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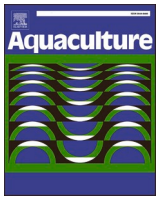

Antioxidant and osteoinductive properties of organic selenium in microdiets for gilthead seabream (*Sparus aurata*) larvae

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ABSTRACT

This study investigated the effect of yeast derived selenium (Se) dietary supplementation on larval performance, whole body mineral contents, lipid peroxidation, fatty acid profiles, expression of antioxidants and bone biomarkers genes, mineralization and skeletal anomalies in gilthead seabream (*Sparus aurata*) larvae. Five experimental microdiets containing 1.4, 6.1, 9.2, 12.0 and 14.0 mg Se/kg diets were fed to triplicate groups of larvae (total length: 7.26 ± 0.83 mm, 30 dph) for 21 days. Larval Se levels increased proportionally to the elevation of dietary Se. Those fed with dietary Se level at 6.1 mg/kg had a higher growth performance than those fed with non-supplemented diet (1.4 mg/kg), then reached a plateau from larvae fed with Se6.1 to Se14 diet although growth was not significantly affected by different diets. In addition, larval survival was not significantly affected. However, larvae fed the lowest Se levels showed a significant increase in lipid peroxidation, the downregulation of antioxidant genes, a reduction in polyunsaturated fatty acids (PUFA) contents and the highest incidence of skeletal anomalies. While increase up to 6.1–12 mg Se/kg, reduced oxidative stress, increased PUFA, vertebral mineralization, up-regulated antioxidant and bone metabolism related genes and contributing to reduce skeletal anomalies of larvae. However, further increase in dietary Se up to 14 mg/kg led to the lowest *gpx, cat* and *alp* expressions, in agreement with a lower mineralization. These findings suggest, dietary Se level at 6.1 mg/kg would be the minimum dietary Se level required in microdiets for gilthead seabream larvae. Thus, the study demonstrates the importance of dietary Se for bone metabolism and mineralization due to its antioxidant and osteo-inductive properties.

1. Introduction

Larval production is a lengthy and complex process that requires a deep understanding of the biology of the species cultivated. During this developmental period, a high incidence of skeletal anomalies may be observed which can increase the mortality rate (Boglione et al., 2013; Hamre et al., 2013; Koumoundouros, 2010). The occurrence of skeletal anomalies in larval culture are due to nutrition, oxidative stress, genetic and other environmental conditions (Dellacqua et al., 2023; Izquierdo et al., 2013; Lewis-McCrea and Lall, 2007; Nguyen et al., 2016; Prestinicola et al., 2013). This uncertain larval quality limits the successful mass production of juvenile fish. During the regular cellular metabolism or defense mechanisms, reactive oxygen species (ROS) are generated as by-products that may play a role in cell signaling for the normal biologic processes. However, the overload of unstable ROS is a potential cause of oxidative damage to lipids, proteins and DNA, may alter the normal cellular function and ultimately lead to cell apoptosis (Bhattacharya, 2015). Lipid peroxidation can be caused by ROS and affects cellular membranes by removing hydrogen from the unsaturated chain of fatty acids. The lipid peroxidation-derived aldehyde malondialdehyde (MDA), is an early product of polyunsaturated fatty acid (PUFA) peroxidation, which may induce mitochondrial toxicity (Bhattacharya, 2015). The oxidative damage causes injuries in fish, including muscular necrosis (Betancor et al., 2012), hydropic degeneration in liver (Domínguez et al., 2020) or intestine inflammation (Long et al., 2022). Moreover, it can increase skeletal anomalies and delay bone mineralization in early stages of seabream larvae (Poudel et al., 2022a). Indeed, marine fish larvae are highly sensitive and prone to increase the risk of

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oxidative stress due to their fast-growth during larval stages, high requirements of long-chain PUFA (LC-PUFA), high metabolic rate, high water content in larvae tissue, and due to the long exposure to water of the microdiets used in larval rearing, which causes a peroxidation of their nutrients (Hamre et al., 2013; Izquierdo et al., 2017). This suggests the necessity of including antioxidant nutrients in the microdiets. Even though, antioxidant nutrients may promote the protection of membrane lipids against the oxidative stress, some nutrients can induce the oxidative stress in fish when fed in with excess or deficiency level. For instance, ascorbic acid (Izquierdo et al., 2019), α-tocopherol (Lozano et al., 2017; Sau et al., 2004), copper (Tseng et al., 2023), selenium (Saleh et al., 2014) or coconut oil (Tseng and Lin, 2020) are all examples of nutrients with antioxidant properties that can have a pro-oxidant effect when fed in high/low levels. Therefore, it is important to supplement an adequate level of antioxidant nutrients in microdiets to avoid the negative effects of oxidative damage in fish larvae.

Selenium (Se) is one of the essential trace minerals that plays an important role in the antioxidant system and prevents or decreases the harmful effects in fish (Antony Jesu Prabhu et al., 2016; Domínguez et al., 2020; Domínguez et al., 2017; Lall and Kaushik, 2021). In all living organisms, Se conducts its physiological functions generally as selenoproteins. One of the selenoproteins, glutathione peroxidases (GPX) is involved in antioxidation, and catalyzes hydrogen peroxide to water and oxygen as well as catalyzing the reduction of peroxide radicals to alcohols and oxygen (Bhattacharya, 2015). Hence, Se, as an integrant of GPX, is a crucial factor in cellular defense against the oxidative stress (Zhang et al., 2020). Optimum dietary Se levels in fish varies with different species, development stages and the form of Se used in diets. In the case of juvenile marine fish species, these dietary levels are 0.7 mg Se/kg (Selenomethionine, SeMet) for malabar grouper (*Epinephelus malabaricus*), 0.79–0.81 mg Se/kg (SeMet) for cobia (*Rachycentron canadum*), 3.98 mg Se/kg (Se-yeast) for meagre (*Argyrosomus regius*), 5.56 mg Se/kg (Se-yeast) for yellowtail kingfish (*Seriola lalandi*) or 1.07 mg Se/kg (seleno-yeast) for olive flounder (*Paralichthys olivaceus*) (Le and Fotedar, 2013; Lee et al., 2009; Lin and Shiau, 2005; Liu et al., 2010; Mansour et al., 2017). For other species of the sparidae family, the recommended Se levels are 0.86 mg Se/kg (Se polysaccharide) for black seabream (*Acanthopagrus schlegelii*) (Wang et al., 2019), 1.34–2.27 mg Se/kg (Se-nanoparticles) for red seabream (*Pagrus major*) (Dawood et al., 2019) or 0.94 mg Se/kg (sodium selenite) (Domínguez et al., 2020) and 0.79 mg Se/kg (hydroxy-SeMet) for gilthead seabream (Tseng et al., 2021). Studies of Se requirement in fish larvae are 1.4 to 3.0 mg Se/kg (DW in rotifer) for cod (*Gadus morhua* L.) larvae, 2.2 μg Se/g (DW in rotifer) for red seabream larvae, and 11.65 mg Se/kg microdiet for gilthead seabream larvae (Kim et al., 2014; Penglase et al., 2010; Saleh et al., 2014). However, such differences of Se level between using live prey and microdiet are related to the high leaching of minerals from the microdiet due to its high surface/ volume ratio (Hamre et al., 2013). Thus, leaching is a complicated issue when using microdiet since 55 to 67% of the initial mineral content can be lost after 5 min of water immersion in seabass larvae diet (Viegas et al., 2023). Similarly, high leaching of minerals also observed seabream larvae microdiet (Tseng et al., 2023).

Previous studies found similar symptoms among different species when fish were fed with either deficient or excess Se levels in diets, including increased lipid peroxidation (Lin and Lin, 2021; Saleh et al., 2014), altered whole body fatty acids composition and reduced antioxidant capacity, growth or survival (Domínguez et al., 2020; Du et al., 2021). A low dietary Se (2 mg/kg, Selenium nanoparticles) induced the spinal curvature, tail malformations and yolk sac edema in zebrafish (*Danio rerio*) embryos (Moges et al., 2021). A dietary Se level of 0.49 mg/kg with Zn level at 149 mg/kg during early seawater phase, which is below the optimal level for Atlantic salmon, increased the risk of vertebral deformities as adults (Antony Jesu Prabhu et al., 2023). Moreover, red seabream larvae fed with non-enriched rotifers (0.0 μg Se/g) shows a reduction of the notochord flexion compared with the larvae fed Se-enriched rotifer (2.2 μg Se/g) (Kim et al., 2014). Furthermore, dietary Se level at 1.73 mg/kg down-regulated the expression of bone-related genes of gilthead seabream larvae, denoting an altered skeletal development (Saleh et al., 2014). Indeed, the Se contents in rotifers (0.08 mg Se/kg) (Hamre et al., 2008) are 3.4 times lower than the recommend levels given by NRC (2011) or several fish species already mentioned previously. This suggests that diets or rotifers deficient in Se modify the larval skeletal development and increase the risk of skeletal anomalies. Additionally, inadequate Se can lead to other bone disorders, such as kashin-beck disease, osteoarthritis, rheumatoid arthritis or osteoporosis in humans (Zhang et al., 2014). Noteworthy, gilthead seabream larvae fed with diets supplemented with microminerals (Se: 4.9 mg Se/kg) have a lower incidence of branchial arch anomalies and higher bone mineralization levels compared with larvae fed a non-supplemented diet (Se: 1.9 mg Se/kg) (Izquierdo et al., 2017). Furthermore, gilthead seabream larvae fed with increasing dietary Se levels up to 11.65 mg Se/kg show the up-regulation of several bone-related genes (Saleh et al., 2014). Therefore, these studies point out the necessity of including Se in early larval feeds for an adequate skeletal development.

However, the previous studies tested a combination of microminerals or did not determine the effect of dietary Se on skeletal anomalies and mineralization levels. Given the involvement of Se in the antioxidant system, and its role skeletal development, it would be interesting to clarify the effects of dietary Se levels on the incidence of skeletal anomalies in fish larvae. Hence, this study aimed to investigate the effects of dietary Se levels on growth, oxidative stress, fatty acids composition and skeletal development in gilthead seabream larvae (*Sparus aurata*) larvae.

