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Threshold of nutritional quality for the Mediterranean production

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Deliverable 4.1

Threshold of nutritional quality for the Mediterranean production

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1. Introduction

Fish meal is being increasingly substituted in diets for gilthead sea bream and Sea bream with ingredients of terrestrial origin. The replacement of Fish Meal (FM) and Fish Oil (FO), as main protein and lipid sources by alternative ingredients, may alter the micronutrient contents in the diets, leading to deficiencies or excess in several micronutrients related to fish growth, feed efficiency and health (Fountoulaki et al., 2016; Dominguez et al., 2019a, 2019b). Nowadays, aquafeeds for Mediterranean species are mostly based on nutritional requirements determined for other species (NRC, 2011) and in many most micronutrients, the requirements have not been yet determined for fish species. Hence, those feeds may be deficient or overdosed in some nutrients and, consequently, negatively affect KPIs (Dominguez et al., 2016). PerformFish WP4 aimed to define the recommended levels for minerals and vitamins in gilthead sea bass and Sea bream diets to produce Species-Specific Nutrient Balanced Formulations and improve KPIs in relation to growth, feed utilization and mortality in Mediterranean aquaculture.

Despite there is scarce information published about nutrients requirements and availability for gilthead sea bream and sea bass (essential fatty acids, protein,…), certain national and EU funded projects produced some information about the recommended dietary levels for specific nutrients (phospholipids, Zn, Vit B5…). This critical nutritional information has been compiled and collected by PerformFish and included in Milestone 4.1. Besides, since the nutritional requirements of some nutrients essential to improve KPIs have not been yet determined for gilthead sea bream and sea bass, Performfish has conducted a series of studies to determine the recommended dietary levels to produce Species-Specific Nutrient Balanced Formulations. In the case of gilthead sea bream, the trials were conducted to define the effect of fat-soluble vitamins (A, K, and D), minerals (Cu, Mn and Se) and B group vitamins (B1, B9, and B12), whereas for Sea bass different profiles of chelated minerals have been tested.

Deliverable 4.1 is mainly composed of the major experimental findings and, thus, the nutrient recommendations to define the threshold of nutritional quality for both fish species as keystone elements of the Mediterranean production.

2. Gilthead sea bream

2.1.Copper

2.1.1. Functions

Copper (Cu) is an essential metal that forms part of metalloenzymes involved in numerous physiological and structural functions in fish including antioxidant protection, such as copper−zinc superoxide dismutase and catalase (CuZnSOD), cellular energy production,

neurotransmitters metabolism or synthesis of collagen synthesis and melanin. Copper tends to accumulate in the liver, eyes, heart and brain of fish.

2.1.2. Requirements

Dietary Cu requirements of most fish species range at concentrations of 3–13 mg Cu kg^{-1} dry diet, whereas this quantity may increase depending on the species or during rapid growth phases of their life cycle (Antony Jesu Prabhu et al., 2016). The highest optimum dietary levels reported (18.0 mg Cu kg^{-1}) are found for blunt snout bream (*Megalobrama amblycephala*) (Shao et al., 2012).

2.1.3. Effects on KPIs and biomarkers

Several KPIs can be affected by dietary levels of copper such as mortalities, final body weight, specific growth rates or food conversion rates. Copper deposition in tissues is also used to determine Cu requirements. Besides, several molecular markers can be affected by Cu, such as regulators of oxidative damage like CuZnSOD, or Cu transporters, like copper transporter 1 and atp7b.

2.1.4. Toxicity

The main concern with Cu levels in aquafeeds has traditionally been related to its potential toxic effects on fish, which can vary from reduced growth, feed ingestion and productivity, to increased cell apoptosis, hepatic lipid peroxidation, damage to gills and necrosis in liver and kidney. Copper may lead to alterations in several physiological functions and markers when its level is inadequate.

2.1.5. Practical aspects

Up to 28 mg Cu kg^{-1} can be found in certain plant ingredients, such as corn gluten or soy bean meal derived feedstuffs. This values are up to 5 fold the amount compared to FM. Therefore, it is important to asses the amount of copper to be supplemented in feeds containing high levels of plant ingredients.

The effects of Cu on gilthead sea bream have been evaluated focusing on Cu transporters (Minghetti et al., 2008), Cu proteins (Minghetti et al., 2010), seasonal Cu tissue changes (Carpené et al., 1999) and the effects of toxic levels of Cu on metallothionein (Ghedira et al., 2010). However, none of these studies had addressed the Cu requirements in gilthead sea bream. PerformFISH evaluated the effect of dietary Cu supplementation (5.5-32 mg kg-1) on growth, productive parameters and health status of gilthead sea bream fed diets containing only 10% FM and 6% FO (Dominguez et al., 2019a).

2.1.6. Dietary levels for sea bream

In gilthead sea bream juveniles (10-40g) and up to 300g, feeding a diet containing 10% FM and several of the plant ingredients containing higher Cu levels than FM (5–6 mg Cu kg^{-1}), such as soya concentrate (23 mg Cu kg^{-1}) or corn gluten (12 mg Cu kg^{-1}), the Cu levels in the non-supplemented diet (5.5 mg Cu kg^{-1}) are enough to cover gilthead sea bream requirements for growth (Domínguez et al., 2019a). Increase in dietary Cu up to 9.3 mg kg^{-1} raises liver Cu contents, contributes to reduce skeletal anomalies incidence by almost 10% and up-regulates *cat* expression and Cu transport related genes, *ctr1* and *atp7b* without negatively affecting growth. These quantitative requirements (5.5-9.3 mg Cu kg^{-1}) are close to those found for Atlantic salmon or malabar grouper, but higher than those determined for carp or tilapia among other species (Domínguez et al., 2019a). Nevertheless, dietary Cu requirements may also be affected by the dietary Cu source used, fish age, welfare status, water Cu levels and, most possibly, dietary levels of other minerals such as iron or zinc (Lall, 2002; Lin et al., 2010).

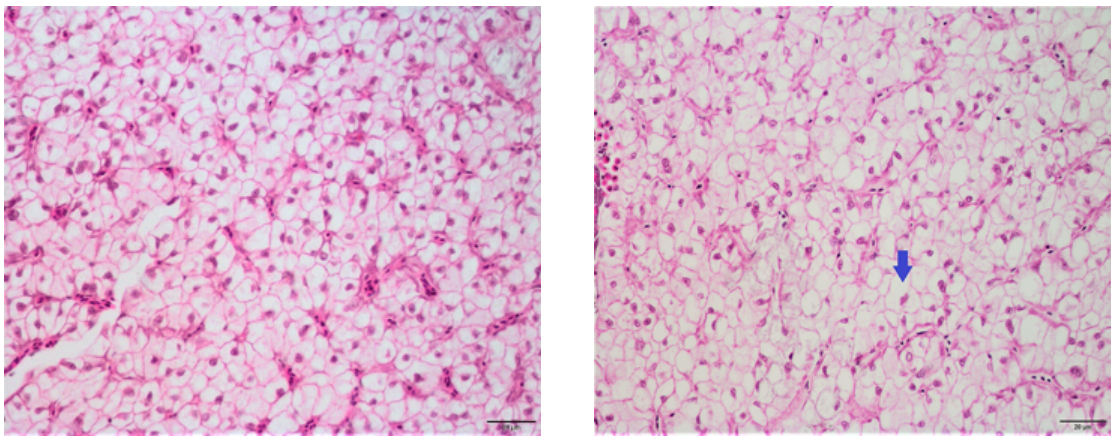


Figure 2.1.2. Microscopic view of liver cell margin (40x). A) well-preserved cell margins. B) Broken cell margins

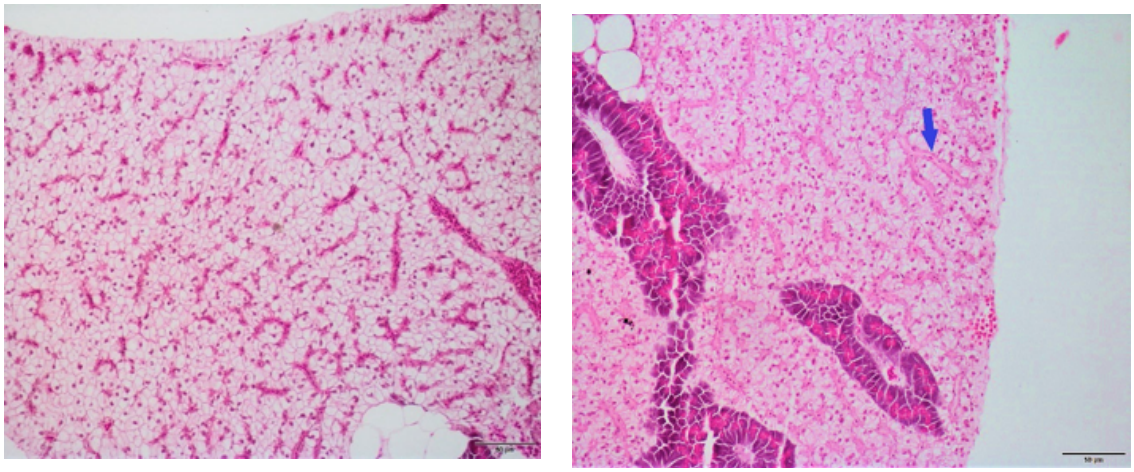


Figure 2.1.2. Microscopic view of liver cell sinusoids (20x). A) Sinusoids with abundant erythrocytes. B) Sinusoids dilated with plasma and without erythrocytes

2.1.7. Toxic levels for sea bream

However, further Cu supplementation up to 11–32 mg Cu kg^{-1} reduces growth by 14%, and increased FCR by 11% in only 1.5 months. Moreover, it increases peroxidative risk, down-regulating cat expression and increasing TBARs, one of the most commonly used indicator of tissue peroxidation, and damaged hepatic tissue, leading to increased broken cell margin and sinusoids dilatation. It is interesting to remark that this hepatic damage found in gilthead sea bream fed 11–32 mg Cu kg^{-1} can be also observed in rainbow trout but exposed at 15 times higher Cu levels (500 mg Cu kg^{-1} Handy et al., 1999) than those tested in gilthead sea bream.

2.1.8. Recommendations

In summary, dietary Cu recommended levels for gilthead sea bream are 5.5-9.3 mg Cu kg^{-1} and since Cu is frequently present in most animal (including blood meal spray) and plant protein sources (5–30 mg Cu kg^{-1}), as well as in the aquatic environment, deficiency symptoms should not appears in commercial farms. Moreover, little or even non Cu supplementation is required in gilthead sea bream fed practical diets based on plant protein sources, if these sources provide between 5.5 and 9.3 mg Cu kg-1. However, gilthead sea bream is sensitive to excessive dietary Cu levels, an increases over 9.3 mg Cu kg^{-1} should be avoid; for example, 11 mg Cu kg^{-1} could trigger hepatic damage and growth reduction. Therefore, even though the maximum dietary Cu content allowed by EFSA (2014) is 25 mg kg^{-1}, dietary contents over 9.3 mg Cu kg^{-1} should be avoided to prevent negative effects on gilthead sea bream growth, increase oxidative risk and hepatic damage and cholestasis.

2.2.Selenium

2.2.1. Functions

Selenium (Se) is an essential element for fish and plays important roles in different biological processes including antioxidant protection or physiological responses to stress (Domínguez et al., 2019b). Selenium forms part of selenoproteins, being the teleost fishes the organisms with the highest number of them. The glutathione peroxidase (gpx) is among the most important selenoprotein family, as it forms part of one of two enzymatic systems present in vertebrates able to metabolize hydrogen peroxide to water. In gilthead sea bream the gpx homologues have been studied.

2.2.2.Requirements

Selenium requirements are high in fast-growing species such as cobia (*Rachycentron canadum*), malabar grouper (*Epinephelus malabaricus)*, meagre (*Argyrosomus regius*) or yellowtail kingfish (*Seriola lalandi*), whereas other species do not require Se supplementation (Dominguez et al., 2019b). Described Se requirements for aquaculture fish range between 0.12 and 7.37 mg Se kg^{-1} (Dominguez et al., 2019b). In the case of Sparids, requirements for red sea bream (*Pagrus major*) and black sea bream (*Acanthopagrus schlegelii*), species phylogenetically closely related to gilthead sea bream, were 1.34 mg Se kg^{-1} (Dawood et al., 2018) and 0.86 mg mg Se kg^{-1} (Wang et al., 2019), whereas optimum Se dietary levels for gilthead sea bream had not been determined yet.

2.2.3.Effects on KPIs and biomarkers

Several KPIs can be affected by dietary levels of selenium such as mortalities, final body weight, specific growth rates or food conversion rates. Besides, Se deficiencies can produce lethargy, diminished appetite, muscle dystrophy, reduced vitamin E levels and low haematocrit, affecting fish health and resistance to infectious diseases. Therefore, the main criteria to evaluate requirements for Se in fish are growth, feed efficiency, tissue retention, antioxidant activity/ expression markers and immune response/haematology (Antony Jesu Prabhu et al., 2016; Khan et al., 2017). The activity and expression of glutathione peroxidase have been used to evaluate Se requirements in fish species. The other enzymatic systems able to dispose of hydrogen peroxide are catalases. Expression of catalase (*cat*) can be used to evaluate oxidative status, and high doses of Se have been observed to increase its activity in several fish species (Dominguez et al., 2019b).

2.2.4.Toxicity

In the case of Se, the margin between requirement and toxicity is very narrow. Selenium in toxic levels associates to sulphur containing amino acids due to its similar properties, thus altering the functional enzyme. Furthermore, under stressful conditions, Se requirements are increased, whereas under normal conditions these levels would be considered toxic. Selenium toxicity curses with reduced growth and feed efficiency, increased oxidative stress mortality,

increased skeletal anomalies, oedema, decreased egg viability, altered immunological functions, necrosis of renal tubules and renal calcinosis.

