species. A dietary Zn supplementation

(from 65 to 90 mg kg⁻¹) to feed rainbow trout (initial weight of 82.3 mg), also led to an increase of survival, though dependent on the Zn form [36]. Nevertheless, a lower survival was observed in gilthead seabream larvae, when Zn levels increased (from 86 to 110 mg kg⁻¹) [22]. Still, in another study on red seabream larvae, Zn supplementation of *Artemia nauplii* (from 119 to 423 mg kg⁻¹ dry weight) had no effect on survival [21]. The potential toxic effect of dietary Zn requires further clarification since higher Zn levels are commonly found in enriched live feeds (copepods and *Artemia*) without inducing any detrimental effects on the survival of several other marine species.

As far as growth performance is concerned, unlike what was found in this study, an increase in dietary Zn levels alone or the combined supplementation with Mn (from 3 to 15 mg kg⁻¹), Zn (from 86 to 110 mg kg⁻¹), and selenium (from 2 to 5 mg kg⁻¹) can result in higher growth of rainbow trout fry [36] and gilthead seabream larvae fed from 20 to 44 DAH. In larval nutrition trials, a scenario of variable survival may cause difficulties in the precise assessment of growth performance. A higher survival of smaller sized sole larvae in treatments with the highest Zn level may have led to an artifactual reduction of DW, even though dietary Zn had no effect on the growth performance of fish. Statistical analysis performed on the best performing 50% cohort of sampled fish

(data not shown) showed that dietary zinc levels had no significant effect on DW of sole larvae, in accordance with non-significant effect on the RGR. Similar results were obtained for red seabream larvae, where Zn supplementation of *Artemia* nauplii had no effect on the growth performance [21]. In zebrafish, graded dietary levels of zinc sulphate up to 120 mg kg⁻¹ had no effect on survival and the total length, while higher dietary levels of Zn (240 and 480 mg kg⁻¹) increased larval mortality [37]. The absence of beneficial or detrimental effects of a Zn supplementation above requirements has also been reported in juvenile fish from several species, such as channel catfish [38], Nile tilapia [39], striped seabass [40], European seabass [41], and yellow catfish [42].

The effect of Mn supplementation has also been studied in other species such as red seabream larvae and the results showed that, unlike this study, Mn supplementation (from 12 to 43 mg kg⁻¹ dry weight) of *Artemia* nauplii significantly increased larval growth [21]. In our study, changes on the dietary Mn levels (from 45 to 90 mg kg⁻¹) had no effect on the growth performance of Senegalese sole larvae. Several other studies with juvenile fish also showed that a dietary Mn supplementation above the estimated requirement level does not result in a growth-promoting effect [43–45].

A meta-analysis of literature data on mineral and trace element requirements of fish showed that vertebral mineral concentration is the most appropriate criterion to assess dietary adequacy of P, Ca, Zn, and Mn [46]. Our data shows that Senegalese sole post-larvae fed higher dietary Mn levels presented significantly higher levels of Mn in vertebral bone as well as in the whole body. This positive relationship between Mn intake and vertebral Mn content is confirmed by studies in juvenile channel catfish [47] and grouper [48].

The influence of Zn supplementation on mineral composition of vertebral bone in Senegalese sole juveniles was only significant for Na, which was increased at high dietary Zn levels. Although some studies have reported an increase of Zn in bone with the increase of dietary Zn [38, 39], our results fall in line with other studies [41, 49, 50] that have shown that Zn concentrations in fish bone remain stable even though dietary Zn levels were increased to levels similar or above the ones tested in this study. To our knowledge, an interaction between a higher intake of Zn and an increase of Na content in vertebral bone has never been reported in fish but due to the complexity of the interactions among minerals and scarce information, further research on the topic of mineral nutrition in fish is needed.

Bone development is critically dependent on the larval period and a majority of skeletal anomalies arise from uncontrolled environmental and nutritional factors occurring at this stage [1]. Despite often being referred to as critical nutrients for skeletal development and bone remodeling, the optimal dietary supply of trace minerals, such as Zn and Mn, in marine fish larvae is still incipient. In osteology, zinc has an anabolic effect on osteogenesis by stimulating cell proliferation,

alkaline phosphatase activity, and collagen synthesis in osteoblastic cells, along with signs of improving Ca deposition in the extracellular bone matrix [51]. Manganese plays an important role in the formation of bone cartilage and bone collagen, as well as in bone mineralization, as it is a component of various enzymes involved in cartilage and bone metabolism. Manganese-dependent superoxide dismutase (Mn-SOD) activity can also favor bone formation over resorption by removing reactive oxygen species (ROS) that are known to modulate osteoclastogenesis [52] and osteoblast-mediated mineralization [53]. Most studies report skeletal malformations or detrimental effects on fish bone, associated with Mn and/or Zn supplementation, are due to sub-optimal levels of these same trace minerals [19]. However, given the typical high incorporation levels of marine ingredients, commercial microdiets for marine fish larvae show a limited risk of nutritional deficiencies regarding trace minerals like Zn or Mn. Our study shows that a dietary increase in both Mn (from 45 to 90 mg kg⁻¹) and Zn (from 100 to 130 mg kg⁻¹) had no effect on vertebral density and mineral apposition rate probably because the dietary levels of Mn and Zn tested in this study were enough to cover the requirements for these trace minerals. In fact, the dietary content of these trace minerals in the feed was well above the requirements for juvenile fish, according to NRC [20]. Nonetheless, the increase in Zn (from 100 to 130 mg kg⁻¹) resulted in a statistically significant decrease in malformations affecting the caudal complex region and caudal fin vertebrae of Senegalese sole post-larvae. Similar results were obtained by Nguyen et al. [21] who reported a decrease of anomalies in the dorsal fin rays of red seabream fed Zn-enriched *Artemia*. These authors suggested that the beneficial effects of Zn supplementation on skeletal development could be due to the stimulation of osteoblastic bone formation and inhibition of osteoclastic bone resorption by Zn, as demonstrated in mammalian cell cultures. Recently, Roberto et al. [37] have shown that osteogenic markers (*runx2*, *sp7*, *oc*) were increased by dietary Zn supplementation (up to 120 mg kg⁻¹), indicating that appropriate amounts of Zn are critical to support normal bone formation in zebrafish larvae. However, care must be taken on further raising Zn levels, since high dietary Zn levels may compromise larval survival, as reported in other fish species [22, 37].

Overall, our study suggests that Mn and Zn supplementation of a commercial microdiet, which generally contains high levels of marine ingredients, for marine fish larvae had no significant effect on the growth performance of Senegalese sole post-larvae. However, higher dietary levels of Mn (90 mg kg⁻¹) and Zn (130 mg kg⁻¹) could improve larval survival, decrease the severity of vertebral malformations, and increase the deposition of Mn in bone that could counteract oxidative stress under certain stressful conditions. Further studies targeting a better understanding of the mechanistic role of trace minerals in bone metabolism in fish larvae are needed.

Additionally, a detailed assessment of the effect of trace minerals on the immune status of fish larvae will add information of paramount importance on fish nutrition requirements.

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Compliance with Ethical Standards

Ethics Approval The experimental protocol was approved by the Animal Welfare Committee (ORBEA) of the Instituto Português do Mar e da Atmosfera (IPMA) (Project LARVAMIX approval no. 17935) and carried out in registered facilities (0421/2018). Experiments were conducted by trained scientists and in full compliance with the European (Directive 2010/63/EU) and Portuguese (Decreto-Lei no. 113/2013, August 7th) legislation on the protection of animals for scientific purposes.

Conflict of Interest The authors declare that they have no conflict of interest.

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