2. Material and methods

2.1. Ethical statement

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

2.2. Experimental microdiets

Five experimental microdiets were supplemented with different Se levels using a yeast-derived Se source. A negative control diet (Se1.4) without Se supplementation contained 1.4 mg Se/kg and four other diets (Se6.1, Se9.2, Se12, Se14) were supplemented with Se and contained 6.1, 9.2, 12.0, 14.0 mg Se/kg, respectively. All microdiets were prepared to contain equal amounts of proteins (68.25%) and lipids (16.55%) (Table 1) and were tested in triplicate groups ($n = 3$). The dietary copper, zinc, manganese, vitamin D_3 and K_3 level was formulated based on previous studies in gilthead seabream larvae (Tseng et al., 2023; Sivagurunathan et al., 2022; Sivagurunathan et al., 2023). Prior to microdiet preparation, all ingredients were ground (ZM 200, RETSCH, Germany) and sieved (Retsch AS200, Germany) below 125 μm. Then all the water-soluble ingredients were mixed, and then, the lipid ingredients and fat-soluble vitamins were added. Finally, gelatin was dissolved in warm water and mixed with all the above ingredients until a stiff dough was obtained. The mix was passed through a mincer (12TS, Fimar, Italy), and the obtained strands were dried in the oven at 37 °C for 24 h. The dried strands were grounded and sieved again into two different particle sizes, < 250 μm and 250–500 μm, and stored at −80 °C until use.

2.3. Fish larvae and rearing system

The experimental gilthead seabream larvae were obtained from

Table 1
Ingredients and proximate composition of the experimental diets.

Ingredients (%)	Se1.4	Se 6.1	Se 9.2	Se 12	Se 14
Squid powder[1]	40.0	40	40	40	40
Casein	30.8	30.8	30.8	30.8	30.8
Krill oil[2]	13	13	13	13	13
Sel-Plex® 2000[3]	0	0.2	0.325	0.45	0.575
Mineral Premix[4]	4.55	4.55	4.55	4.55	4.55
Vitamin Premix[5]	5.42	5.42	5.42	5.42	5.42
Attractants[6]	3	3	3	3	3
Gelatin	3	3	3	3	3
Proximate composition (%)					
Total lipid	16.44	16.53	16.50	16.48	16.56
Crude protein	68.14	68.21	68.26	68.30	68.35
Mineral contents					
Se (mg/kg)	1.4	6.1	9.2	12.0	14.0
Mn (mg/kg)	190	220	230	240	210
Zn (mg/kg)	100	110	120	120	110
Cu (mg/kg)	12	14	15	16	14
Fe (mg/kg)	400	480	540	590	510
Ca (g/kg)	4.4	5.4	5.8	6.2	5.7
P (g/kg)	14	15	15	16	15
Ca:P	0.31	0.36	0.39	0.39	0.38

[1] Bacarel Express, United Kingdom.
[2] Aker BioMarine, Fjordalléen, Norway.
[3] Alltech, Lexington, KY, Contained 2000 mg Se/kg.
[4] Mineral premix (mg/100 g) supplied for 100 g diet: NaCl, 215.133 mg; $MgSO_4.7H_2O$, 677.545 mg; $NaH_2PO_4.H_2O$, 381.453 mg; K_2HPO_4, 758.949 mg; $Ca(H_2PO_4).2H_2O$, 671.610 mg; $FeC_6H_5O_7$, 146.884; $C_3H_5O_3.1/2Ca$, 1617.210 mg; $Al_2(SO_4)3.6H_2O$, 0.693 mg; $ZnSO_4.7H_2O$, 17.590; $CuSO_4.5H_2O$, 0.330 mg; $MnSO_4.H_2O$, 55.40 mg; KI, 0.742 mg; $CoSO_4.7H_2O$, 10.706 mg.
[5] Vitamin premix (mg/100 g) supplied for 100 g diet: Cyanocobalamin, 0.03 mg; Astaxanthin, 5.0 mg; Folic acid, 5.44 mg; Pyridoxine-HCL, 17.28 mg; Thiamine-HCL, 21.77 mg; Riboflavin, 72.53 mg; Calcium Pantothenate, 101.59 mg; 4-Aminobenzoic acid, 145 mg; Nicotinic acid, 290.16 mg; myo-inositol, 1450.90 mg; L-Ascorbic acid, 180.00 mg; Choline chloride, 2965.80 mg; Retinoic acid, 0.24 mg; Cholecalciferol, 0.0063 mg; Menadione, 17.28 mg; α-Tocopherol acetate, 150 mg.
[6] Attractants premix (mg/100 g) supplied for 100 g diet: Inosine 5-monophosphate, 500 mg; Betaine, 660 mg; L-Serine, 170 mg; L- Tyrosine, 170 mg; Phenylalanine, 250 mg; DL- Alanine, 500 mg; L-Aspartic acid sodium, 330 mg; L-Valine, 250 mg; Glycine, 170 mg.

natural spawns of the gilthead seabream selected broodstock (PROGENSA, Spanish National Seabream Breeding Program) (Afonso et al., 2012) from ECOAQUA Institute facilities at the University of Las Palmas de Gran Canaria (ULPGC, Spain). Fish larvae were fed rotifers (*Brachinous plicatilis*) enriched with Ori-Green (Skretting, France) from 3 dph until 30 dph.

Initial larvae at 30 dph (total length: 7.26 ± 0.83 mm; dry weight: 0.33 ± 0.05 mg) were individually distributed into 15 tanks at the density of 940 larvae per tank (170 L fiberglass cylinder tank). Microdiets were manually fed to larvae every 45 min from 8:00 am to 20:00 pm for 21 days until 51dph. All larvae were reared in tanks with filtered UV-sterilized seawater (37‰) at an increasing rate of 0.3 to 1.0 L/min. Water inflowed continuously from the bottom of tank then outflowed from top throughout the entire experiment. To maintain water quality, dissolved oxygen (5–8 g/L) and saturation (60%–80%) were monitored daily. Photoperiod was kept at 12 h light: 12 h dark. Tanks were siphoned daily at 14:00 to remove the uneaten microdiets. Water temperature was determined daily (21.54 ± 0.24 °C).

2.4. Larval performance

A total of 30 larvae per tank were randomly collected at all sampling points (day 7, 37 dph; day 14, 44 dph; day 21, 51 dph) then washed with distilled water for measuring the total length by stereoscope (Leica, M125, Wetzlar, Germany), while the body weight was assessed by drying the larvae in an oven at 105 °C until constant weight. Mean mortality was daily checked by removing and counting the total dead larvae per tank. At the end of the trial (day 21, 51 dph), all live larvae per tank were individually counted to assess the final survival rate. The survival rate (%) was calculated using the formula: (n° final live larvae / n° initial larvae) *100. Biomass was determined by multiplying the dry weight of the larvae by the number of remaining live larvae. Subsequently, the remaining live larvae per tank were collected, anesthetized in cold seawater, washed with distilled water, and equally stored in different sample bags. All the sample bags with pooled larvae samples were then stored at −80 °C for further analysis, including mineral, lipid peroxidation, and biochemical analysis.

2.5. Mineral composition

The mineral composition in diets, and whole body fish larvae collected from the final sampling were performed by inductively coupled plasma MS (ICP-MS) (Silva et al., 2019). The mineral analysis was conducted at the Institute of Marine Research (IMR) in Bergen, Norway. Approximately 0.20 to 0.25 g of the ground diets and freeze-dried homogenates whole body fish larvae, were digested by using 2 mL of nitric acid (HNO_3) in an ultrawave digestion system (UltraWAVE, Milestone, Sorisole, Italy). The samples were sealed and introduced into the ultrawave system with a container of 130 mL of Milli-Q® water and 5 mL of H_2O_2. The digested samples were then diluted to 25 mL with Milli-Q® water, then introduced into the nebulizer tube of the ICP-MS (iCapQ ICP-MS, Thermo Scientific, Waltham, USA) with an auto sampler (FAST SC-4Q DX, Elemental Scientific, Omaha, USA).

2.6. Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) values were analyzed from the extracted total lipid of whole-body larvae at the concentration of 10 mg per mL (Burk et al., 1980). Briefly, to 200 μL of lipid sample were added 50 uL of 0.2% (*w/v*) butylated hydroxytoluene (BHT), 0.5 mL of 10% (w/v) trichloroacetic acid (TCA), and 0.5 mL of 0.288% (w/v) thiobarbituric acid (TBA) freshly prepared. Then the mix solution was kept in a water bath at 100 °C for 20 min. After cooling, the precipitate was removed by centrifugation at 2000*g*, the supernatant was determined by ELISA reader (Thermo Scientific™) at 532 nm. The concentration of TBARS, expressed as nmol malondialdehyde (MDA)/g lipid was calculated using the absorption coefficient 0.156 μM^{-1} cm^{-1}.

2.7. Biochemical composition

Experimental microdiets and whole-body larvae (51 dph) were analyzed for moisture, ash, and crude protein content according to the method of AOAC (1995). Total lipids were extracted by using chloroform:methanol solution (2:1) following the methodology described by Folch et al., 1957. Fatty acid methyl esters were obtained by transmethylation of lipids as described by (Christie, 1989), separated by gas chromatography (GC), quantified by flame ionization detection (FID) (GC-14 A; Shimadzu, Tokyo, Japan) following the conditions previously described (Izquierdo et al., 1989) and identified by comparison with previously characterized standards. Mineral compositions were determined by ICP-MS at the Institute of Marine Research (IMR), Bergen, Norway.

2.8. Gene expression

Samples (30 larvae/tank) were randomly collected at each sampling point: after 7 (37 dph), 14 (44 dph) and 21 (51 dph) days of feeding. All samples were preserved in RNA Later (Sigma-Aldrich) overnight at 4 °C, then transferred to −80 °C until RNA extraction and analysis. Total RNA

of whole body was extracted from pool samples (30 larvae per tank) by TRI Reagent Solution (Sigma-Aldrich.) and purified using RNeasy Mini kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. The quantity of RNA was calculated by using NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The integrity of RNA was assessed with Gel Red™ staining (Biotium Inc., Hayward, CA) on 1.4% agarose gel by electrophoresis. A total of 1 μg RNA per tank was conducted used with the iScript cDNA Synthesis Kit (Bio-Rad) following the instructions of the manufacturer in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA).