2.2.5. Practical aspects

In commercial aquafeeds for carnivorous fish, such as gilthead sea bream, FM has traditionally been the main source of Se (Domínguez et al., 2019b). Selenium concentrations on plant ingredients vary greatly depending on plant species and soil, and certain areas have been observed to contain Se in levels toxic to livestock, while others are considered deficient for animal nutrition. Still, feeds formulated with ingredients with low levels of Se have little margin for Se supplementation since regulations in the European Union are strict and account for a maximum of 0.2 mg kg^{-1} for organic Se (Regulations [EU] No 427/2013; 445/2013; 121/2014; 847/2014 and 2015/489) and 0.5 mg kg^{-1} feed for total Se in animal feeds including fish (EC 1831/2003 and amendments). Indeed, regulations for Se supplementation in feeds for certain fish species may be below the levels considered as required. Regulations in the use of Se as a supplement in feeds contribute to reduce discharges of Se to the environment and keeping its levels below toxic.

There was a scarce knowledge available for selenium nutrition in gilthead sea bream and its essentiality and possible effects as contaminant, highlights the importance of further research in this area. For this reason, PerformFISH evaluated the effect of dietary NaSe supplementation (0.45-1.7 mg Se kg^{-1}) on growth, productive parameters and health status of gilthead sea bream fed diets containing only 10% FM and 6% FO (Dominguez et al., 2019b).

2.2.6. Dietary levels for sea bream

In gilthead sea bream juveniles (10-40g), the increase in dietary Se contents up to 0.94 mg Se kg-1, supplementing NaSe, improves growth measured as final standard length and body weight, increasing SGR and TGC by 12-15% in only 1.5 months (Dominguez et al., 2019b). This improvement in growth is also followed by the up-regulation of *gpx1a* gene. Selenium deposition in liver increases linearly with increasing levels of dietary selenium, whereas whole body Se contents are increased until reaching a plateau that denotes an optimum dietary Se level of 0.94 mg Se kg^{-1}. Despite dietary Se may affect the deposition of other minerals in liver, such as copper or zinc, neither these microminerals nor iron or manganese contents in liver are affected by the dietary increase in Se up to 1.7 mg kg^{-1}. Based on growth performance, *gpx1a* gene expression and Se deposition in whole body, an optimum dietary level of 0.94 mg Se kg^{-1}supplemented as NaSe was determined (Dominguez et al., 2019b). Besides, when NaSe supplementation is compared to OH-SeMet, supplementation of OH-SeMet up to 0.2 mg kg^{-1} (around 1 mg Se kg^{-1} diet), improved growth, hepatic morphology and protection against acute or chronic stress and against oxidative stress in fish muscle (Mechlaoui et al., 2019). This requirement is similar to that described for other sparids, higher than the requirement described for carp and lower than that established for fast growing fish species.

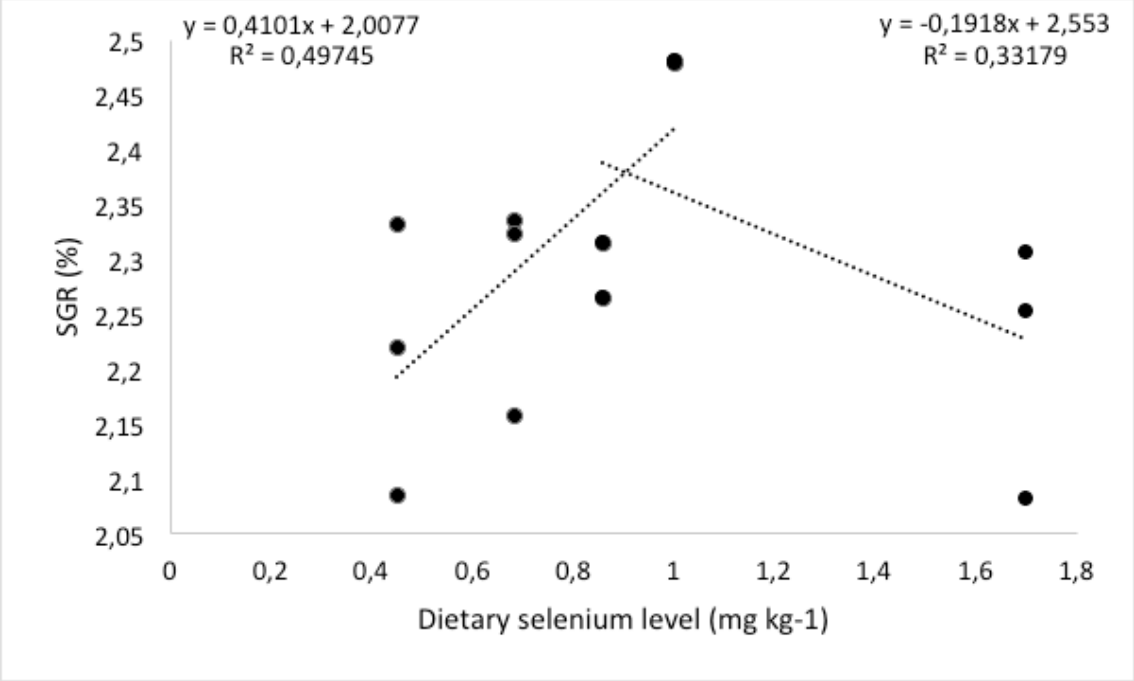


Figure 2.2.1. Relationship between dietary selenium level and SGR of gilthead sea bream fed increasing levels of dietary selenium for 42 days

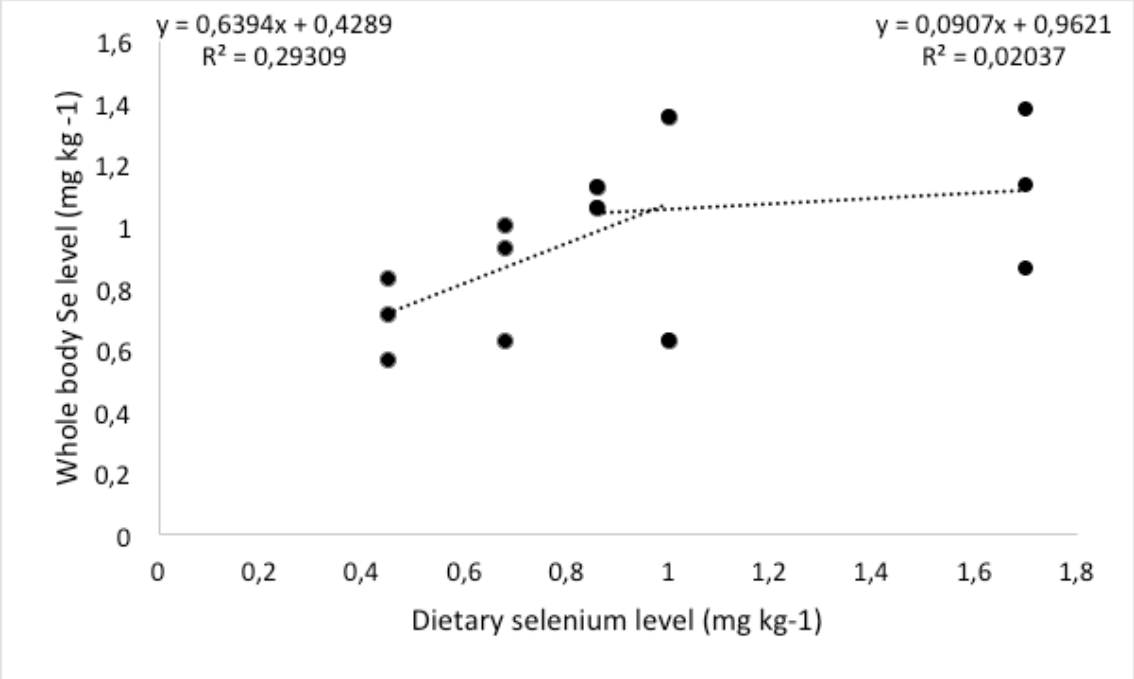


Figure 2.2.2. Relationship between dietary selenium level and whole body selenium content of gilthead sea bream fed increasing levels of dietary selenium for 42 days

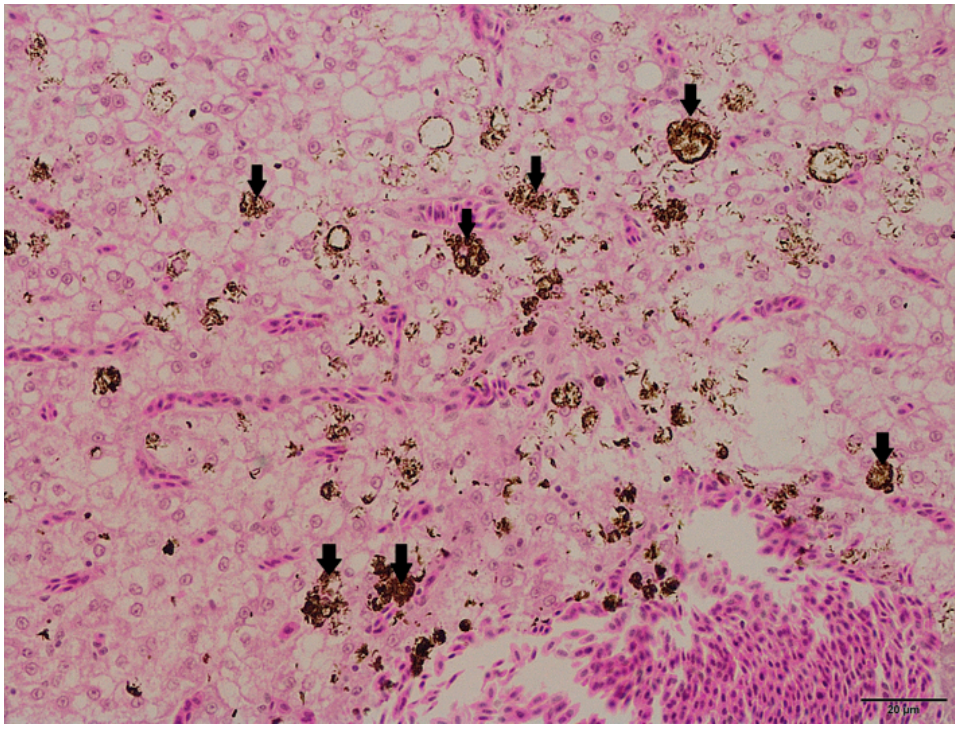


Figure 2.2.3. Microscopic view of liver cell nucleus (40x) presenting melanomacrophages aggregates (black arrows).

2.2.7.Toxic levels for sea bream

However, elevation of selenium up to 1.70 mg Se kg^{-1} supplemented as NaSe increases oxidative stress and reduces growth to levels comparable to those fish fed a diet without Se supplementation. Moreover, excess of dietary Se in gilthead sea bream also negatively affects hepatic tissue morphology, including pathological signs such as hydropic degeneration in kidney, whereas liver morphology does not show pathological effects. Therefore, dietary levels of 1.70 mg Se kg^{-1}, supplemented as NaSe, are high enough to induce oxidative stress and reduce growth, but not sufficient to cause large damage in hepatic tissues. However, when Se is supplemented as OHSeMet, dietary Se levels as high as 1.4 mg Se kg^{-1} only cause an increase in HSI and liver lipid content, but do not negatively affect growth (Mechlaoui et al., 2019).

2.2.8.Recommendations

In summary, in diets with 100 g/kg FM, which contain a basal level of 0.45 mg Se kg^{-1}, the optimum dietary level of total Se for gilthead sea bream is around 0.94 mg Se kg^{-1} to promote

growth and health of gilthead sea bream juveniles. This level may be reached by supplementation with 0.5 mg NaSe kg^{-1} diet or with 0.2 mg OH-SeMet kg^{-1} diet. However, dietary levels of 1.70 mg Se kg^{-1} are excessive and cause negative effects such as growth reduction, increased catalase expression and hydropic degeneration, and should be avoided in gilthead sea bream formulated feeds containing low levels of marine ingredients.

2.3.Manganese

2.3.1. Functions

Manganese is a transition metal essential for life that acts as a cofactor for metalloenzymes. Thus, Mn is involved in several enzyme complexes including the Mn superoxide dismutase (MnSOD), which intervenes preventing the initiation of the radical chain reaction (Dominguez et al., 2020a). Besides, Mn also intervenes in carbohydrate, lipid, and protein metabolism (Lall, 2002). Mn can be found in high concentrations in bone, but other tissues with high levels of this mineral are liver, muscle, kidney, gonadal tissues, and skin (Lall, 2002). Despite water dissolved Mn can be absorbed by fish, diet borne Mn is the main source of uptake.

2.3.2.Requirements

Manganese requirements described for aquaculture fish species range from 2 -22 mg Mn kg^{-1}, being higher in fast growing fish such as cobia or grouper and lower in channel catfish (Dominguez et al., 2020a). However little is known about Mn nutrition in gilthead sea bream juveniles (Dominguez et al., 2017).

2.3.3.Effects on KPIs and biomarkers

Manganese dietary levels may have diverse effects on growth and feed utilization, as well as on survival, but most frequently can markedly compromise fish health and fish quality in relation to skeletal anomalies or intestinal immunity (Dominguez et al., 2020a). Deficiency in Mn may alter a wide range of biomarkers due to its ubiquity in the different tissues and its involvement in carbohydrate, lipid, and protein metabolism. The main criterion to assess Mn requirements is considered to be vertebral Mn concentration (Antony Jesu Prabu et al., 2016). However, other markers can be strongly affected by a deficiency, such as MnSOD activity in heart and liver. Other deficiency symptoms include dwarfism, skeletal anomalies, cataracts, mortality, reduced growth and equilibrium disorders (Dominguez et al., 2020a).