All primers sequences were listed in Table 2. The expression of bone-related and antioxidant enzyme genes was determined by using iQ5 Multicolor Real-Time PCR detection system (Bio-Rad). Beta-actin (β-actin) was used as the housekeeping gene, and the stability of this gene was assessed by BestKeeper (Pfaffl et al., 2004; Zhou et al., 2018). The final reaction mixture contained 0.6 μL (10 mM) for both forward and reverse primers, 5 μL of cDNA, 7.5 μL of SYBR Green (Bio-Rad), and 1.3 μL of MilliQ water. The RT-PCR conditions were: 95 °C for 3 min 30 s, followed by 40 cycles of 95 °C for 15 s, then 30 s of annealing temperature for each primer (Table 2), and 72 °C for 30 s, 95 °C for 1 min, a final denaturing step from 58 °C to 95 °C for 10 s. The comparative $2^{-\Delta\Delta}$CT method described by Livak and Schmittgen (2001) was used to analyze the fold expression of each gene.

2.9. Skeletal mineralization and anomalies

At the end of trial, a total number of 51 larvae per tank (51dph) were randomly collected to determine skeletal anomalies and the axial vertebral mineralization level. Specimens were fixed and stored in the fixative (4% formalin in phosphate, pH 7.2, 0.1 M) and stained with alcian blue and alizarin red following the method of Dingerkus and Uhler (1977) and Taylor and Van Dyke (1985). Larvae samples were examined under a stereomicroscope (Leica, M125, Germany) and imagined by Leica DFC295 digital camera using the Leica application suite. The skeletal anomalies were classified according to different regions and types (Prestinicola et al., 2013). Fish were divided into 5 size classes based on the total length (mm): (1) 11.26–12.06, (2) 12.07–12.87, (3)12.88–13.68, (4) 13.69–14.49, (5) 14.50–15.3. The axial vertebral mineralization level was evaluated considering the percentage of fully mineralized vertebral bodies within larval size class.

2.10. Statistical analysis

Each experimental diets were tested as replicate group ($n = 3$ per treatment) and all data were presented as mean ± SE. Levene's test was used to test data normality and homogeneity of variances. All data were analyzed through a one-way ANOVA of variance by using IBM SPSS Statistics software (21.0 version: SPSS Inc., Chicago, IL, USA). In the case of statistical differences ($P < 0.05$) among the treatment, a Duncan's new multiple-range test was applied to determine among means per group. Linear and quadratic regression models were used to determine the effect of dietary Se on different parameters. All parameters were considered with significance difference when the P value was below 0.05 ($P < 0.05$). The heatmap representation of the bone biomarker and antioxidant genes was designed using GraphPad Prism.9 (GraphPad Software, San Diego, California).

3. Results

3.1. Larval performance

Larvae accepted well all the experimental microdiets, and there was a very high survival rate (>83%) at the end of the feeding trial (Table 3). Growth in terms of both total length and body weight was exceptional throughout the entire feeding trial, with no significant differences among fish fed the different Se dietary levels (Fig. 1). Therefore, after 21 days of feeding, when larvae had increased 10 times their initial weight, there were no significant differences in total length, body weight, specific growth rate or biomass among larvae fed with different levels of dietary Se (Table 3). However, there was a tendency ($P = 0.08$) for higher growth in larvae fed the Se 6.1 diet compared to those fed with Se1.4 diet, and growth reached a plateau in fish fed with Se6.1 to Se14.0 diet (Fig. 2).

3.2. Biochemical composition

Total lipid, crude protein and ash contents in larvae were not affected by dietary Se levels (Table 4). Noteworthy, the larval lipid peroxidation TBARS values were significantly ($P = 0.03$) highest in larvae fed the Se1.4 diet and were lower in fish supplemented with Se (6.2 to 14.0 mg Se/kg).

Table 3
Survival and biomass of gilthead seabream larvae fed diets containing different Se levels for 21 days (mean ± SD, $n = 3$).

Parameters	Diets					One-way ANOVA P value
	Se1.4	Se6.1	Se9.2	Se12	Se14	
Survival (%)	86.4 ± 5.53	84.4 ± 1.83	84.0 ± 3.23	83.2 ± 3.16	84.1 ± 4.21	0.87
Biomass (g)	2.0 ± 0.34	3.1 ± 0.69	2.7 ± 0.53	2.6 ± 0.53	2.9 ± 0.49	0.18

Data expressed as mean ± SD (n = 3), the statistical analysis was performed by using one-way ANOVA, the statistical significance was set as $P < 0.05$.

Table 2
Primer pair, and GenBank accession numbers of specific primers[1].

Gene[1]	5′–3′ primer sequence		Tm (°C)	GenBank accession no.
	Forward	Reverse		
bmp2	GTGGCTTCCATCGTATCAACATTTT	GCTCCCCGCCATGAGT	60	JF261172.1
runx2	GCCTGTCGCCTTTAAGGTGGTTGC	TCGTCGTTGCCCGCCATAGCTG	61	AJ619023
opn	AAGATGGCCTACGACATGACAGAC	CCTGAAGAGCCTTGTACACCTGC	61	AY651247
on	AAAATGATCGAGCCCTGCATGGAC	TACAGAGTCACCAGGACGTT	61	AY239014
alp	AGA ACG CCC TGA CGC TGC AA	TTC AGT ATA CGA GCA GCC GTC AC	61	AY266359
enpp1	GCAACGCTCCGCTGAACA	CCTCCGCTCTCATCTTTGC	60	XM_030405802.1
oc	AGCCCAAAGCACGTAAGCAAGCTA	TTTCATCACGCTACTCTACGGGTT	58.1	AF048703
cat	ATGGTGTGGGACTTCTGGAG	AGTGGAACTTGCAGTAGAAAC	58.1	JQ308823
gpx	TCCATTCCCCAGCGATGATGCC	TCGCCATCAGGACCAACAAGGA	58.1	DQ524992
β-actin[2]	TCTGTCTGG ATC GGAGGCTC	AAGCATTTG CGGTGGACG	58.1	X89920

[1] *bmp2*, bone morphogenetic protein 2; *runx2*, runt-related transcription factor 2; *opn*, osteopontin; *on*, osteonectin; *alp*, alkaline phosphate; *enpp1*, ectonucleotide pyrophosphatase/phosphodiesterase family member 1-like, *oc*, osteocalcin; *cat*, catalase; *gpx*, glutathione peroxidase.
[2] Housekeeping gene.

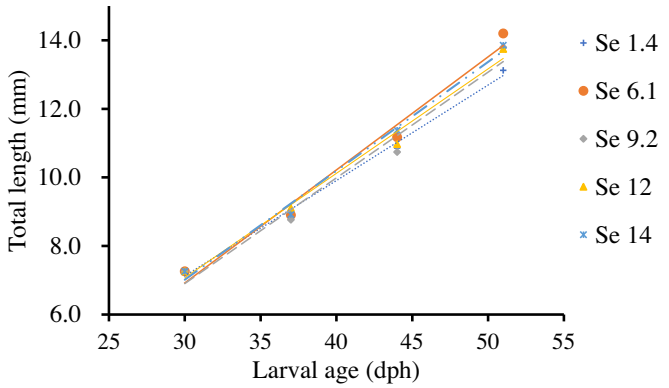

Fig. 1. Development of the total length of gilthead seabream larvae fed different diets Se1.4 ($y = 0.28\times - 1.23$, $R^2 = 1$), Se 6.1 ($y = 0.33\times - 2.98$, $R^2 = 0.98$), Se 9.2 ($y = 0.31\times - 2.35$, $R^2 = 0.97$), Se 12 ($y = 0.30\times - 2.07$, $R^2 = 0.99$), and Se 14 ($y = 0.32\times - 2.51$, $R^2 = 0.99$) from the initial (30 dph) to final point (51 dph) a total of 21 days of feeding.

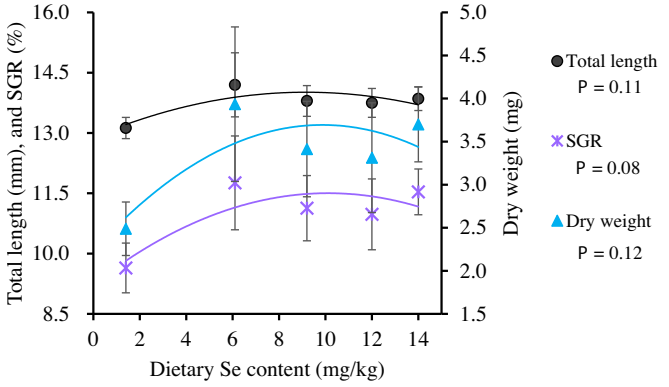

Fig. 2. Growth performance of gilthead seabream larvae fed different diets after 21 days. Data are presented as mean ± SD. Symbols presented different parameters: total length (mm), $y = -0.01 \times ^2 + 0.24\ x + 12.89$, $R^2 = 0.31$, $P = 0.11$; specific growth rate, SGR (%), $y = -0.02 \times ^2 + 0.45\ x + 9.24$, $R^2 = 0.37$, $P = 0.08$; dry weight (mg), $y = -0.02 \times ^2 + 0.31\ x + 2.23$, $R^2 = 0.29$, $P = 0.12$. The lines represent quadratic regression analysis of the growth on different dietary Se level (x-axis), the statistical significance was set as $P < 0.05$.

Table 4
Whole-body proximate composition and TBARS value of gilthead seabream larvae fed diets containing different Se levels for 21 days.

	Diets					One-way ANOVA *P* value
	Se1.4	Se6.1	Se9.2	Se12	Se14	
Total lipid[1] (%)	14.9 ± 0.67	15.6 ± 1.04	14.0 ± 0.84	15.0 ± 0.80	15.8 ± 0.61	0.14
Crude protein[1] (%)	78.2 ± 1.65	77.3 ± 2.08	76.8 ± 0.70	77.6 ± 1.02	76.8 ± 1.02	0.15
Ash[1] (%)	13.6 ± 0.30	12.8 ± 0.33	13.2 ± 0.26	13.3 ± 0.57	12.8 ± 0.17	0.22
TBARS (nmol MDA/g)[2]	76.1 ± 7.82[b]	59.9 ± 9.67[a]	56.7 ± 2.47[a]	56.1 ± 8.43[a]	54.8 ± 9.08[a]	0.03

Data expressed as mean ± SD (n = 3), the statistical analysis was performed by using one-way ANOVA, the statistical significance was set as $P < 0.05$.
[1] Expressed in dry weight, including total lipid, crude protein and ash.
[2] Thiobarbituric acid reactive substances (TBARS); Malondialdehyde (MDA).

3.3. Mineral composition

After 3 weeks of feeding trial, the elevation of Se content in larval whole body was significantly ($P < 0.05$) increased with increasing dietary Se content (Table 5). The Se content in larvae fed the Se14 diet showed the significantly ($P < 0.05$) highest Se content, being 2.9 times higher than in larvae fed the non-supplemented diet (1.4 mg Se/kg). However, other minerals including Fe, Cu, Zn, Mn, Ca, P and Ca:P ratio in larval whole body did not significantly differ ($P > 0.05$) among larvae fed different diets (Table 5).

3.4. Whole body fatty acid composition

Increase in dietary Se levels significantly ($P < 0.05$) raised the PUFA and n-3 PUFA contents in the larvae, with the highest values found in larvae fed the Se14 diet (Table 6). Similarly, DHA was significantly ($P < 0.05$) increased with dietary Se level, and larvae fed Se14 showed the highest content (Table 6). The EPA contents in larvae were also significantly ($P < 0.05$) increased by dietary Se levels although in a lower extend than DHA. Accordingly, ARA contents in larvae showed a tendency to increase with the elevation of dietary Se levels following a significant linear regression ($y = 0.02\ x + 1.08$, $R^2 = 0.95$, $P = 0.01$). Therefore, the significant high level of n-3 PUFA was mainly due to the elevation of EPA, DHA, ARA and larvae fed Se14 showed the highest EFAs and DHA/EPA ratio among all fish groups. Moreover, n-3/n-6 and EPA/ARA ratio were significantly ($P < 0.05$) increased with increasing dietary Se, with the highest values being found in larvae fed with both Se12 and Se14 diets. Interestingly, the significant ($P < 0.05$) highest Δ6 desaturated fatty acids, γ-linolenic acid (GLA, 18:3n-6) was found in larvae fed with Se12, and stearidonic acid (STA, 18:4n-3) found in larvae fed with both Se12 and Se14 diets. Although larvae fed with different diets did not show significant differences in the 18:4n-3/18:3n-3 ratio, larvae fed with Se12 have high 18:4n-3/18:3n-3 ratio and 18:4n-3 in whole body larvae, following a significant linear regression (18:4n-3/18:3n-3 ratio: $y = 0.03\times + 0.48$, $R^2 = 0.77$, $P = 0.01$; 18:4n-3: $y = 0.03\times + 0.46$, $R^2 = 0.80$, $P = 0.04$). On the contrary, MUFA and n-9 fatty acids were significantly ($P < 0.05$) reduced in larvae fed with dietary Se from 9.2 to 14 mg/kg, mostly due to the reduction of 14:1n-7, 18:1n-7 and 18:1n-9. In addition, SFA fatty acids were significantly ($P < 0.05$) reduced in larvae fed with dietary Se 14 mg/kg, due to the low 16:0 and

Table 5
Whole-body mineral composition (wet wt) of gilthead seabream larvae fed diets containing different Se levels for 21 days.

	Diet					One-way ANOVA *P* value
	Se1.4	Se6.1	Se9.2	Se12	Se14	
Se (mg/kg)	1.7 ± 0.10[a]	2.8 ± 0.15[b]	3.6 ± 0.10[c]	4.0 ± 0.25[d]	4.9 ± 0.10[e]	0.00
Cu (mg/kg)	5.8 ± 0.31	5.7 ± 0.00	5.7 ± 0.32	5.1 ± 0.25	5.4 ± 0.38	0.13
Zn (mg/kg)	91.3 ± 4.16	88.7 ± 2.08	90.3 ± 2.08	87.3 ± 1.15	91.3 ± 2.52	0.31
Mn (mg/kg)	6.0 ± 1.13	5.6 ± 0.06	6.1 ± 0.26	5.6 ± 0.35	6.2 ± 0.25	0.43
Fe (mg/kg)	31.5 ± 6.36	40.3 ± 2.89	43.0 ± 4.58	39.7 ± 5.13	39.7 ± 3.06	0.15
Ca (g/kg)	23.3 ± 3.51	19.3 ± 0.58	19.7 ± 1.53	20.0 ± 0.00	19.7 ± 0.58	0.09
P (g/kg)	20.7 ± 0.58	19.7 ± 0.58	20.0 ± 0.0	20.0 ± 1.0	20.3 ± 0.58	0.42
Ca: P	1.1 ± 0.20	0.98 ± 0.06	0.98 ± 0.08	1.00 ± 0.05	0.97 ± 0.03	0.34

Data expressed as mean ± SD (n = 3), the statistical analysis was performed by using one-way ANOVA, the statistical significance was set as $P < 0.05$.

Table 6
Final whole-body fatty acid profile of gilthead seabream larvae fed diets containing different Se levels for 21 days (% of total fatty acids, mean ± SD, n = 3).*

	Diets				
Fatty acids	Se1.4	Se6.1	Se9.2	Se12	Se14
14:0	1.46	1.53	1.50	1.67	1.12
14:1n-7	0.07^{b}	0.07^{b}	0.04^{a}	0.03^{a}	0.02^{a}
14:1n-5	0.07	0.08	0.08	0.09	0.05
15:0	0.22	0.21	0.21	0.23	0.16
15:1n-5	0.03	0.03	0.02	0.02	0.02
16:OISO	0.09^{b}	0.09^{ab}	0.09^{ab}	0.09^{b}	0.07^{a}
16:0	23.20^{b}	21.68^{b}	21.32^{b}	21.64^{b}	17.13^{a}
16:1n-7	3.17	3.12	3.07	3.35	2.50
16:1n-5	0.42	0.44	0.43	0.46	0.32
16:2n-4	0.09	0.11	0.10	0.13	0.12
17:0	0.16	0.16	0.13	0.15	0.14
16:3n-4	0.38^{b}	0.37^{b}	0.36^{b}	0.37^{b}	0.27^{a}
16:3n-3	0.24	0.21	0.22	0.20	0.19
16:3n-1	0.55	0.53	0.62	0.69	0.57
16:4n-3	0.25	0.24	0.28	0.34	0.34
16:4n-1	0.13	0.14	0.18	0.17	0.17
18:0	11.72^{c}	10.32^{b}	10.24^{b}	9.20^{a}	8.78^{a}
18:1n-9	21.97^{c}	21.70^{bc}	19.57^{ab}	18.92^{a}	18.17^{a}
18:1n-7	6.79c	6.35^{bc}	6.39^{bc}	5.50^{a}	5.76^{a}
18:1n-5	0.34	0.32	0.33	0.33	0.26
18:2n-9	1.64	1.75	1.83	1.73	1.47
18:2n-6	2.51	2.91	2.23	2.20	2.59
18:2n-4	0.15	0.13	0.14	0.15	0.14
18:3n-6	0.17^{a}	0.19^{b}	0.20^{b}	0.22^{c}	0.19^{ab}
18:3n-4	0.08	0.08	0.07	0.08	0.06
18:3n-3	0.92	1.07	0.91	0.98	0.99
18:3n-1	0.02	0.01	0.01	0.01	0.01
18:4n-3	0.49^{a}	0.61^{ab}	0.68^{bc}	0.84^{c}	0.76^{c}
18:4n-1	0.04	0.04	0.04	0.06	0.08
20:0	0.45^{b}	0.40^{ab}	0.39^{ab}	0.30^{a}	0.36^{ab}
20:1n-9	0.16	0.15	0.12	0.11	0.15
20:1n-7	1.37	1.30	1.10	0.96	1.27
20:1n-5	0.43	0.37	0.35	0.30	0.37
20:2n-9	1.31	1.36	1.40	1.14	1.19
20:2n-6	0.21	0.20	0.16	0.15	0.21
20:3n-9	0.03	0.03	0.05	0.04	0.04
20:3n-6	0.35	0.35	0.37	0.31	0.33
20:4n-6	1.12	1.15	1.24	1.29	1.33
20:3n-3	0.27^{bc}	0.30^{c}	0.21^{a}	0.23^{ab}	0.24^{ab}
20:4n-3	0.55	0.62	0.67	0.67	0.75
20:5n-3	6.88^{a}	8.06^{ab}	9.34^{bc}	10.43^{cd}	11.73^{d}
22:1n-11	0.16	0.17	0.12	0.11	0.21
22:1n-9	0.86	0.85	0.76	0.55	0.79
22:4n-6	0.04	0.04	0.05	0.04	0.06
22:5n-6	0.08	0.11	0.11	0.12	0.14
22:5n-3	1.00^{a}	1.17^{a}	1.37^{a}	1.40^{a}	1.86^{b}
22:6n-3	7.37^{a}	8.92^{ab}	10.91^{b}	12.01^{b}	16.50^{c}
ΣSFA[1]	37.31^{b}	34.37^{b}	33.87^{b}	33.27^{b}	27.76^{a}
ΣMUFA[1]	35.84^{b}	34.94^{b}	32.37^{a}	30.74^{a}	29.90^{a}
ΣPUFA[1]	26.85^{a}	30.68^{ab}	33.76^{b}	35.99^{b}	42.35^{c}
Σn − 3	17.97^{a}	21.20^{ab}	24.58^{bc}	27.11^{c}	33.37^{d}
Σn − 6	4.48	4.95	4.37	4.33	4.85
Σn − 9	25.97^{b}	25.84^{b}	23.72^{a}	22.49^{a}	21.81^{a}
n − 3/n − 6	4.01^{a}	4.35^{a}	5.59^{ab}	6.28^{b}	7.01^{b}
n-3 LC-PUFA[1]	16.07^{a}	19.07^{ab}	22.50^{bc}	24.74^{c}	31.09^{d}
DHA/EPA[1]	1.07^{a}	1.11^{a}	1.16^{a}	1.14^{a}	1.41^{b}
EPA/ARA[1]	6.13^{a}	6.99^{ab}	7.47^{abc}	8.27^{bc}	8.86^{c}
18:2n-6/18:3n-6	14.59	15.57	11.43	10.09	13.79
18:4n-3/18:3n-3	0.53	0.58	0.76	0.86	0.79
ΣEFAs[1]	15.36^{a}	18.13^{ab}	21.49^{bc}	23.73^{c}	29.56^{d}

[1] SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ARA: arachidonic acid; EFAs: essential fatty acids (ARA, EPA, DHA).