2.3.4.Toxicity

Effects of manganese toxicity are rare when supplemented in the diet except for altered intestinal immunity (Jiang et al., 2015). In fact, EFSA establishes a maximum total content of Mn in complete feed for fish at 100 mg Mn kg^{-1} (EFSA, 2016). However, Mn intoxication through

water is more common and can course with severe hepatic damage as denoted by histopathological disorders including pycnotic degeneration of hepatocytes, congestion and dilatation in the sinusoids, mild necrosis, nuclear degeneration and hyper-vacuolization (Dominguez et al., 2020a).

2.3.5. Practical aspects

Manganese is one of those minerals whose content in marine or terrestrial plant ingredients is markedly different. Therefore, changes in aquafeed ingredients may markedly affect Mn levels. For instance, corn gluten has only 6-8 mg Mn kg^{-1}, whereas soybean meals may have a high content in Mn (31 mg Mn kg^{-1}), which is similar to those found in different fish meals (35-40 mg Mn kg^{-1}).

There were few studies dealing with Mn nutrition in gilthead sea bream, but given its importance and the high variations that may appear in the diet when different ingredients are included, PerformFISH evaluated the effect of dietary Mn oxide supplementation (19-66 mg Mn kg^{-1}) on gilthead sea bream fed diets containing only 10% FM and 6% FO (Dominguez et al., 2020b).

2.3.6. Dietary levels for sea bream

The combined supplementation of Mn, Zn and Se in inorganic rather than in organic forms in low FM diets for gilthead sea bream juveniles lead to an improved growth (Dominguez et al., 2017). However, Mn content in vertebrae, was not affected by dietary increase in Mn 22-52 mg Mn kg^{-1}, suggesting that such growth improvement was more related to dietary Zn or Se contents as it was later demonstrated (Dominguez et al., 2019c, 2020a). In a second study, increase in dietary Mn from 30 to 60 mg Mn kg^{-1} together with amino acid chelated Zn did not affect gilthead sea bream growth, whole body or vertebrae Mn contents (Dominguez et al., 2019c). However, in comparison to amino acid chelated Mn, Mn oxide up-regulated the expression of superoxide dismutase gene (*Mnsod*) (Dominguez et al., 2019c). Also, dietary supplementation of Mn oxide from 21-73 mg Mn kg^{-1} as part of an increased nutrient package, did neither increased Mn contents in vertebrae, but affected *Mnsod* expression (Dominguez et al., 2020b).

Increase in dietary manganese from 19 to 66 mg Mn kg^{-1} supplemented as Mn oxide did not affect growth or feed utilization parameters in gilthead sea bream juveniles fed practical diets with high levels of plant ingredients (Dominguez et al., 2020a). These results suggest that 19 mg Mn kg^{-1} are sufficient to cover manganese requirements for growth in gilthead sea bream and that novel diets with high FM replacement by terrestrial plant ingredients basal levels of manganese may be enough to cover gilthead sea bream requirements for growth. These values are close to those described as optimum for other species such as rainbow trout (19 mg Mn kg^{-1}, Satoh et al., 1991) or cobia (21.72 mg Mn kg^{-1}, Liu et al., 2013). Nevertheless, tissue contents in manganese for vertebrae and whole body have been also used as biomarkers to assess manganese requirements in different fish species and, in agreement, in sea bream there is also positive correlation between dietary manganese and vertebrae contents. Moreover, increase in Mn supplementation up to 30 mg Mn kg^{-1} reduced the oxidative risk, reducing TBARs values and down-regulating biomarkers of oxidative stress. Therefore, despite the basal

dietary Mn levels (19 mg Mn kg^{-1}) were sufficient to cover growth requirements, the overall reduction in oxidative risk in fish fed the 30 mg Mn kg^{-1} diet denotes a higher requirement of this mineral for antioxidant protection in gilthead sea bream.

Figure 2.3.1. Effect of dietary manganese levels on incorporation of Mn in vertebra of gilthead sea bream.

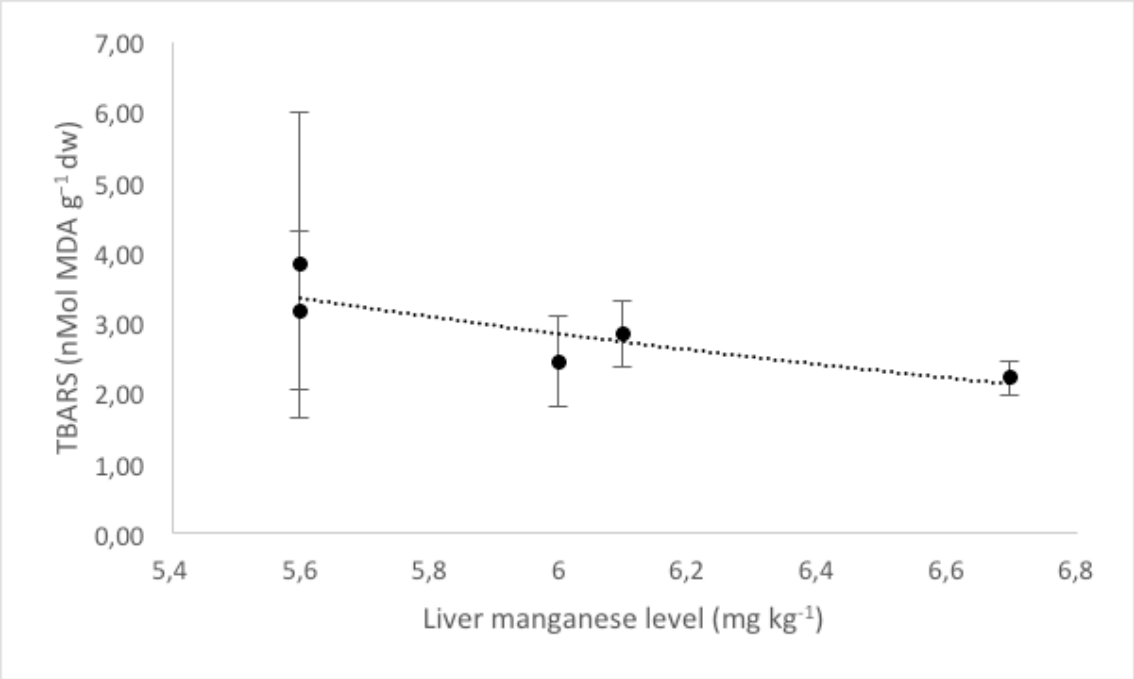


Figure 2.3.2. Effect of dietary manganese levels on TBARs in liver of gilthead sea bream.

2.3.7.Toxic levels for sea bream

Despite interactions between manganese and other minerals, such as copper, iron or zinc, have been observed in several species, mineral contents in gilthead sea bream tissues were not affected by manganese supplementation and no interactions have been observed in sea bream under the dietary mineral levels tested in PerformFISH. Dietary Mn levels did neither affected whole body lipid, protein or ash content or hepatic morphology. Morphological alterations in liver alterations due to excess Mn have been found mostly in fish from highly polluted rivers, or submitted to very high manganese intoxications, while no hepatic alterations are mentioned in trials conducted on fish with dietary manganese. Therefore, no Mn intoxication would be expected from practical diets containing between 19 to 66 mg Mn kg^{-1}.

2.3.8.Recommendations

In summary, the presence of manganese in higher concentrations in plant ingredients than animal sources suggests that practical diets based on plant ingredients may contain sufficient manganese to cover the requirements for gilthead sea bream fingerlings. In this study from PerformFISH, markers for growth, feed utilization, whole body chemical composition or hepatic morphology were not affected by manganese supplementation, suggesting that the manganese content present in the basal diet (19 mg Mn kg^{-1}) was sufficient to cover the requirements in gilthead sea bream fed practical plant-based diets, which remains lower than the maximum tolerable levels established by EFSA (2016) for fish (100 mg Mn kg^{-1}). Nevertheless, an overall reduction in oxidative risk in fish fed 30 mg Mn kg^{-1} suggests the need to increase up to this level the dietary contents for antioxidant protection.

2.4.Vitamin A

2.4.1.Functions

Vitamin A is directly involved in preserving the epithelium, cell growth and differentiation, reproduction, rhodopsin formation and regeneration, and maintaining resistance to infection (Dominguez et al., submitted a). Besides, vitamin A is necessary for osteoblast differentiation, it regulates osteoclasts activity and intervenes in chondrocytes development, hence playing a role in skeletogenesis and prevention of bone anomalies (Lall and Lewis-McCrea, 2007). Deficiency in vitamin A has been associated to defective remodelling of intramembranous bone. Besides, vitamin A is also involved in the synthesis of mucopolisacharides that are components of cartilage and bones. Thus, vitamin A deficiencies may lead to disorganized bone growth and subsequent anomalies.

2.4.2.Requirements

Optimum vitamin A dietary levels respond to a series of criteria which have been used in several fish species and include weight gain, maximum vitamin A liver storage and the lack of

deficiency signs. Optimum vitamin A levels described in the literature to promote growth in the different aquaculture species range from as low as 1,700 IU vitamin A kg^{-1} to as high as 40,000 IU vitamin A kg^{-1} for European sea bream (Villeneuve et al., 2005). Despite the marked importance of deficiencies or excess of this vitamin, the requirements have not been yet defined for gilthead sea bream.

2.4.3. Effects on KPIs and biomarkers

Dietary vitamin A levels and molecular forms can markedly affect several KPIs, particularly, growth, food conversion rates and fish quality. Since liver is a main storage organ for vitamin A, this criteria has been also used as a marker to define optimum dietary levels. Vitamin A can also influence the lipid composition of different tissues and stress-related parameters (Hemre et al., 2004). Low levels of vitamin A in the diet often cause poor growth and vision, hemorrhage, keratinization of the epithelia and abnormal bone development (Dominguez et al., submitted a). In relation to its important role in regulation of bone cells differentiation and growth, the expression of several genes of bone development-related proteins, such as bone morphogenic protein, osteocalcin or matrix Gla protein, which are regulated by dietary vitamin A levels, are also considered as valid biomarkers of the vitamin A status in fish (Fernández et al., 2012).

2.4.4. Toxicity

Conversely, an excess of vitamin A leads to similar clinical signs as deficiencies, including keratinization of epithelia, abnormal growth and skin lesions, but also hepato- and splenomegaly, and skeletal anomalies (Lall and Lewis-McCrea, 2007; NRC, 2011). In gilthead sea bream excess vitamin A led to changes in bone homeostasis and structure, increasing the deposition of oscteocalcin and matrix Gla protein (Fernández et al., 2012). Other signs of vitamin A excess can be found in the liver, such as the pale and fragile liver characteristics, suggesting a liver steatosis. Since vitamin A is mainly stored in the liver, any histological observations made in this tissue related to dietary vitamin A may shed light on the effects of excess supplementation of this vitamin in this tissue.

2.4.5. Practical aspects

Vitamin A can be found as retinol, retinal and as retinoic acid. Several sources of vitamin A are used in the aquaculture industry (retinol, retinyl acetate, retinyl palmitate, retinyl propionate, carotenoids, etc.). These sources however, have different conversion ratios to retinol, for this reason IU kg^{-1} have been adopted in many studies. Diets high in FM and FO contain retinyl esters or vitamin A precursors from xanthophylls including astaxanthin as sources of vitamin A, whereas plant based diets may contain different types of carotenoids.

The need to substitute ingredients derived from fisheries by more sustainable sources has driven the feed industry to use ingredients with different vitamin A compositions. However, most studies aiming to determine vitamin A requirements have been based on purified diets or

practical diets based on fish meal and oil, whereas, the use of diets based on alternative ingredients to define vitamin A requirements has been very limited (Fontagné-Dicharry et al., 2010). Assuming the present replacement of FM and FO by plant sources in diets for gilthead sea bream, it is necessary to understand the effects of dietary vitamin A levels in this type of diets. Therefore, the increased use of alternative ingredients, which may affect basal vitamin A levels and forms, requires to define optimum dietary levels in practical formulations. Given the important effect of either deficient or excess dietary levels of this vitamin on KPIs, and the completely lack of information regarding optimum dietary levels for gilthead sea bream, PerformFISH evaluated the effect of dietary vitamin A levels (24,000-37,000 IU kg^{-1}) supplemented as retinil acetate in gilthead sea bream growth, productive parameters and health status, when dietary levels of FM-FO are low.

2.4.6. Dietary levels for sea bream

Increase in dietary vitamin A levels from 24,000 to 27,000 IU kg^{-1} in the form of retinyl acetate, improves sea bream growth in terms of final body weight by 6-12% in only 70 days of feeding. However, further supplementation does not significantly improve growth. Besides, retinol contents in liver tend to increase linearly with dietary levels of vitamin A. Moreover, vitamin A deficiencies may lead to disorganized bone growth and subsequent anomalies and in sea bream fed the lowest vitamin A levels (24,000 IU kg^{-1}) the incidence of anomalies was at least double than in fish fed 27,000 IU kg^{-1} of vitamin A.