* Different letters (a, b, c, d) indicate significant differences among larvae fed different diets ($P < 0.05$).

18:0.

Overall, larvae fed with the inclusion of Se in diet showed an increase of these essential fatty acids in whole body, whereas a reduction was found in the larvae fed with non-supplemented diet (1.4 mg Se/kg) (Table 6). Thus, the larval whole body absolute fatty acids including PUFA, DHA, EPA, ARA, 18:4n-3, 18:4n-3/18:3n-3 ratio, n-3/n-6 ratio all increase with increasing Se content in whole body (Fig. 3).

3.5. Gene expression

To understand the effect of dietary Se on antioxidant genes expression in seabream larvae, the expression of two related antioxidants genes, *cat* and *gpx*, were analyzed after 7, 14 and 21 days of feeding. After 7 days of feeding, there were no significant differences in *cat* and *gpx* expression among larvae (37 dph) fed different diets (Supplementary Table 1). After 14 days of feeding, *gpx* expression was up-regulated in larvae (44 dph) fed the non-supplemented diet (1.4 mg Se/kg) and down-regulated by the increase in dietary Se, following a highly correlated quadratic regression ($R^2 = 0.96$, $P = 0.04$) (Supplementary Table 2). After 21 days of feeding, no significant differences were found in larvae (51 dph) (Supplementary Table 3). However, *gpx* expression was progressively increased by the elevation of Se content in larvae up to 12 mg Se/kg and further increase in Se levels led to the lowest *gpx* expression ($y = -0.18 \times ^2 + 1.13\times$-0.44, $R^2 = 0.66$, $P = 0.34$) (Fig. 4). In addition, *cat* expression followed a similar tendency ($y = -0.53 \times ^2 + 3.56\times$ - 3.53, $R^2 = 0.93$, $P = 0.07$) (Fig. 4).

After 7 days of feeding the expression of bone biomarkers did not differ among larvae fed different dietary Se levels (Supplementary Table 1). After 14 days, there was a tendency ($P = 0.08$) for a higher *runx2* expression in larvae fed diets Se6.1 and Se12 (Supplementary Table 2). Besides, *oc* expression also tended to be higher in larvae fed diets Se6.1, Se 9.2 and Se12, following a polynomial regression with the Se contents in the larvae ($y = -0.19 \times ^2 + 1.35\times - 0.73$, $R^2 = 0.98$, $P = 0.02$) (Fig. 5). After 21 days of feeding, despite there were not significant differences in bone related genes expression among larvae fed different dietary Se levels (Supplementary Table 3), expression of *alp* tend to be higher in larvae fed Se6.1, Se 9.2 and Se12 diets, following a polynomial regression with the Se contents in the larvae ($y = -0.31 \times ^2 + 2.06\times - 1.59$, $R^2 = 0.94$; $P = 0.06$) (Fig. 5). Indeed, the heat map representing bone related gene expression shows the highest expression in larvae fed diet Se9.2 at day 21st, whereas in general the lowest expressions at 7, 14 and 21 days of feeding were found in larvae fed diets Se1.4 and Se14 (Fig. 6).

3.6. Skeletal mineralization and anomalies

The mineralization level of vertebrae axial skeleton was calculated as the percentage of mineralized vertebrae found in larvae fed with

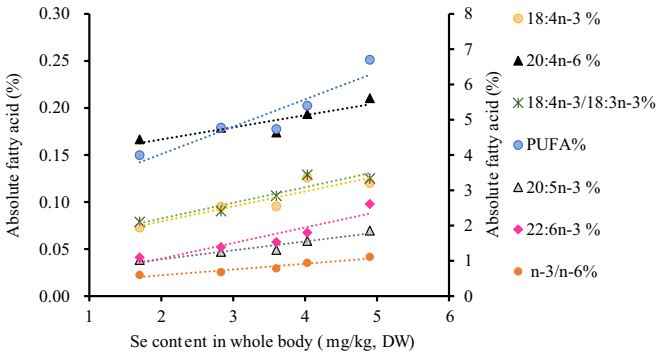

Fig. 3. Relation between Se content in larvae (mg/kg) and absolute fatty acids of gilthead seabream larvae fed diets containing different Se levels for 21 days. Fatty acids: 18:4n-3 ($y = 0.02$ x + 0.05, $R^2 = 0.82$, $P = 0.03$), 20:4n-6 ($y = 0.01$ x + 0.14, $R^2 = 0.81$, $P = 0.09$), 18:4n-3/18:3n-3 ($y = 0.02$ x + 0.05, $R^2 = 0.87$, $P = 0.04$), PUFA ($y = 0.78$ x + 2.48, $R^2 = 0.86$, $P = 0.02$), 20:5n-3 ($y = 0.25$ x + 0.54, $R^2 = 0.93$), 22:6n-3 ($y = 0.44$ x + 0.18, $R^2 = 0.86$, $P = 0.02$), n-3/n-6 ($y = 0.1634\times + 0.2644$ $R^2 = 0.93$, $P = 0.01$).

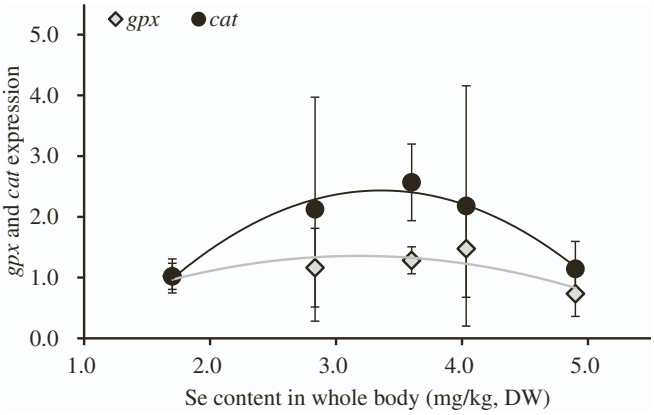

Fig. 4. Relation between whole body Se content (mg/kg) and *gpx, cat* expression in gilthead seabream larvae fed diets containing different Se levels for 21 days. ◇ *gpx*: y = −0.18 × ^{2} + 1.13 x - 0.44, R^2 = 0.66, P = 0.34; ● *cat*: y = −0.53 × ^{2} + 3.56 x - 3.53, R^2 = 0.93, P = 0.07.

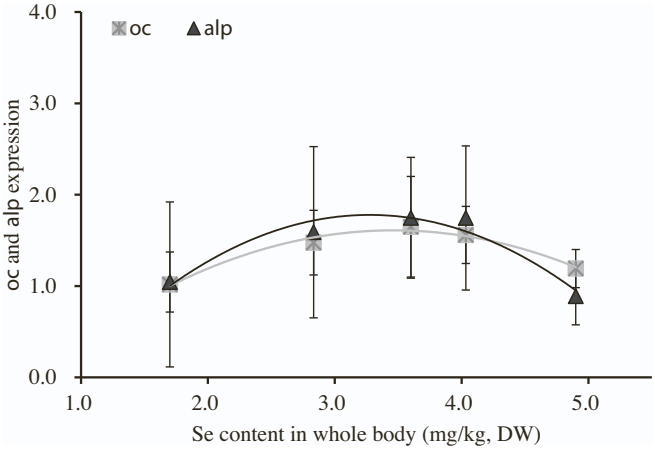

Fig. 5. Relation between whole body Se content (mg/kg) and *oc, alp* expression in gilthead seabream larvae fed diets containing different Se levels for 14, 21 days, respectively. ▩*oc*: y = −0.20 × ^{2} + 1.35 x - 0.73, R^2 = 0.98, P = 0.02; ▲*alp*: y = −0.31 × ^{2} + 2.05 x - 1.58, R^2 = 0.94, P = 0.06.

different dietary Se levels for each size class (Fig. 7). The mineralization level for larvae fed each experimental diet was increased with size class, following a high linear regression (Se1.4: R^2 = 0.77; Se6.1: R^2 = 0.79; Se9.2: R^2 = 0.92; Se12: R^2 = 0.99; Se14: R^2 = 0.72) (Fig. 8). However, the slope of those lineal regressions for mineralized vertebrae was increased in fish fed with Se6.1 diet, resulting the highest in Se9.2, 12 diets, and was lowest in larvae fed with the highest level of dietary Se (14 mg/kg), followed by that of larvae fed the lowest dietary Se (1.4 mg/kg). Besides, the slopes of the regression for mineralization were followed a significantly linear regression with *alp* expression (y = 8.52×-1.63, R^2 = 0.83, P = 0.03) (Fig. 8).

After 21 days of feeding, the frequency of total skeletal anomalies (percentage of fish bearing at least one anomaly) was reduced with increasing dietary Se levels, following a linear regression (y = −0.67× + 41.42, R^2 = 0.93, P = 0.08) (Fig. 9).Whereas, the anomalies of intramembranous bones were significantly ($P < 0.05$) reduced in larvae (51 dph) fed with increasing dietary Se levels up to 12 mg Se/kg, and a slight increase at Se 14 diet was observed, following a linear regression (y = −0.09× + 3.48, R^2 = 0.95, P = 0.01) (Fig. 9).

The skeletal anomalies described in present study were abdominal vertebrae: kyphosis (B1), partial vertebral fusion (B3), total vertebral fusion (B3*), vertebral anomaly (B4), anomalous neural arch and/or

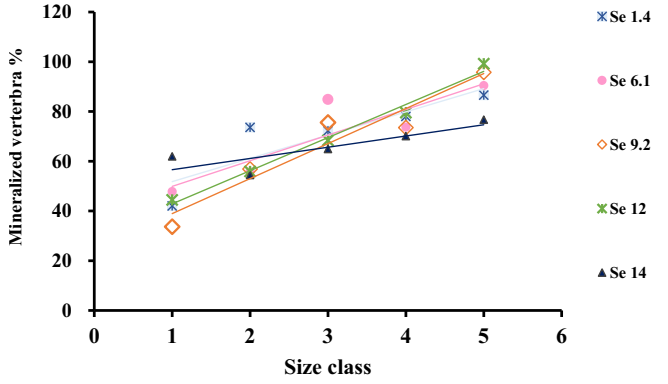

Fig. 7. Percentage of mineralized vertebrae in gilthead seabream larvae fed diets containing different Se levels for 21 days. Diets: Se1.4 (y = 9.35 x + 42.44, R^2 = 0.77, P = 0.05), Se6.2 (y = 10.31 x + 39.58, R^2 = 0.79, P = 0.04), Se9.2 (y = 14.08 x + 24.86, R^2 = 0.92, P = 0.01), Se12 (y = 13.32 x + 29.55, R^2 = 0.99, P = 0.00), Se14 (y = 4.51 x + 52.09, R^2 = 0.72, P = 0.06).

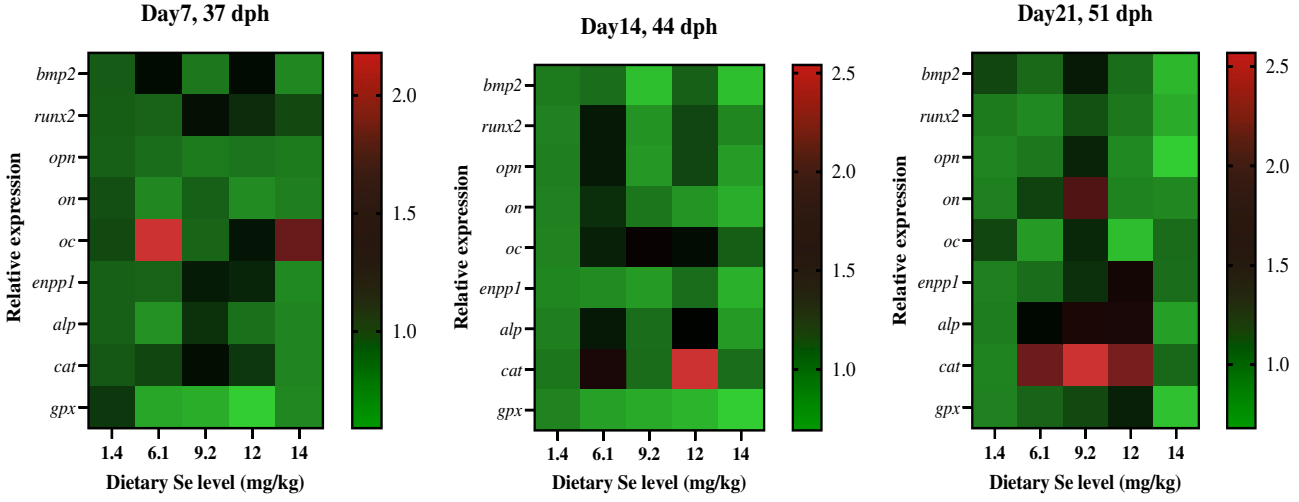

Fig. 6. Heat map representing the antioxidant and bone relative gene expression of seabream larvae fed different diets at different stages. Column in the heat map presented as dietary Se levels. Rows presented different biomarkers. Light green color indicated the lowest expression, and red color indicated the highest expression levels as shown in the scale bar of the figure. Data presented as mean value of each triplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

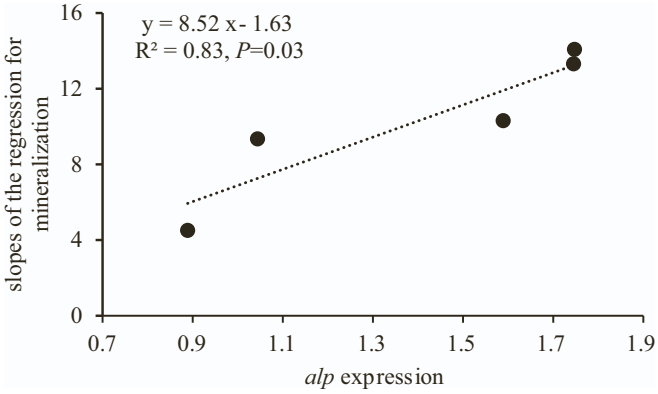

Fig. 8. Relation between slopes of the regression for mineralization (average number of mineralized vertebra in each size class) and the expression of *alp* in gilthead seabream larvae fed different levels of dietary Se after 21 days.

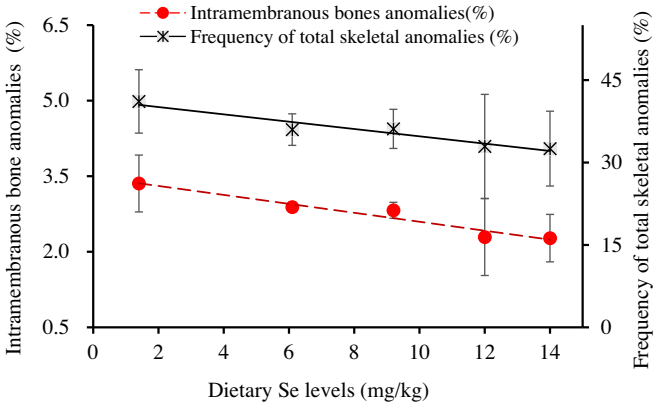

Fig. 9. Frequency of total skeletal anomalies (%) and of intramembranous bone anomalies (%) in gilthead seabream larvae fed diets containing different Se levels for 21 days. Frequency of total skeletal anomalies (%): $y = -0.67\times + 41.42$, $R^2 = 0.93$, $P = 0.08$. Intramembranous bone anomalies (%): $y = -0.09\times + 3.48$, $R^2 = 0.95$, $P = 0.01$.

spine (B5); Haemal vertebrae: vertebral anomaly (C4), anomalous neural arch and/or spine (C6), Supernumerary haemal elements/absence of haemal elements (C6*); Caudal vertebrae: partial vertebral fusion (D3), total vertebral body fusion (D3*), vertebral anomaly (D4), anomalous neural arch and/or spine (D5), anomalous neural arch and/or spine (D6), Supernumerary haemal elements/absence of haemal elements (D6*); Caudal fin**:** anomalous hypural (G9), supernumerary bone (G26); Anomalous maxillary and/or pre-maxillary (14); Anomalous dentary (15); Anomalous, absent, fused branchiostegal ray (17*).

The frequency of skeletal anomalies per regions (Table 7) did not show significant differences among larvae (51 dph) fed with different dietary Se levels. According to the observations made in the different regions, the mean prevalences of skeletal anomalies were found to be higher in cranium (18.12%), abdominal vertebrae (16.33%) caudal vertebrae (23.80%), and lower in caudal fin (5.88%), cephalic vertebrae (0.13%) and haemal vertebrae (1.35%), respectively (Table 7.).

A reduced tendency of anomalies in the abdominal vertebrae region was found in larvae fed with increasing dietary Se levels, following a significant quadratic regression ($y = -0.01 \times {}^{2}\text{–}0.94\times + 25.03$, $R^2 = 0.97$, $P = 0.04$) (Fig. 10A). This result was mostly due to the significant reduction of kyphosis ($R^2 = 0.97$, $P = 0.03$) (Fig. 10B) and abdominal vertebral anomaly ($R^2 = 0.97$, $P = 0.03$) (Fig. 10C) followed by quadratic regression. In addition, no anomalies were found in cephalic vertebrae when larvae were fed with Se supplemented in diets ($R^2 = 0.95$, $P = 0.04$) (Fig. 10D). Noteworthy, larvae fed with dietary Se level

Table 7
Frequency of skeletal anomalies in different regions of seabream larvae (51 dph) fed different dietary Se levels for 21 days (150 larvae/treatment).

Anomalies detected per region (%)	Diets					One-way ANOVA *P* value
	Se1.4	Se6.1	Se9.2	Se12	Se14	
Cranium	17.4 ± 7.47	22.9 ± 6.13	18.0 ± 0.93	18.8 ± 8.12	13.5 ± 0.87	0.43
Cephalic vertebrae	0.7 ± 1.15	ND	ND	ND	ND	0.45
Abdominal vertebrae	24.0 ± 10.60	18.1 ± 1.85	15.9 ± 2.56	14.2 ± 9.36	9.5 ± 2.60	0.16
Haemal vertebrae	1.3 ± 1.15	ND	1.4 ± 2.36	2.0 ± 2.00	2.1 ± 2.08	0.62
Caudal vertebrae	29.5 ± 13.51	26.1 ± 21.58	26.1 ± 7.50	13.4 ± 3.09	23.8 ± 10.84	0.62
Caudal fin	4.5 ± 3.12	8.2 ± 4.25	9.9 ± 1.51	2.7 ± 3.05	4.0 ± 1.96	0.06

Data expressed as mean ± SD ($n = 3$), the statistical analysis was performed by using one-way ANOVA, the statistical significance was set as $P < 0.05$; ND, not detected.

at 12 mg/kg showed low anomalies in caudal vertebrae (13%) in comparison with other diets (>20%) (Table 7). Some skeletal anomalies presented in Fig. 11.