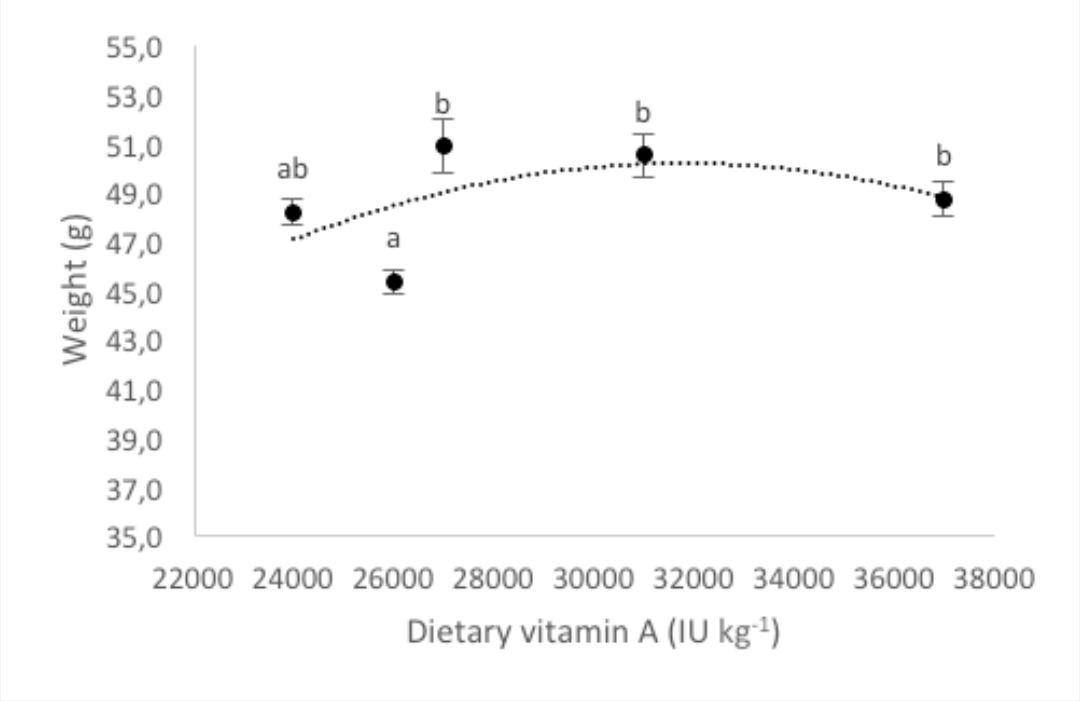


Figure 2.4.1. Effect of dietary vitamin A levels on weight of gilthead sea bream.

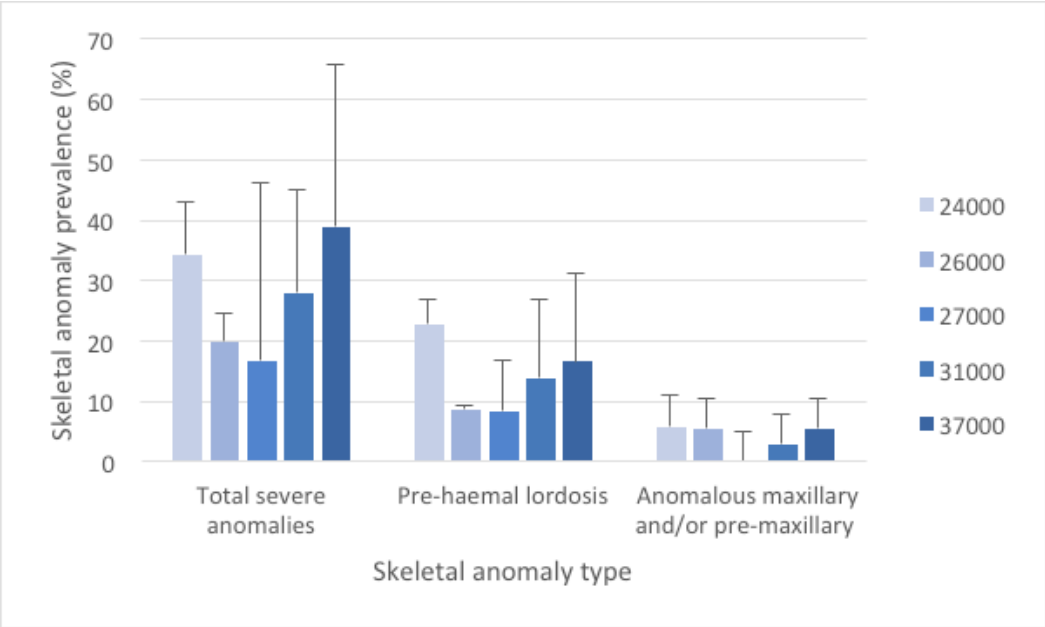


Figure 2.4.2. Effect of dietary vitamin A levels on skeletal anomalies presence in gilthead sea bream.

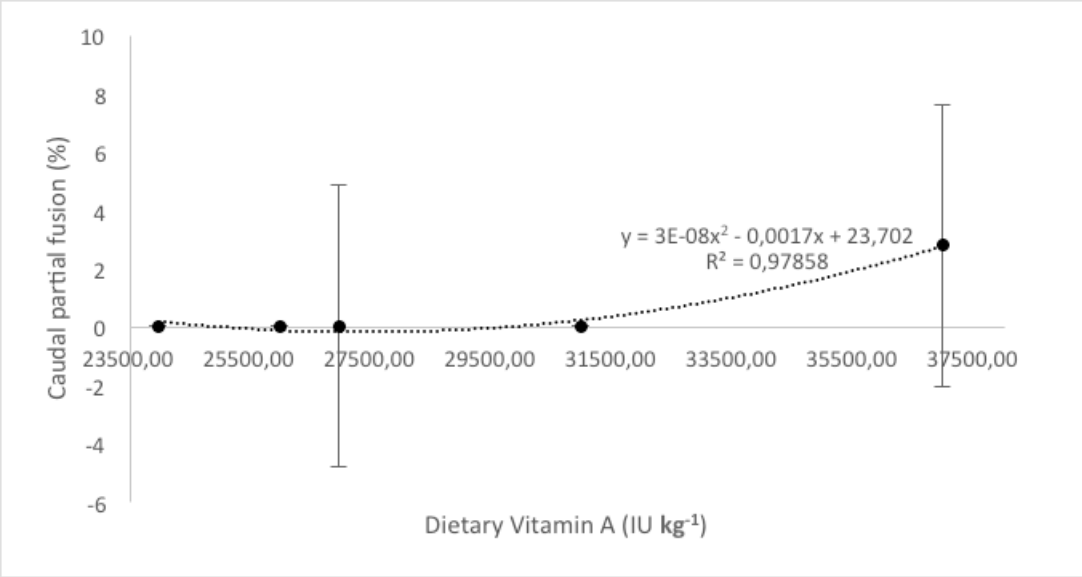


Figure 2.4.3. Effect of dietary vitamin A levels on occurrence of caudal partial fusions in gilthead sea bream

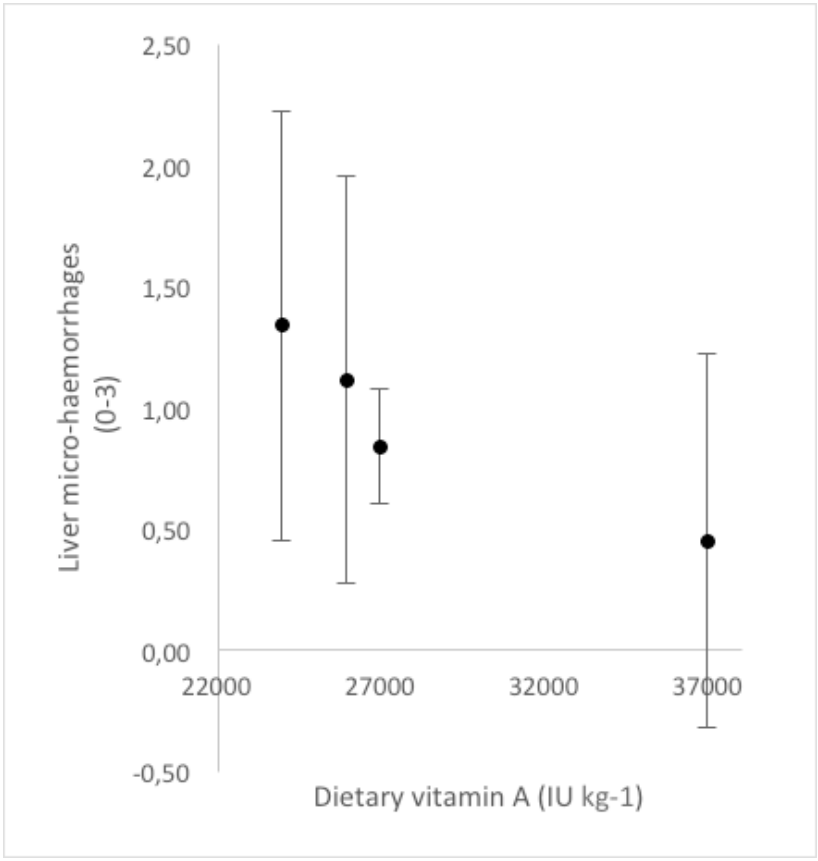


Figure 2.4.4. Widespread presence of micro-hemorrhages in the liver of gilthead sea bream fed increasing levels of dietary vitamin A for 70 days.

Besides, no anomalous maxillary and/or pre-maxillary were found in fish fed 27,000 IU kg^{-1}, denoting the importance of correct dietary vitamin A levels for endochondral bones such as the sea bream cranial bones. Moreover, Vitamin A induces osteoblast differentiation, but require bone morphogenetic proteins to stimulate both osteoblast differentiation and bone formation. Sea bream fed 27,000 IU kg^{-1} vitamin A showed the highest expression of *bmp2* in vertebrae and the expression of this gene was inversely correlated to the frequency of anomalies. Therefore, based on growth, skeletal anomalies and molecular markers, a requirement of 27,000 IU vitamin A kg^{-1} diet seems to be needed for this species when low FM and low FO diets are supplemented with retinyl acetate. This requirements are lower than those suggested for European seabass (40,000 IU vitamin A kg^{-1} diet) but higher than those described for other species such as hybrid tilapia (5,850 IU vitamin A kg^{-1}) (Hu et al., 2006).

2.4.7. Toxic levels for sea bream

Although vitamin A is necessary for bone formation, the excess of retinoic acid may promote bone resorption, reduce bone formation and lead to bone anomalies. In gilthead sea bream, hypervitaminosis A causes skeletal malformations by affecting both compact and trabecular bone layers and their calcification pattern, which in turn, may cause skeletal anomalies (Fernández et al., 2012). Thus, the incidence of skeletal anomalies in sea bream fed the highest vitamin A levels (37,000 IU vitamin A kg^{-1} diet) also doubled that of fish fed 27,000 IU vitamin A kg^{-1} diet. Moreover, increase of dietary vitamin A was particularly related to the increase in caudal partial fusion of vertebrae. Therefore, event though previous studies showed that 2,300,000 IU vitamin A kg^{-1} diet causes hypervitaminosis in gilthead sea bream, even a dietary vitamin A level of 37,000 IU vitamin A kg^{-1} diet may already caused hypervitaminosis signs in this species. Indeed, in sea bream vertebrae, a down-regulation of *bmp2* together with the up-regulation of *alp* was related to the increase in skeletal anomalies found in fish fed excessive (37,000 IU vitamin A kg^{-1} diet) levels of dietary vitamin A.

2.4.8.Recommendations

The optimum dietary levels for gilthead sea bream determined by PerformFISH in practical diets for containing only 10% FM and 6% FO are 27,000 IU vitamin A kg^{-1} diet, obtained by supplementation of the dietary basal levels (24,000 IU vitamin A kg^{-1} diet) with retinyl acetate. This level allowed to improve growth, skeletal anomalies and liver health, in agreement with the regulation of molecular markers. However, levels of even 37,000 IU vitamin A kg^{-1} diet may produce the first signs of toxicity and should be avoid. It is important to highlight that optimum dietary levels of vitamin A for European sea bass have been proposed to be 40,000 IU vitamin A kg^{-1} diet and, despite they may be correct for sea bass, would be already toxic for sea bream.

In practical diets, there is a basal content of vitamin A inherent to the ingredients used, and the presence of other nutrients or ingredients may affect vitamin A availability. It is important to consider this basal level to avoid the appearance of toxicity in gilthead sea bream.

2.5.Vitamin K

2.5.1.Functions

Vitamin K is a fat-soluble vitamin required in very little amounts in animal feeds and its requirements in fish have been scarcely determined. Vitamin K comprises three major forms, K1 (phylloquinone) obtained from plants, K2 (menaquinone) synthesized by bacteria and the synthetic form K3 (menadione). Menadione is frequently used in animal nutrition, but may be unstable during feed processing and storage, reaching to critically low levels when fed to fish. Some scientists have also questioned its availability in salmonids feed. Vitamin K is involved in blood clotting, bone mineralization and resorption, collagen formation, postranscrpition regulation, reproduction and activation of certain vitamin-K dependent proteins (Dominguez et al., submitted b).

2.5.2. Requirements

Optimum dietary vitamin K levels have been less addressed than other fat-soluble vitamins, therefore there are fewer species with their requirements defined. Overall the requirements range between 0.2 and 3.45 mg vitamin K kg^{-1} diet, whereas requirements for sea bream have never been addressed (Dominguez et al., submitted b).

2.5.3. Effects on KPIs and biomarkers

Severe deficiency in vitamin K may depress growth and food utilization related KPIs. Since this vitamin intervenes in a wide array of biological functions, several markers are commonly employed to evaluate vitamin K requirements. Besides growth parameters, vitamin retention in tissues, blood clotting time, prevalence of skeletal anomalies skeletal anomalies or bone mineralization have been used.

Deficiency curses with increased blood clotting times, anaemia, haemorrhages in several tissues and increased prevalence of skeletal anomalies. Lower vitamin K dependent (VKD) enzymatic activities or degree of VKD protein carboxylation are also considered as markers for suboptimal vitamin K nutrition.

2.5.4. Toxicity

Effects derived from an excess of vitamin K supplementation are rare. Even increasing dietary levels by thousand times toxic effects may not be encountered. Only a slight growth reduction has been observed in both Atlantic cod (Grahl-Madsen and Lie, 1997) and large yellow croaker (Cheng et al., 2015) when optimum dietary vitamin K levels are surpassed.