4. Discussion

The necessity of including Se in fish diets to improve fish growth has been found in several studies (Le and Fotedar, 2013; Lin and Shiau, 2005; Liu et al., 2010; Kim et al., 2014; Mansour et al., 2017), including gilthead seabream juveniles (Domínguez et al., 2020). In the present study, neither growth nor survival of gilthead seabream were significantly affected by the dietary Se levels. These results agree well with previous studies in gilthead seabream larvae, where a dietary Se increase from 1.73 to 11.65 mg/kg did not affect total length or body weight (Saleh et al., 2014). However, in this previous study, survival was significantly improved by the elevation of dietary Se up to 8 mg/kg (Saleh et al., 2014). In another study (Betancor et al., 2012), after 21 days of feeding European seabass (*Dicentrarchus labrax*) larvae with microdiets containing two Se levels, the increase in dietary Se from 1.33 to 6.27 mg/kg significantly improved larval total length by 3.5% but did not affect body weight or survival. In agreement, in the present study, increase in dietary Se in microdiets from 1.4 to 6.1 mg/kg did not affect survival or body weight, and also increased total length by 10%, although the large standard deviation did not allow to obtain significant differences. Therefore, it seems that the dietary Se levels tested in these 3 studies (1.33–11.65 mg/kg) may have little influence in larval growth. Interestingly, in those previous studies the TBARs contents ranged between 282 and 2402 and 130–230 nmol MDA/g larvae (Betancor et al., 2012; Saleh et al., 2014), being much higher than in the present study (54.79–76.06 nmol MDA/g larvae) and denoting a much higher peroxidation risk and the unbalance among pro- and antioxidant nutrients. In agreement, in the present study larval survival was very high (83.16–86.38%), whereas in the previous studies it was lower than 55% (Saleh et al., 2014), despite similar larval age, Se source, feed proximate composition and feeding periods. Therefore, in the present study the lack of effect of Se on survival could be related to a better antioxidant defenses status on the larvae of the present study in comparison to the previous ones.

Increase in dietary Se significantly increased whole body Se, following a lineal regression in agreement with previous studies in gilthead seabream (Saleh et al., 2014). Moreover, increase in dietary Se

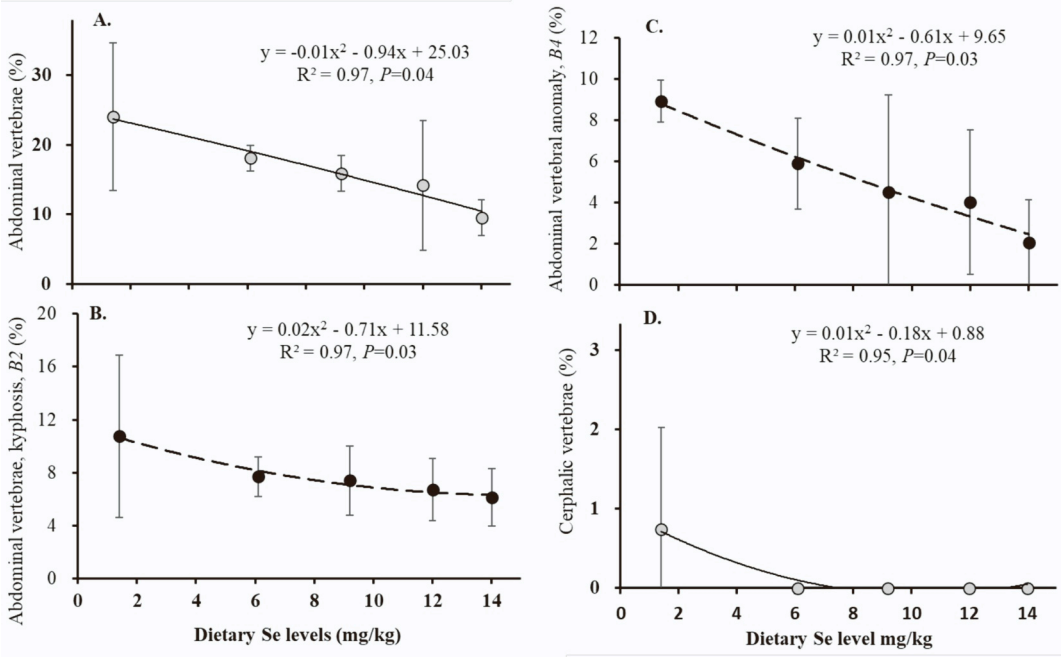

Fig. 10. Frequency of skeletal anomalies % in different regions of gilthead seabream larvae fed diets containing different Se levels for 21 days.

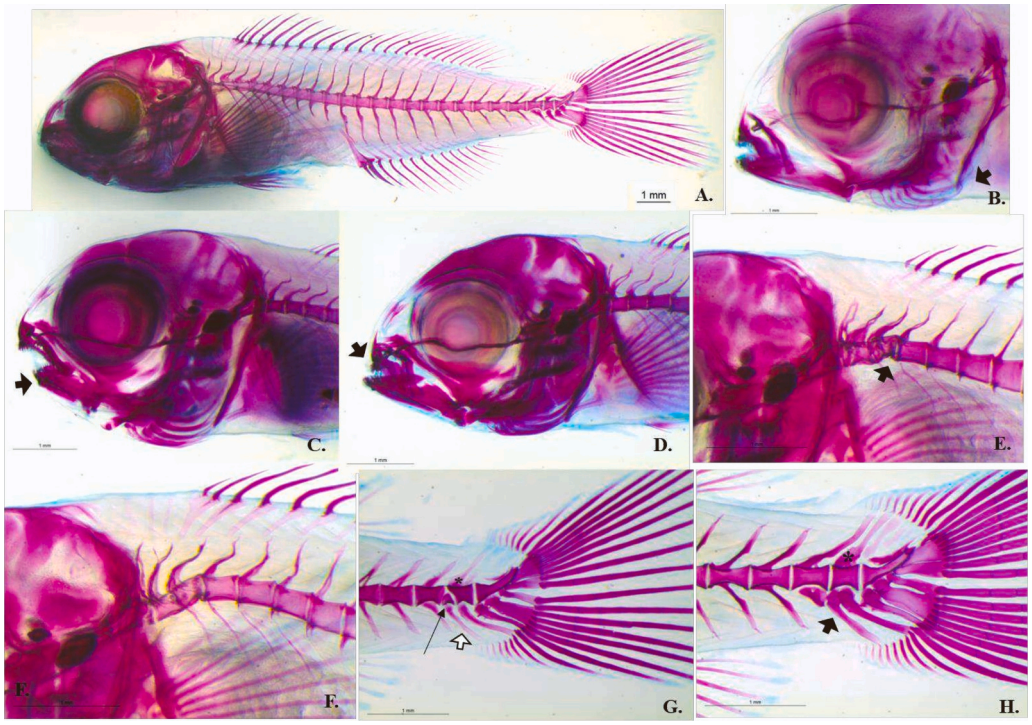

Fig. 11. Typology of skeletal anomalies in gilthead seabream larvae (51 dph). A: Fish larvae with normal vertebrae column; B: Anomalous branchiostegal rays (17*); C: Anomalous dentary (15); D: Abdominal premaxillary (14); E: Vertebral anomaly (B4), vertebra compression; F: Anomalous vertebrae kyphosis (B1); G: Anomaly caudal vertebrae, an extra vertebra hemivertebrae (black arrow) (D4) with heterotopic haemal element (D6*) (white arrow) and heterotopic neural element (D5*) (*); H: heterotopic, mineralized skeletal element in the haemal spine in caudal vertebral region (D26) (black arrow), and heterotopic neural element (D5*) (*).

from 1.33 to 6.27 mg Se/kg diet increased Se contents in whole body of sea bass larvae from 1.11 to 2.65 mg Se/kg (Betancor et al., 2012), very close to the Se contents observed in the present study (1.70 to 2.83 mg Se/kg) when feeding similar dietary Se levels (1.4 and 6.1 mg Se/kg diet). In agreement with the results of the present study, in cod larvae, fish fed Se enriched rotifers showed a higher Se content in larvae tissue (3.99 mg Se/kg) and the Se levels did not affect larval growth (Penglase et al., 2010). On the contrary, other sparidae species, as red seabream (*Pagrus major*), increase in Se levels in rotifers from 0 to 2.2 mg/kg elevated the Se contents from 1.3 to 9.5 mg/kg in larval whole body together with a growth improvement (Kim et al., 2014). Such higher body content in Se in comparison to the present study could be a species-specific difference or be related to a better utilization of dietary Se when this highly water-soluble mineral is fed through the protection of the

rotifer body instead of a microbounded diet.

Even though growth and survival were not significantly reduced in gilthead seabream larvae fed the lowest dietary Se level (1.4 mg/kg), these larvae tended to reduce growth presented symptoms of oxidative stress and the highest lipid peroxidation. Thus, increase in dietary Se from 1.4 to 6.1 mg/kg and its increase in larval whole body significantly reduced TBARS values, evidencing a reduction in oxidative stress. These results agree well with the reduction in TBARS values obtained by the increase in dietary Se levels in other species such as giant grouper (*Epinephelus lanceolatus*) (0.3–1.0 mg Se/kg, Lin and Lin, 2021) or meagre (0.77–3.98 mg Se/kg, Mansour et al., 2017). Besides, improved protection against lipid peroxidation has been also found in other fish studies (Betancor et al., 2012; Lin and Lin, 2021; Mechlaoui et al., 2019; Saleh et al., 2014). Actually, Se incorporated within selenoproteins plays a major role in the antioxidant system, such as GPX, thioredoxin reductase (TRXR) and methionine sulphoxide reductases (MSR). Fish fed diets with Se inclusion presented an enhanced expression of these antioxidants (Fontagné-Dicharry et al., 2020; Lin and Shiau, 2005; Pacitti et al., 2016; Antony Jesu Prabhu et al., 2020). In agreement in the present study, Se increase up to 12 mg/kg in microdiets and up to 4 mg/kg in larval whole body, linearly up-regulated *gpx* expression. Moreover, the elevation of dietary Se up to 6.1 mg Se/kg increased the larval antioxidant capacities linked with the up-regulated expression of the Fe-dependent catalase gene (*cat*) ($R^2 = 0.97$, $P = 0.03$). The increase of Se content in larvae, *cat* expression and reduced TBARS value, suggested a strengthened antioxidant system, in agreement with previous studies in fish (Du et al., 2021; Jingyuan et al., 2020) and humans (Fatima et al., 2021).