2.5.5. Practical aspects

Being a fat-soluble vitamin, required in low amounts, it may be present in alternative ingredients containing fat. Vitamin K is present in both FO and FM, and whereas it is absent from most alternative protein ingredients, it may be present in alternative oils. For instance, rapeseed or soybean oils may have even higher vitamin K contents than fish oil. Therefore, it is important to determine the requirements of sea bream for this vitamin and to consider the concentration of vitamers in the feed ingredients used. PerformFISH addressed the effect of different vitamin K levels (0.1-23 mg vitamin K kg^{-1}) by supplementing low FM and low FO diets with menadione and determining their effect on growth, productive parameters and health related issues.

2.5.6. Dietary levels for sea bream

Increase in dietary vitamin K contents up to 12 mg vitamin K kg^{-1} diet improves growth performance by 10% in only 2.5 months of feeding, whereas further supplementation did not yield a further increase in growth despite the level of vitamin K1 continued to be deposited in a linear manner in the liver (Dominguez et al., submitted b). Besides, expression of the gene for Gla-rich protein, which is vitamin K dependent, is increased by the elevation of dietary vitamin K contents. On the contrary, increase in vitamin K levels from 0.1 to 23 mg vitamin K kg^{-1} does not affect biomarkers of bone development neither improves skeletal anomalies incidence. Therefore, optimum vitamin K levels for gilthead sea bream have been determined at 10-13 mg vitamin K kg^{-1} diet. These results are similar to those determined for Atlantic salmon, based on on weight and other parameters such as mortality, blood coagulation time and bone deformities (Krossøy et al., 2009), but higher than those for other species.

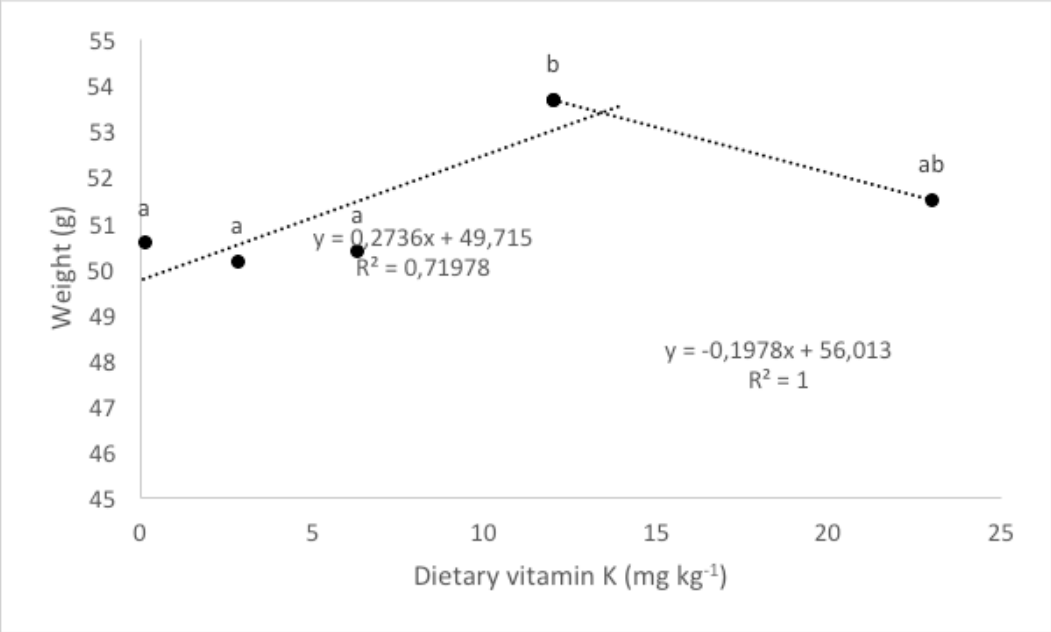


Figure 2.5.1. Effect of dietary vitamin K on growth of gilthead sea bream juveniles

2.5.7. Toxic levels for sea bream

Increase in dietary vitamin K levels up to 23 mg vitamin K kg^{-1} slightly reduced growth performance as described in Atlantic cod fed 21.5 mg menadione sodium bisulphite kg-1 (Grahl-Madsen and Lie, 1997), suggesting a possible toxic effect of excess vitamin K in sea bream. Besides, sea bream fed 23 mg vitamin K kg^{-1} also showed an increase in the occurrence of anomalies of the caudal vertebrae, in relation to the up-regulation of *bmp2*, a gene that codifies for a bone morphogenic protein involved in bone and cartilage development.

2.5.8.Recommendations

In practical diets for sea bream it is important to consider the basal contents in vitamin K derived from alternative oils. Gilthead sea bream seems to be able to use menadione properly in view of the increase in vitamin K found in body tissues. Moreover, dietary supplementation with menadione to rise vitamin K levels up to 12 mg vitamin K kg-1 diet is recommended as improves growth performance, up-regulates molecular markers of vitamin K status and tend to reduced prevalence of skeletal anomalies. On the contrary, dietary vitamin K levels of 23 mg vitamin K kg-1 should be avoided to prevent some signs of toxicity such as slightly reduced growth or increase caudal anomalies occurrence.

2.6.Vitamin D

2.6.1.Functions

Vitamin D is mainly involved in Ca homeostasis, acting in synergy with calcitonin and parathyroid hormone. Together they regulate Ca uptake and liberation from bone intervening in bone remodeling (Dominguez et al., submitted c). Fish are unable to synthesize vitamin D, and so require absorbing it directly from the diet. Once it is absorbed, deposition takes place in liver, intestine, kidney, spleen, gills, skin and muscle. Besides its relevance for bone formation, vitamin D also plays important roles in muscle function and cardiovascular physiology. Several forms of vitamin D can be found including ergocalciferol (vitamin D2), cholecalciferol (vitamin D3) and calcitriol (1,25-dihydroxychelecalciferol).

2.6.2.Requirements

Vitamin D requirements markedly vary among species. Optimum dietary levels have been reported to be as low as 0.00004 mg vitamin D kg^{-1} diet and as high as 125 mg vitamin D kg^{-1} (Dominguez et al., submitted c). Recent studies define the optimum levels for Atlantic salmon to be in the range of 0.06-0.09 mg vitamin D kg-1 as part of a practical approach using a multi-nutrient package with reduced levels of fish ingredients (Antony Jesu Prabhu et al., 2019), suggesting that slightly higher levels of supplementation are needed when feeds are based on ingredients alternative to fish meal and fish oil.

2.6.3.Effect on KPIS and biomarkers

Inadequate levels of vitamin D reduce growth and negatively affect fish health. Bone mineralization has been used as a biomarker for vitamin D status. Bone formation is tightly regulated by a series of molecular markers that affect cell differentiation and mineralization, especially at early developmental stages. These markers include *runx2*, bone morphogenic proteins (*bmp*), alkaline phosphatase (*alp*) or osteocalcin (*oc*). Regarding fish health, inadequate doses of vitamin D in fish may cause tetany, alteration of thyroid hormone levels,

thin epidermis, muscle necrosis, hypocalcaemia, erosion of fins, and increased liver and muscle lipid deposition.

2.6.4.Toxicity

Despite toxic effects of vitamin D are very rare, toxicity symptoms can cause reduced growth, hypercalcaemia, and elevated haematocrit levels.

2.6.5.Practical aspects

Vitamin D in fish is found mostly as vitamin D3, which seems to have a higher bioavailability than vitamin D2. However, vitamin D2 is the form in which this vitamin is present in plant ingredients. Therefore, replacement of marine ingredients by terrestrial plant feedstuffs may affect not only the total content of vitamin D in the basal diet, but also its availability.

In gilthead sea bream, dietary vitamin D3 has been found to stimulate immune system and therefore it may be important to contribute to prevent diseases in commercial farms. However, little knowledge is available at the moment regarding the essentiality of this vitamin in gilthead sea bream. Therefore, PerformFISH studied the effect of dietary vitamin D levels (5.8-26 mg vitamin D kg^{-1} diet) by supplementing cholecalciferol in practical diets with low FM (10% and low FO (6%) levels on growth performance, proximate composition, and morphology of bone, liver and heart of gilthead sea bream.

2.6.6.Dietary levels for sea bream

Feeding gilthead sea bream juveniles with a range of vitamin D3 levels between 0.15-0.65 mg vitamin D3 kg^{-1} diet for 2.5 months until fish double their weight, does not affect gilthead sea bream growth, FCR, mortality or any other KPI related parameter, suggesting that the basal dietary levels of 0.15 mg vitamin D3 kg^{-1} diet are sufficient to cover the requirements of this species for growth. Increase dietary vitamin D3 significantly raised the liver contents in vitamin D3 in a dose-dependent manner following a potential regression, denoting a nonspecific accumulation of the vitamin even when fed at high dietary doses. Interestingly increase in dietary vitamin D3 up to 0.30 mg vitamin D3 kg^{-1} diet is associated to the lowest incidence of skeletal anomalies. These levels are close to those recommended for Atlantic salmon, but higher than those recommended for other species.

2.6.7.Toxic levels for sea bream

Elevation of dietary vitamin D over 0.40 mg vitamin D3 kg^{-1} diet up to increases the incidence of skeletal anomalies in gilthead sea bream, denoting an effect of excess supplementation related to the bone catabolic effects. Being bones a main store of calcium phosphate, vitamin D directly affects both osteoblast activity and osteoclast formation to maintain calcium homeostasis. Indeed expression of *bmp2* and *alp*, biomarkers of osteoblast differentiation and mineralization, increase in sea bream in relation to dietary vitamin D3 showing a high lineal

correlation to caudal and maxillary anomalies. Moreover, the increase of dietary vitamin D3 up to 0.5 mg vitamin D kg^{-1} diet significantly increased cardiac congestion and swollen of cardiac muscle, signs of myocarditis. These, together with the increased occurrence of skeletal anomalies in sea bream fed 0.5 0.3-0.4 mg vitamin D3 kg^{-1} diet, suggest initial signs of hypervitaminosis D, despite growth was not negatively affected.

2.6.8.Recommendations

The use of FM and other sources of animal ingredients (including blood meal spray) may provide practical diets for sea bream with sufficient vitamin D3 levels to cover the requirements for growth (0.15 mg vitamin D3 kg^{-1} diet). Nevertheless, supplementation up to 0.3-0.4 mg vitamin D3 kg^{-1} diet would contribute to reduce the incidence of skeletal anomalies. On the contrary, dietary vitamin D levels of 0.5 mg vitamin D3 kg^{-1} diet may negatively affected cardiac tissue and skeletal anomalies incidence. Thus, the recommended dietary levels for gilthead sea bream juveniles would be between 0.15-0.30 mg kg-1 vitamin D3. In practice, it is desirable to produce diets for gilthead sea bream containing sufficient levels of vitamin D3 in the basal ingredients since EU legislation restricts the supplementation of vitamin D3 in aquafeeds.

2.7.Vitamin B1

2.7.1.Functions

Thiamin, vitamin B1, serves as a cofactor for several enzymes in energy metabolism, including pyruvate dehydrogenase and a -ketoglutarate dehydrogenase, forming part of the coenzyme cocarboxylase (thiamin pyrophosphate), required in the decarboxilation of alfa-cetoacids derived from carbohydrates, fatty acids and aminoacids. Therefore, thiamin is necessary for the catabolism of these nutrients, taking part, particularly, in the direct oxidative pathway of glucose. Therefore, since body organs and systems use glucose as energy, their functioning is susceptible to be impaired by thiamin deficiency, particularly the nervous system.

2.7.2.Requirements

Vitamin B1 requirements to optimize growth range between 0.6-1.5 mg vitamin B1 kg^{-1} diet, whereas in order to achieve thiamin saturation levels on whole body or liver, it is necessary to increase dietary vitamin B1 up to 2.5-18.2 mg vitamin B1 kg^{-1} diet (Izquierdo et al., in prep.). In general thiamin requirements have been claimed to be higher for freshwater fish and for warm-water species.

2.7.3.Effect on KPIS and biomarkers

Thiamin deficiency impairs carbohydrate metabolism and therefore reduces growth preformance. It also induces anorexia, hence affecting to feed intake and food utilization. Since thiamin pyrophosphate is depleted earlier in brain than in muscle, thiamin deficiency may progress from anorexia to strong neurological signs such as convulsions, nervous paralysis,

shock susceptibility or trunk-winding symptoms. Other deficiency symptoms include haemorrhages, high mortality, congested fins or dark pigmentation (NRC, 2011; Izquierdo et al., in prep). Thiamin contents in tissues respond to the dietary increase until reaching a plateau at sufficient dietary levels, being an indicator of thiamin requirements. Since thiamine is a coenzyme for the transketolase, the activity of this enzyme and the expression of its gene are considered as biomarkers for thiamine status.

2.7.4. Practical aspects

Thiamin is found in dietary ingredients including whole grains, enriched cereals, meat and poultry products, which may contribute to the total dietary thiamin content. For instance, thiamin contents may be up to 10 times higher in soybean meals than in FM. Nevertheless, thiamin bioavailability can be compromised by polyphenolic and sulfite compounds present in the diet, such as thiaminases and antithiamin factors, or food processing. For instance, thiamin is sensitive to high temperatures during food processing forming maillard products. There is a lack of evidence that dietary thiamin may cause any negative symptoms. Despite the importance of thiamin for energy ad carbohydrate metabolism, and its potential effect in different KPIs, the requirements for this vitamins has not been yet stablished for gilthead sea bream. PerformFISH evaluated the effects of dietary vitamin B1 levels (1.6-7.9 mg vitamin B1 kg^{-1} diet), in combination to dietary levels of vitamin B9 and B12, in growth, productive parameters and health status of in gilthead sea bream fed diets low in FM-FO.

2.7.5. Dietary levels for sea bream

Increase in dietary thiamin from 1.6 to 7.9 mg vitamin B1 kg^{-1} diet does not affect sea bream growth or any other KPI. Feeding sea bream a diet without thiamin supplementation (1.6 mg vitamin B1 kg^{-1} diet) reduces vitamin B1 contents in whole body, whereas increase in dietary thiamin up to 6 mg vitamin B1 kg^{-1} raised vitamin levels in whole body and up-regulated *GlutR* expression in liver.

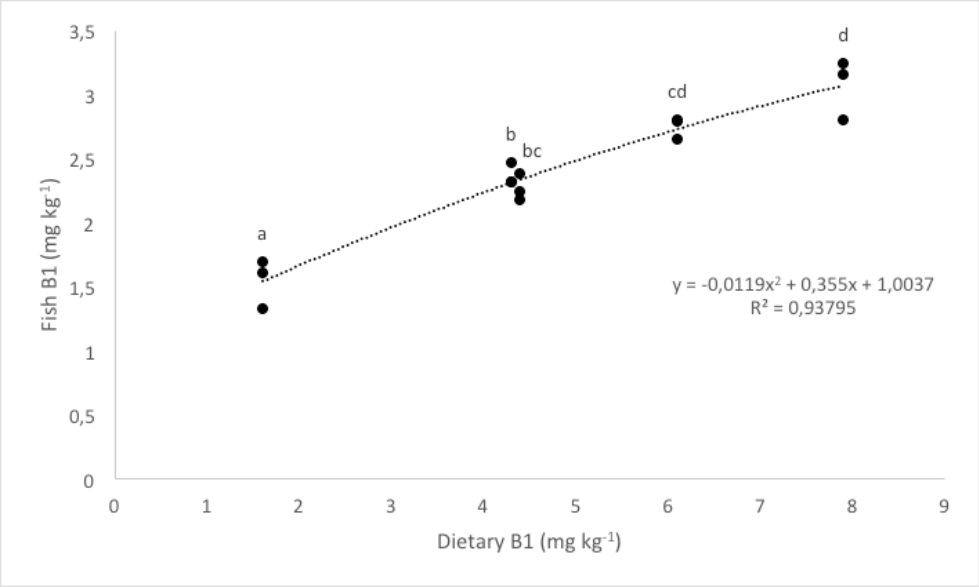


Figure 2.7.1. Effect of dietary vitamin B1 on thiamin body contents of gilthead sea bream

2.7.6.Recommendations

Gilthead sea bream requirements for thiamin to obtain maximum growth are covered by 1.6 mg vitamin B1 kg^{-1} diet, which may be already present in the basal diets containing grain derived ingredients. However, liver contents in vitamin B1 are depleted down to 50% the initial levels and, therefore, to ensure the correct tissue levels of thiamin, dietary levels should be risen up to 6 mg vitamin B1 kg^{-1} diet. Therefore, as in other fish species thiamin requirements in sea bream are higher to maintain vitamin body contents than to promote growth.

2.8.Vitamin B9

2.8.1.Functions

Folic acid, vitamin B9, is a general methyldonor. It acts as a coenzyme taking part in many one-carbon metabolism systems such as amino acids synthesis and conversion or pyrimidine synthesis. Besides, folic acid is necessary for blood cells formation and therefore, megaloblastic anaemia is the most distinctive symptom of vitamin B9 deficiency, resulting from reduced erythrocyte formation due to impaired thymidine synthesis (NRC, 2011, Izquierdo et al., in prep.).

2.8.2.Requirements

Studies on dietary requirements for folic acid exist only for few fish species. The requirements stablished for optimum growth are around 0.5-1.5 mg vitamin B9 kg^{-1} diet, whereas to reach

saturation levels of vitamin B9 in liver they rise to 1-10 mg vitamin B9 kg^{-1} diet (Izquierdo et al., in prep.).

2.8.3. Effect on KPIS and biomarkers

Insufficient levels of folic acid reduce growth performance, feed intake, food utilization and fish health. For instance, folic acid deficiency produce lethargy, anorexia or anemia. Other more extreme signs of folic acid deficiency include dark skin, fragile fins, and infarction of spleen. In salmonids, a low (2.3%) occurrence of senile erythrocytes with misshapen nuclei has been an indicator of folic acid deficiency.

2.8.4. Toxicity

There are no evidences of toxicity caused by high levels of folic acid in practical diets. However, high doses of dietary folic acid could mask vitamin B12 deficiency since megaloblastic anemia is also a symptom of vitamin B12 deficiency. Interactions are also found with dietary vitamin C, and only the dietary increase in both vitamins prevents the occurrence of morphologically abnormal blood cells.

2.8.5. Practical aspects

Folic acid is present in many alternative raw materials, their contents being even higher in soybean meals than in fish meal. Besides, in certain fish, particularly in warm-water species, folic acid may be synthesized by the gut microbiota, contributing at least partly to cover the vitamin B9 requirements. Despite its importance for fish health, requirements for sea bream have not been yet determined. PerformFISH evaluated the effects of dietary vitamin B9 levels (0-14.6 mg vitamin B9 kg^{-1} diet), in combination to dietary levels of vitamin B1 and B12, in growth, productive parameters and health status of in gilthead sea bream fed diets low in FM-FO.

2.8.6. Dietary levels for sea bream

Increase in dietary folic acid from 0 to 14.6 mg vitamin B9 kg^{-1} diet does not affect sea bream growth or any other KPI, but increase up to 5.5 mg vitamin B9 kg^{-1} diet reduces hepatosomatic index and body lipid contents. Feeding sea bream a diet without folic acid supplementation (0 mg vitamin B9 kg^{-1} diet) only slightly reduces vitamin B9 contents in whole body, whereas increase in dietary thiamin up to 6.8 mg vitamin B9 kg^{-1} raises vitamin levels in whole body and up to 3.5 mg vitamin B9 kg^{-1} up-regulates molecular markers of vitamin B9 status (*Slc19a*). Besides, elevation of dietary vitamin B9 kg^{-1} markedly reduces the occurrence of erythrocytes with irregular nucleus in gilthead sea bream.

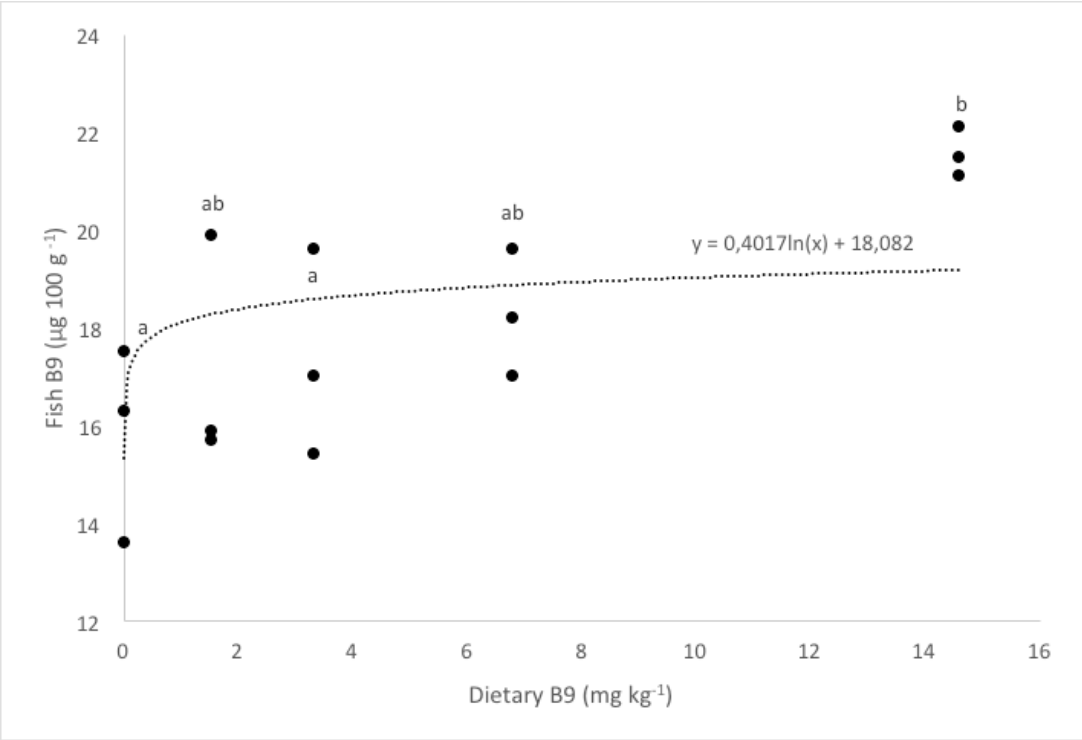


Figure 2.8.1. Effect of dietary vitamin B9 on folic acid body contents of gilthead sea bream

2.8.7.Recommendations

Basal dietary levels of folic acid in diets containing alternative ingredients such as soybean meal may be high enough to cover the vitamin B9 requirements of gilthead sea bream for growth. However, in order to maintain folic acid levels in body tissues and promote fish health, avoiding the occurrence of abnormal erythrocytes, it would be necessary to increase dietary vitamin B9 up to 6.8 mg vitamin B9 kg^{-1} .

2.9.Vitamin B12

2.9.1.Functions

Cobalamin, vitamin B12, is a coenzyme that contains cobalt necessary for the remethylation of homocystein to methionine, and the production of succinyl-CoA and the isomerization of methyl aspartate to glutamate. Together with folic acid, vitamin B12 intervenes in hemopoiesis. Besides, it takes part in cholesterol metabolism, in purine and pyrimidine biosynthesis, and in the metabolism of glycols. Vitamin B12 also regulates the availability of methyl donors such as methionine.

2.9.2. Requirements

Requirements for vitamin B12 have been established for few species and seem to be more species specific than other essential nutrients. Given the availability of non-dietary sources of vitamin B12, some studies found that certain fish such as juvenile tilapia *Oreochromis niloticus × O. aureus* may not require vitamin B12 supplementation, or that growth is only slightly reduced if vitamin B12 is not supplemented in the diet. However, in other species including salmonids and fast growing marine fish, vitamin B12 supplementation in practical diets with plant ingredients is necessary for optimum growth and expression of molecular markers. In these species the requirements range between 0.02 and 0.12 mg vitamin B12 kg^{-1} diet (Izquierdo et al., in prep.).

2.9.3. Effect on KPIS and biomarkers

The potential effect of dietary vitamin B12 on KPIs seems to be species specific. Main deficiency symptoms include anorexia, poor growth and macrocytic anemia, including the occurrence of multiple aberrant forms of erythrocytes. Vitamin B12 is stored for long periods in fish tissues reducing the occurrence of deficiencies. In other vertebrates, vitamin B12 deficiency causes sub-acute degeneration of different tissues, including demyelination of the spinal cord affecting the functioning of the nervous system. Genes codifying for proteins that couple with vitamin B12 have been considered as biomarkers of vitamin B12 deficiency. A single soluble cobalamin-binding protein has been identified in fish and proposed to be used as a biomarker for vitamin B12 status.

2.9.4. Practical aspects

Despite some vitamin B12 is found in FM, alternative plant ingredients are devoid of this vitamin. Moreover, although the fish microbiota may produce cobalamin, replacement of FM by alternative ingredients qualitatively and quantitatively changes gilthead sea bream microbiota. These changes may lead to a reduction in the microbiota production of vitamin B12 and even to the production of forms of cobalamin that reduce vitamin B12 availability for fish species. It is also remarkable that dietary cobalt levels or requirements influence the optimum levels of vitamin B12. Nevertheless, fish accumulate vitamin B12 in the liver what reduces the risk to develop vitamin B12 deficiency.

Requirements of vitamin B12 for gilthead sea bream, had not been determined and PerformFISH evaluated the effects of dietary vitamin B12 levels (0.03-0.41 mg vitamin B12 kg^{-1} diet), in combination to dietary levels of vitamin B1 and B9, in growth, productive parameters and health status of in gilthead sea bream fed diets low in FM-FO.

2.9.5. Dietary levels for sea bream

Increase in dietary cobalamin from 0.03 to 0.41 mg vitamin B12 kg^{-1} diet does not affect sea bream growth or any other KPI. However, increase up to 0.09 mg vitamin B12 kg^{-1} diet re duces

hepatosomatic index and body lipid contents, up-regulating *MMCoA* expression. Besides, elevation of dietary vitamin B12 up to 0.41 mg vitamin B12 kg^{-1} diet markedly reduces t he occurrence of erythrocytes with irregular nucleus in gilthead sea bream.

2.9.6.Recommendations

Cobalamin requirements for optimal growth in gilthead sea bream seem to be as low as 0.03 mg vitamin B12 kg^{-1}. Despite PerformFISH studies showed an improvement in erythrocyte morphology by increase dietary vitamin B12, such an effect could have been related to the dietary folic acid levels. Therefore further studies are needed to confirm the optimum levels of cobalamin in practical diets for gilthead sea bream.

3. European Sea Bass

3.1. Organic versus inorganic minerals

3.1.1. Background

Despite the importance of European sea bass, *Dicentrarchus labrax*, in the Mediterranean marine finfish production, studies on minerals requirements for this species when fed low FM diets are rather scarce. Therefore, further research is required to elucidate the effects of different forms of certain minerals when fish are fed on diets containing low Fish meal (FM) content. In a previous project (ARRAINA FP7) in order to evaluate requirements for certain minerals (Fe, Mn, Zn, Se, Cu) it was used a nutrient package (NP) at increased levels (0 to 400%) based on NRC (2011) recommendations for salmonids and in particular rainbow trout. The NP contained the inorganic form of the above minerals and it was added in low fish meal based diets. Evaluations were based on various parameters such as growth, feed utilization, immune response, oxidative status etc. Results showed that the inclusion of the NP at 150% was the most adequate for best performance, FCR, immune status etc.