Because of the reduction in lipid peroxidation, there was an increase in PUFAs, particularly in DHA, EPA and ARA, when dietary Se was increased in gilthead seabream larval diets in the present study. Indeed, dietary mineral supplementation may affect lipid metabolism in terms of increased lipogenesis or alteration of the fatty acids' profiles in fish and mammals (Knez et al., 2022; Tseng et al., 2023; Tseng et al., 2021; Zheng et al., 2015). Thus, the results of the present study agree well with the increased in DHA and n-3 LC-PUFA content found in whole body of seabass and seabream larvae when dietary Se was increased in microdiets (Betancor et al., 2012; Saleh et al., 2014). Conversely, the elevation of dietary EPA + DHA levels can also increase the Atlantic salmon whole-body contents in Se, as well as in another two antioxidant minerals, Zn and Mn (Selvam et al., 2022). Both DHA and EPA are essential fatty acids for marine fish species since they have a low ability to convert α-linolenic acid (ALA, 18:3n-3) to LC-PUFAs (Izquierdo et al., 2015; Izquierdo et al., 2008; Turkmen et al., 2017). In the n-3 LC-PUFA biosynthetic pathway, stearidonic acid (SA, 18:4n-3) is the first metabolite constituted from the initial precursor (18:3n-3) by delta 6 fatty acid desaturase (D6 desaturase). Then 20:4n-3, the elongation product from 18:4n-3 by elongase 5 (Elovl-5). Therefore, DHA and EPA can be synthetized through several steps of elongation, desaturation and β-oxidation. Interestingly, in the present study the elevation of dietary Se significantly enhanced 18:4n-3 (first metabolite of ALA), EPA, end-product DHA and the total EFAs, suggesting that the inclusion of Se in diets induced the n-3 PUFA biosynthesis activity of fish in agreement with our previous study in seabream at the juvenile stage (Tseng et al., 2021). This positive effect of Se on the activation of n-3 PUFA synthesis in fish could be related to a protective effect of Se against oxidative damage to the fatty acids, in larvae fed with adequate dietary Se level. Besides its antioxidant effect, Se supplementation in a mice diet also up-regulated the expression of peroxisome proliferator-activated receptor alpha gene (*Ppar-a*), a key regulator of β-oxidation of FA in peroxisomes and mitochondria (Nido et al., 2016), suggesting the role of Se in fatty acid metabolism. Hence in present study, the enhanced EFAs level in larvae tissues on fish fed with increasing dietary Se levels appears to be the result of the antioxidant protection against the attack from reactive oxygen species (ROS) to polyunsaturated membrane lipids but could also be related to a potential role of Se in fatty acid metabolism in seabream larvae.

Excessive production of ROS caused by deficient Se can induce the apoptosis of osteoblasts or osteocytes, thereby promoting the osteoclast formation and strengthening the bone resorption by nuclear factor-kB (NF-kB) activation (Domazetovic et al., 2017). In turn, this would promote the production of pro-inflammatory cytokines in osteoclasts, such as tumor necrosis factor-α (TNF-α), and interleukins (IL)-1, IL-6, IL-17, IL-23 (Seong et al., 2016). Besides, excess of ROS stimulates RANKL (receptor activator of nuclear factor kappa-B ligand) expression in osteoblast lineage cells, the important signal mediator for osteoclast differentiation, thus may induce the osteoclast activity (Poudel et al., 2022b). Therefore, the imbalance between bone formation and bone resorption caused by oxidative stress results in abnormal bone homeostasis and development of bone disorders. In agreement, gilthead seabream larvae fed with non-supplemented diets (1.4 mg Se/kg), apart from a high lipid peroxidation, showed the highest percentage of fish with skeletal anomalies. A higher incidence of bone anomalies has been also found in seabream larvae fed a diet without supply Se (1.9 mg Se/kg), in comparison to those fed diet with containing organic Se (4.9 mg Se/kg diet) together with other microminerals (Izquierdo et al., 2017). Thus, in the present study, the larvae fed the lowest dietary Se levels (1.4 mg Se/kg), showed the highest incidence of anomalies in intramembranous bones. Particularly in cephalic and abdominal vertebrae and abdominal kyphosis, in agreement with the low vertebral mineralization. Mineralization of the vertebral column in gilthead seabream occurs at early stage (5.7 to 6.0 mm) and it is directly initiated from notochord sheath in craneo-caudal direction (Faustino and Power, 1998). In the present study, at size class 5 (14.50–15.3 mm TL), larvae fed 12 and 9.2 mg Se had around 100% of their vertebrae mineralized, whereas in smaller larvae fed lower dietary Se contents even <50% of the vertebrae were mineralized. Therefore, abdominal vertebrae were in the process of getting mineralized during the first days of the trial (7.26 mm initial larval TL) and the oxidative stress caused by low dietary Se may have disrupt mineralization leading to a higher incidence of anomalies in this region.

Moreover, Se regulates bone biomarkers expressions, which are essential for maintaining the normal skeletal development (Kupsco and Schlenk, 2016; Saleh et al., 2014; Yang et al., 2022). Among bone biomarkers, BMP2 plays an important role in mesenchymal stem cells (MSCs), osteoblasts differentiation, and induces bone and cartilage formation. Besides, it also upregulates the key transcription factor Runx2 as the master regulator of other downstream osteoblastogenic markers such as *alp*, *enpp1*, *opn*, *on* and *oc* (Amarasekara et al., 2021). In the present study, increase in dietary Se up to 6.1 to 12.0 mg/kg up-regulated *oc* expression after two weeks of feeding in agreement with previous studies in gilthead seabream larvae of the same age (44 dph) fed a Se supplemented microdiet (4.9 mg/kg) (Terova et al., 2018). Besides, after 3 weeks of feeding, increase in dietary Se up to 6.1 to 12.0 mg/kg also up-regulated *alp* expression. Furthermore, larvae fed the 9.2 mg Se/kg diet showed the highest values of expression for *bmp2, opn, on* and *oc*. Whereas *oc* promotes mineral deposition in the extracellular matrix of bone, *opn* and *on* are closely related matrix proteins with calcium-binding properties and *alp* regulates bone mineralization by hydrolyzing pyrophosphate, an inhibitor of mineralization (Riera-Heredia et al., 2019). Therefore, the high expression of these bone biomarkers resulted in high mineralization in larvae fed with dietary Se levels at 6.1 to 12 mg/kg in agreement with the previous study found in seabream larvae fed with inclusion of Se diet (8.47–11.65 mg Se/kg) (Saleh et al., 2014). Indeed, after 21d of feeding, a significant lineal regression was found between *alp* expression and vertebral mineralization in the present study. These results agree well with the good skeletal development denoted by the advanced notochord flexion in red seabream larvae fed with Se enriched rotifers (9.5 mg Se/kg), in comparison with the non-enriched rotifers (1.3 mg Se/kg) (Kim et al., 2014). Adequate Se levels also enhance expression of bone biomarkers (*ALP, RUNX2, ON, OPN, COL1A1*) and increased mineralization in humans

(Fatima et al., 2021).

Therefore, the elevation of dietary Se up to 6.1–12 mg Se/kg, reduced oxidative stress, increased EFA contents and up-regulated antioxidant and bone metabolism related genes in larvae, contributing to reduce skeletal anomalies. Indeed, adequate levels of dietary Se protect MSCs from the ROS-induced inhibition of osteoblastic differentiation (Zeng et al., 2013). Dietary supplementation of other antioxidants such as resveratrol or the antioxidant compound from tunicates, also promotes bone mineralization and reduces skeletal anomalies in seabream (Poudel et al., 2022a) and zebrafish larvae, respectively (Carletti et al., 2022; Poudel et al., 2022b). Besides a well-balanced ratio of pro-oxidant and anti-oxidant nutrients such as DHA (Izquierdo et al., 2013), vitamin A (Fernández et al., 2008), vitamin D_3 (Sivagurunathan et al., 2022), vitamin E (El-Sayed and Izquierdo, 2022), vitamin K (Richard et al., 2014; Sivagurunathan et al., 2023), vitamin C (Izquierdo et al., 2019), copper (Tseng et al., 2023) and microminerals (Zn, Se, Mn) (Izquierdo et al., 2017) reduces the incidence of skeletal anomalies in fish larvae.

However, further increase in dietary Se up to 14 mg/kg led to the lowest *gpx*, *cat* and *alp* expressions, in agreement with a lower mineralization. These results of reduction in antioxidant enzymes concur with those found in coho salmon (*Oncorhynchus kisutch*) alevins (Du et al., 2021) and suggest a potential adverse effect of excessive dietary Se levels in young fish. Toxic effects of excessive dietary Se contents, such as growth or survival reduction, have been found in previous fish studies (Domínguez et al., 2020; Le and Fotedar, 2013; Lin and Shiau, 2005; Liu et al., 2010; Mansour et al., 2017). In the present study, despite the downregulation of these antioxidant and bone metabolism related genes and reduced mineralization, increase in dietary Se levels up to 14.0 mg/kg did not negatively affected growth or survival.

5. Conclusions

In the present study, feeding gilthead seabream larvae for 3 weeks with a diet containing only 1.4 mg Se /kg diet did not prevent oxidative risk, leading to the lowest whole-body Se and EFA contents and the highest TBARS and skeletal anomalies. Although growth was not significantly affected by diets, dietary Se level at 6.1 mg/kg had a higher growth performance than those fed with non-supplemented diet (1.4 mg/kg), between Se6.1 to Se14 diet reached a plateau level. Besides, increase up to 6.1–12 mg Se/kg, reduced oxidative stress, increased whole body EFA contents and vertebral mineralization, up-regulated antioxidant and bone metabolism related genes in larvae and contributing to reduce skeletal anomalies. However, further increase in dietary Se up to 14 mg/kg led to the lowest *gpx*, *cat* and *alp* expressions, in agreement with a lower mineralization. These findings suggest, dietary Se level at 6.1 mg/kg would be the minimum dietary Se level required in microdiets for gilthead seabream larvae. The results demonstrate the important role of Se for bone metabolism and mineralization due to its properties of antioxidation and osteoinduction.

CRediT authorship contribution statement

Yiyen Tseng: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marisol Izquierdo:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **U. Sivagurunathan:** Writing – review & editing, Visualization, Investigation, Data curation. **Antony Jesu Prabhu Philip:** Writing – review & editing, Resources, Methodology, Data curation. **María Jesús Zamorano:** Writing – review & editing, Methodology. **David Dominguez:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741097.

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