Based on these results, for the needs of the present trial it was chosen the concentration of the aforementioned minerals contained at the 150% level of the NP, in the form of inorganic and organic form in low fish meal based diets. Two more diets were tested contained a lower and a higher level of organic minerals together with two control diets, a negative (plant based no minerals) and a positive (FM based and inorganic minerals) completed the experimental design.

The aim of the present study was to evaluate the effects of organic vs inorganic mineral concentrations in low fish meal based diets based on KPI's such as growth performance, feed utilization, body and tissue mineral content, immune status, antioxidant activity, and organoleptic characteristics of the end product in juvenile and commercial sized European sea bass (*Dicentrarchus labrax*).

3.1.2. Material and methods

3.1.2.1. Experimental diets

Trial 1 Juvenile sea bass

Six isonitrogenous and isoproteic diets were formulated and produced at the installations of the Applied Nutrition lab of the Institute of Marine Biology Biotechnology and Aquaculture in Athens, with an experimental extruder (CLEXTRAL) and oil was added by an experimental coater (DINISSEN). A low FM diet (10% FM and 2.5% krill meal) was used as the basis for the evaluation of a mineral premix containing Fe, Mn, Zn, Se, Cu in inorganic (Diet INORG) and organic (Diet ORG) forms at certain concentrations (Table 1) based on results of a previous project (ARRAINA FP7). In order to evaluate if lower concentrations of organic minerals are sufficient for KPI's improvement, a diet (Diet ORGlow) with 65% less mineral premix than diet ORG, was also tested together with an over dose diet (Diet ORGhigh) containing 50% more premix than diet ORG. Two control diets were also added to the experimental design: a negative control diet (CTRL-) consisting of the low FM basal diet where no mineral premix was added and a positive control diet, high in FM (30%) (CTRL+) containing inorganic premix. Levels of vitamins and other key-nutrients such as amino-acids, cholesterol etc was kept constant in all diets. Formulation and proximate analysis of the experimental diets is given in Table 1.

Trial 2 Commercial size sea bass

From the results of trial 1 three diets were chosen and tested to commercial sized fish. The diets were: the control diet (CTRL) high in fish meal (20%) containing inorganic mineral premix, the low FM diet (10% FM and 2.5% krill meal) containing the optimum level of mineral premix inclusion (diet ORG) of the minerals Fe, Mn, Zn, Se, Cu and 65% lower level than the optimum (Diet ORG low). Diet formulation, gross composition and fatty acid profile in given in Table 2. All diets were isonitrogenous and isoenergetic. Levels of EPA&DHA in all diets were at adequate % to cover EFA requirements (1.7% to 1.8%).

3.1.2.2. Fish rearing and sampling

Trial 1 Juvenile sea bass

European sea bass of an initial average weight of 20.18 ± 3.37g were obtained from a commercial fish farm in Greece and transferred to the facilities of the laboratory of Applied Fish Nutrition, Institute of Marine Biology, Biotechnology & Aquaculture, of the Hellenic Centre for Marine Research in Athens. Fish were distributed in groups of 25 fish in 24 tanks of 170-L capacity, quadruplicates tanks per treatment and left to acclimate for two weeks feeding on a commercial type diet, prior to the beginning of the experiment. During the experiment, the photoperiod was 12L: 12D light/dark, seawater salinity was 38g L-1 and temperature was 22 ± 1oC. Temperature and dissolved oxygen (DO) were recorded daily. Total ammonia (NH3/NH4+), nitrate (NO3 -) and nitrite (NO2-) were measured weekly using API tests (aquarium pharmaceuticals) and never exceeded 0.25, 0.25 and 1.0 ppm, respectively. Fish were fed the experimental diets at 1.6% of their body weight on the average by hand three times per day at the begging and until they reach an average weight of 40gr (0:900, 12:00 15:00) and then two

times per day (09:00, 15:00 hours) and until the end of the trial. The growth study was undertaken for 94 days. Feed conversion, specific growth rate, daily growth index, feed and protein utilization was calculated at the end of the experimental period. Feed intake and mortalities were recorded daily.

All fish were anaesthetized using diluted clove oil and weighed individually at the beginning, after 30 days and at the end of the experimental period. At the end of the experimental period 6 fish per tank were anaesthetized and blood was taken by caudal vein puncture. Twenty µl of fresh blood were heparinised for direct use in the respiratory burst assay whereas the remaining non-heparinised blood was kept overnight in the fridge to allow coagulation. The next day, samples were centrifuged at 20 000g for 10 min and serum was kept at -80°C until analysis of the different humoral immune parameters. Ten fish from each tank were sampled for gross body composition as well as, Fe, Zn, Se, Mn, Cu determination. Five to six fish were sampled for mineral terminations in selected tissues as follows: vertebrae and skin for Zn and Mn, muscle and liver for Se and spleen for Fe. Fish for gross composition and mineral determinations were kept at -20oC until analysis. Liver samples from five fish per tank (15 samples /treatment) were also excised and snap frozen in liquid nitrogen. Samples were kept in -80ºC until they were analysed for hepatic glutathione (GSH) and glutathione-related enzyme activities (SOD, Total GPx, Se-GPx, non Se-GPx and GST). Samples of gut, liver and gills were removed from 3 fish of each replicate tank, fixed in 10% buffered formalin and processed for paraffin histology.

Trial 2 Commercial size sea bass

European sea bass of an initial average weight of 217.7 ± 44.3 g were obtained from a commercial fish farm in Greece and transferred to the facilities of the laboratory of Applied Fish Nutrition, Institute of Marine Biology, Biotechnology & Aquaculture, of the Hellenic Centre for Marine Research in Athens. Fish were distributed in groups of 25 fish in 9 tanks of 1m3 capacity, triplicates tanks per treatment and left to acclimate for two weeks feeding on a commercial type diet, prior to the beginning of the experiment. During the experiment, the photoperiod was 12L: 12D light/dark, seawater salinity was 38g L-1 and temperature was 22 ± 1oC. Temperature and dissolved oxygen (DO) were recorded daily. Total ammonia (NH3/NH4+), nitrate (NO3 -) and nitrite (NO2-) were measured weekly using API tests (aquarium pharmaceuticals) and never exceeded 0.25, 0.25 and 1.0 ppm, respectively. Fish were fed the experimental diets at 0,8% of body weight (according to feeding tables provided by the industry) by hand two times per day (09:00, 15:00 hours). The growth study was undertaken for 93 days. Feed conversion, specific growth rate, daily growth index, feed and protein utilization was calculated at the end of the experimental period. Feed intake and mortalities were recorded daily.

All fish were anaesthetized using diluted clove oil and weighed individually at the beginning, and at the end of the experimental period. Six fish from each tank were sampled for gross body composition while four fish were kept for mineral composition in selected tissues. Five fish were sampled for fillet gross composition and fatty acid profile. Finally five fish were taken for organoleptic tests. Fish and fillets for gross composition and mineral determinations were kept at -20oC until analysis.

3.1.2.3. Biochemical analysis

Proximate analysis of the experimental diets and body and fillet gross composition was determined according to AOAC (1990).

Diets whole body and tissues were analyzed for Fe Zn, Mn and Se, concentrations using flame atomic absorption spectophotometry AOAC 999.10-2005 in order to evaluate bioavailability of the different forms of the mineral premix used (inorganic vs orghanic).

3.1.2.4. Analysis of GSH content and GSH-related enzymes activities

Livers from 15 fish per diet (5 fish from each replicate tank) were collected in liquid N2 and stored at -80 oC for the analysis of GSH and GSH-related enzyme activities (i.e. SOD, tGPx, Se-GPx, non Se-GPx and GST). GSH analysis was performed according to Rahman et al. (2006). SOD activity was measured by using xanthine/xanthine oxidase model as the source of superoxide radicals according to Peskin and Winterbourn (2000). tGPx, Se-dependant GPx and non Se-GPx were estimated according to McFarland et al (1999). The substrates for the enzymes were cumene hydroperoxide and H2O2. The loss of NADPH was monitored as the reaction progressed. The GST assay was based on Habing and Jakoby (1981) modified for a 96-well format and using CDNB as a conjugation substrate. The analysis is still on going.

3.1.2.5. Immunological analysis

The haemoglobin concentration was measured using the Drabkin reagent as described before (Rigos et al., 2010). The spontaneous and zymosan-triggered respiratory burst activities were assessed in whole blood using the chemiluminescence assay (Henry et al., 2009). The antibacterial activity of serum was assessed against a Gram-positive bacterium (lysozyme activity) and against a Gram negative bacterium (complement E.coli killing activity) (Kokou et al., 2012). The ceruloplasmin oxidase activity was measured to assess the inflammatory status of the fish and the serum antiprotease activity was used as an indicator of the fish capacity to inhibit the trypsin produced by pathogens (Henry and Fountoulaki, 2014). The alkaline phosphatase activity was measured using the methods described by Guardiola and colleagues (2014) and the total protein concentration was measured using the Bradford method (Bradford, 1976).

3.1.2.6. Histological analysis

Standard histological methods (heamatoxyline and eosin staining) were used. Briefly, tissues were transferred to ethanol 70%, dehydrated in ascending ethanol concentrations to 100%,

embedded in paraffin (56 1C), and sections (5 mm) (microtome Leica RM 2255, Nussloch, Germany) were stained with haematoxylin and eosin (Leica Auto Stainer XL, Nussloch, Germany) and examined under light microscopy (Olympus VANOX-T, NJ, USA) equipped with a digital camera (Infinity, Lumenera, Ontario, Canada. Sections of the anterior, and posterior gut were examined for the appearance of absorptive vacuoles in the mucosal enterocytes and the integrity of mucosa and submucosa. Measurements of the goblet cells as well as villi width and height was measured using the programme Imag G. Goblet cells were counted in three fields per intestinal tract at 40× magnification. Liver sections were evaluated on lipid degeneration level and the integrity of hepatocytes. Gills were checked for alterations in primary and secondary lamellae. Photos were processed by a Image analysis software (Digital Image Systems, Athens, Greece).

3.1.2.7. Statistical analysis

All values are presented as means ± standard deviations and differences present at 5% level were considered significant. Normal distribution and homogeneity of variance were checked using Kolmogorov-Smirnov and Levene tests, respectively. One-Way ANOVA and Tuckey's post hoc test were performed. For some of the immune parameters, when appropriate (i.e., population was not normal as for the respiratory burst and ceruloplasmin activity), data were Ln- transformed and when homogeneity of variance was not obtained (i.e. haemoglobin and protein concentrations),Welch test was applied. Kruskal-Wallis and Tamhane test were performed for lysozyme and anti-protease activity. SPSS 13.0 software was used for all statistical analysis.

Table 3.1. Diet formulation and proximate composition of the experimental diets in trial 1 sea bass juveniles, in g kg-1

	CTRL+	CTRL-
Fish meal 68	30	10
Krill meal		2.5
Wheat meal	16.4	13.4
Wheat Gluten	15	22
Corn gluten	10	10
Soy bean Conc 60	15	25
Fish Oil	7	8
Rapeseed Oil	4	4
Vitamin premix	0.086	0.3
Mineral premix*	0,05	3
Monocalcium Phosphate		
Lysine	0.4	0.9
Methionine	0.2	0.4
Cholesterol		0.2
Choline Chloride	0.2	0.2
Histidine		0.1

* theoretical concentration per mineral in the diet (mg/kg) Se 0.67, Fe 248.0, Cu 17.3, Mn 59.7, Zn 169.0 the inorganic form consisted of Sodium Selenite, Iron sulphate, Copper sulphate, Manganeous oxide and Zinc oxide. The organic mineral consisted of the chelated form which is the mineral linked to an aminoacid.

	CTRL -	INORG	ORGlow	ORG	ORGhigh	CTRL +
Dry matter	91.98	91.79	92.01	91.80	92.13	91.60
Protein	48.37	48.18	47.68	48.33	48.19	49.42
Fat	16.34	15.92	16.26	16.26	16.00	15.90
Ash	5.65	5.63	5.69	5.62	5.71	6.58
Starch	13.22	11.1	11.05	11.09	13.68	14.98
NFE	16.43	19.17	19.33	18.70	16.42	13.13
Carohydrates	29.65	30.27	30.38	29.79	30.10	28.11
Energy kj/kg	23.00	22.90	22.93	22.98	22.90	22.81

Mineral content (measured by analysis) in the diets in mg/kg diet

	CTRL -	INORG	ORGlow	ORG	ORGhigh	CTRL +
Mn	34.0	43.9	35.8	45.3	87.9	44.6
Zn	68.0	155.0	106.1	166.6	257.0	172.7
Cu	6.4	19.4	13.1	18.7	26.6	11.1
Fe	166.1	203.1	172.0	215.1	267.1	237.8
Se	0.25	0.65	0.39	0.76	0.98	0.82

Table 3.2. Diet formulation and proximate composition of the experimental diets of trial 2, sea bass commercial size in g kg-1

	CTRL	Low FM
Fish meal 68	20	10
Krill meal	0	2.5
Wheat meal	18.52	16.16
Wheat Gluten	18	18
Corn gluten	10	10
Soy bean Conc 60	17	25
Fish Oil	10	10
Rapeseed Oil	4	4
Vitamin premix	0.086	0.3
Mineral premix	0.05	0.05
Monocalcium phosphate	1.5	3
Lysine	0.4	0.7
Methionine	0.2	0.3
Cholesterol		
Choline Chloride	0.2	0.2

% Gross composition & Fatty acids Analysis			
	CTRL	ORG	ORGlow
Moisture	6.28	5.87	6.21
Ash	5.57	5.93	5.93
Protein	46.54	45.37	46.15
Fat	16.99	17.03	16.91
Starch	14.16	12.95	11.36
NFE	24.62	25.81	24.80
Carbohydrates	38.78	38.76	36.16
Energy	24.40	24.14	23.83
∑ Saturates	19.18	19.98	19.19
∑Monounsaturates	47.89	47.91	47.90
∑ω9	34.24	34.71	34.47
∑ω6	16.59	16.27	16.71
ω3	16.34	15.84	16.21
EPA	5.03	4.85	5.01
DHA	5.60	5.37	5.33
ω3/ω6	0.99	0.97	0.97

Table 3.3. Growth performance parameters and feed utilization of sea bass juveniles fed different experimental diets

	CTRL-	INORG	ORGlow	ORG	ORGhigh	CTRL+
Initial body weight (g)	20.21±0.31	19.92±0.53	20.22±0.32	20.23±0.41	19.85±0.35	20.46±0.74
Final body weight (g)	69.59±1.1	69.95±3.3	71.40±1.1	74.44±2.5	70.30±3.9	72.76±2.4
Weight increase (g)	49.47±0.92	50.03±2.8	51.19±1.35	54.22±2.15	50.45±3.74	52.30±1.7
[1]SGR	1.30±0.03^{a}	1.31±0.02^{ab}	1.32±0.03^{ab}	1.36±0.02^{b}	1.32±0.04^{ab}	1.33±0.01^{ab}
[2]DGI	1.48±0.02^{a}	1.50±0.05^{ab}	1.52±0.04^{ab}	1.58±0.04^{b}	1.51±0.07^{ab}	1.53±0.02^{ab}
[3]FCR	1.19±0.02	1.21±0.04	1.18±0.05	1.14±0.02	1.20±0.04	1.19±0.04
[4]PER	1.74±0.03	1.71±0.05	1.78±0.08	1.82±0.04	1.73±0.06	1.71±0.05
[5]ANPU	27.93±0.41	27.59±1.8	29.93±1.1	30.51±2.1	28.26±1.4	27.42±0.9
[6]Hep. index	2.12±0.38	2.40±0.41	2.16±0.4	2.29±0.51	2.09±0.41	2.39±0.43

Data are presented as means of four replicates accompanied by STD Standard deviation. Different letters at the same row indicate significant difference between diets.

[1] **SGR (Specific growth rate (% day^{-1}) = 100x (ln final weight - ln initial weight)/days**

[2] **DGI (Daily growth index)**= (Final BW^{3})-(Initial BW^{3})*100/days of rearing

[3] **FCR (Feed conversion ratio)** = consumed dry feed (g) / weight gain (g).

[4] **PER (Protein efficiency ratio)=** weight increase (g) / protein consumed (g)

[5] **ANPU (Apparent net protein utilization)=** {(protein body final - protein body initial)*100}/ protein consumed

[6] **Hepatosomatic index**= weight fish(g)/weight liver (g)*100

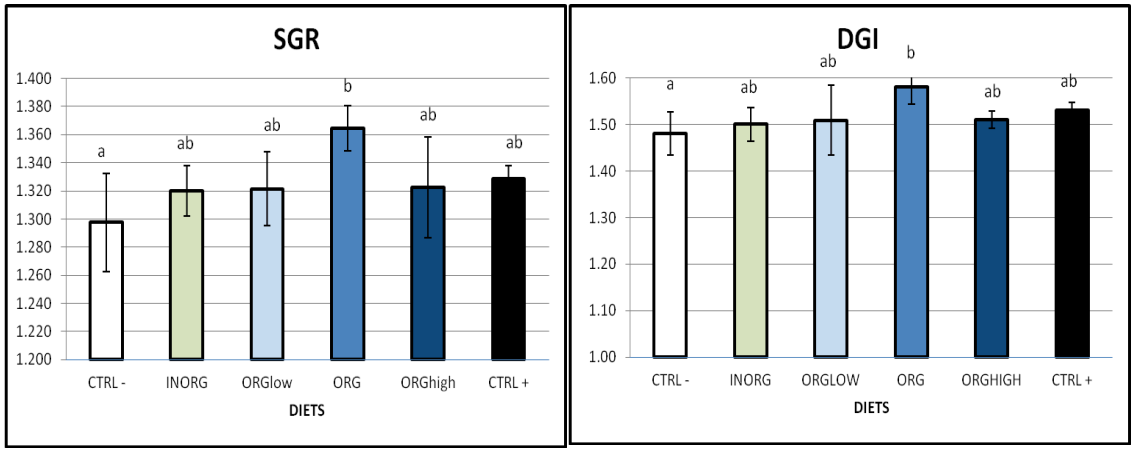


Figure 3.1. Specific growth rate and feed conversion ratio in juvenile Sea bass fed on different forms of mineral premix supplemented diets

3.1.3. Results

Growth-feed efficiency

At the start of the experiment, there were no significant differences between means and range of sea bass initial body weights for the eight diets (Table 3, Figure 1). No mortalities (survival rate 98%) were observed during the whole experimental period. Growth parameters measured, like mean final weight, weight increase, showed an improvement when the organic mineral was added in the low FM diet compared to the inorganic mineral premix, although differences were not significant due to deviations between replicate tanks of the same diet. SGR and DGI showed significant difference between the ORG diet and the CTRL- diet while with the rest of the diets differences were not significant. However diet ORG showed a tendency for better performance that diet INORG and the same was true also for CTRL+, ORGlow and ORGhigh.

Feed conversion ratio (FCR) didn't showed significant differences between diets (Table 3) however the lowest value (best) was achieved in those groups fed on diet ORG. The same was true for protein efficiency expressed as PER and ANPU, but since deviations between replicate tanks were high differences were not significant. Although fish fed on diet ORG achieved higher values than the rest of the diets (PER 1.82, ANPU 30.51). The lowest value 27.43 was shown in diet CTRL+ followed by diets INORG 27.59 and CTRL- 27.93.

Whole body composition

Whole body composition is given in Table 4. There were no significant differences in any of the components measured. Moisture content ranged from 63,5. to 64.9%, ash from 3.6 to 4.1%, protein from 15.7 to 16.3% and fat from 14.7 to 16.3%. Liver glycogen and fat didn't show differences between diets and values ranged from 7.8% to 10.2% and 27.4% to 31.8% respectively. A tendency to lower fat content was evident in all diets compared to the CTRL+ diet (FM based diet). While fat and glycogen content correlate negatively.

Whole body mineral content

Whole body Mn, Zn, Cu, Fe, Se are given in Table 5 and values are expressed in wet material. Mn, Zn and Cu concentrations differed significantly between diets; Mn level in the CTRL- diet was the lowest while in ORG diet was the highest although not significantly different from the rest of the diets. Zn levels were significantly the lowest in the CTRL- and the ORGlow while the highest concentration was measured in the CTRL+, the ORGhigh and the ORG diet. Zn concentrations significantly correlate with dietary Zn concentrations (r2 0.82). Values for the ORG diet were higher than the INORG although differences were not significant. Cu values were low in the CTRL- followed by the ORGlow and the CTRL+, intermediate values were given by the rest of the diets. Fe whole body levels were higher in the ORG diet but differences were not significant because deviations between replicate tanks were high. Se concentrations didn't showed significant differences although values for diets ORG, ORGhigh and CTRL+ tented to be higher.

Trial 2 Growth feed utilization

At the end of the experimental period fish reached the same final weight in all diets. Final weight SGR and DGI, of diets ORG and ORGlow were the same with the CTRL+, and the same was true also for feed utilization (FCR). Body and fillet composition Body composition in all the components measured didn't show significant differences between diets, and the same was true also for fillet composition. Fillet fat content was lower in the ORG and ORGlow diets compared with the CTRL. Fatty acid profile in main groups of fatty acids didn't show significant differences. The percentages measured reflected that of the diets.

Table 3.4. Whole body and liver composition of sea bass juveniles fed on different forms of mineral premix supplemented diets

	Initial. Population	CTRL-	INORG	ORGlow	ORG	ORGhigh	CTRL+
Moisture	71.68	64.93±0.47	64.56±0.13	64.29±0.56	63.55±1.31	64.50±0.62	64.33±0.61
Ash	4.62	3.97±0.10	3.94±0.65	4.08±0.39	4.07±0.81	3.59±0.42	3.56±0.11
Protein	14.65	16.01±0.72	15.70±0.45	16.19±0.33	16.17±0.67	15.83±0.60	15.94±0.62
Fat	8.76	14.68±0.34	15.55±0.39	15.30±0.55	15.69±0.81	15.39±0.79	16.31±1.01
				Liver composition			
Glycogen		10.16±1.13	9.10±1.02	9.68±0.76	9.29±0.98	8.19±1.14	7.79±1.03
Fat		29.99±1.5	30.06±3.2	27.37±4.7	29.12±1.3	30.32±3.8	31.79±0. 7

Table 3.5. Whole body mineral content (mg/kg) of sea bass juveniles fed on different forms of mineral premix supplemented diets (values are expressed on wet material)

	Mn	Zn	Cu	Fe	Se
CTRL-	1.49±0.28^{a}	12.69±1.63^{a}	0.48±0.10^{a}	6.17±2.15	0.17±0.02
INORG	1.70±0.21^{ab}	16.66±1.83^{b}	0.63±0.07^{b}	7.35±2.86	0.17±0.01
ORGlow	1.84±0.29^{b}	12.65±1.72^{a}	0.58±0.13^{ab}	7.03±2.28	0.17±0.02
ORG	1.87±0.22^{b}	17.93±1.40^{bc}	0.65±0.18^{b}	7.81±2.25	0.21±0.06
ORGhigh	1.70±0.26^{ab}	18.97±1.63^{c}	0.64±0.11^{b}	7.52±1.88	0.21±0.02
CTRL+	1.81±0.18^{b}	18.56±2.31^{c}	0.61±0.11^{ab}	6.72±0.95	0.20±0.01

Pvalue Mn 0.003, Zn 0.000, Cu 0.012

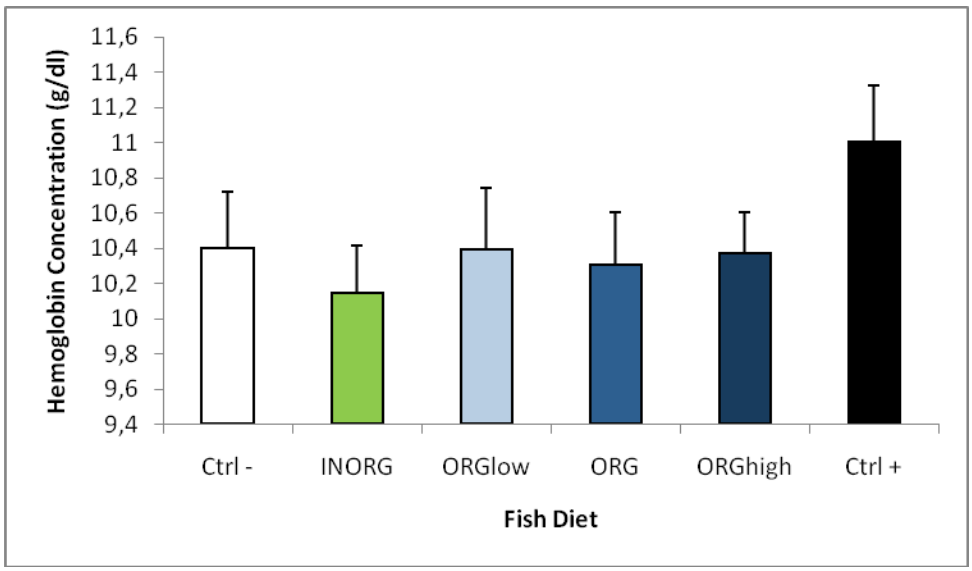


Figure 3.2. Hb concentration (g/dl) in fish fed the 6 experimental diets. Bars represent mean ± SEM. n=17-24.

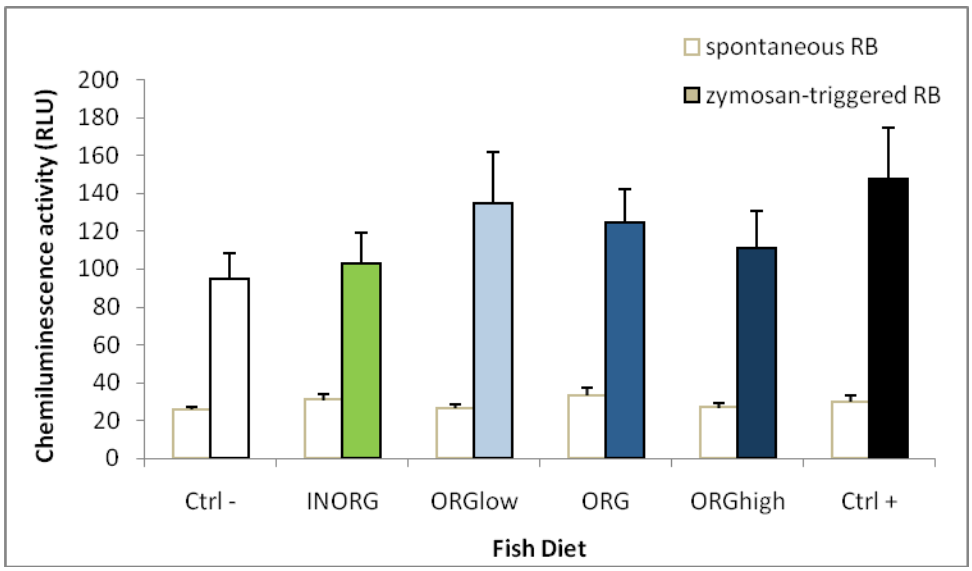


Figure 3.3. Spontaneous (white bars) and zymosan-triggered (coloured bars) respiratory burst activity in the blood of European sea bass fed FM-based diet (Ctrl+) or a plant-meal-based diet (Ctrl-) enriched with inorganic (INORG) or organic (ORG) minerals at 100% of predefined requirement levels or at 65% (ORGlow) or at 150% (ORGhigh) of the organic minerals premix. Bars represent mean ± S.E.M. There was no significant difference between dietary treatments (P>0.05). n=18-24.

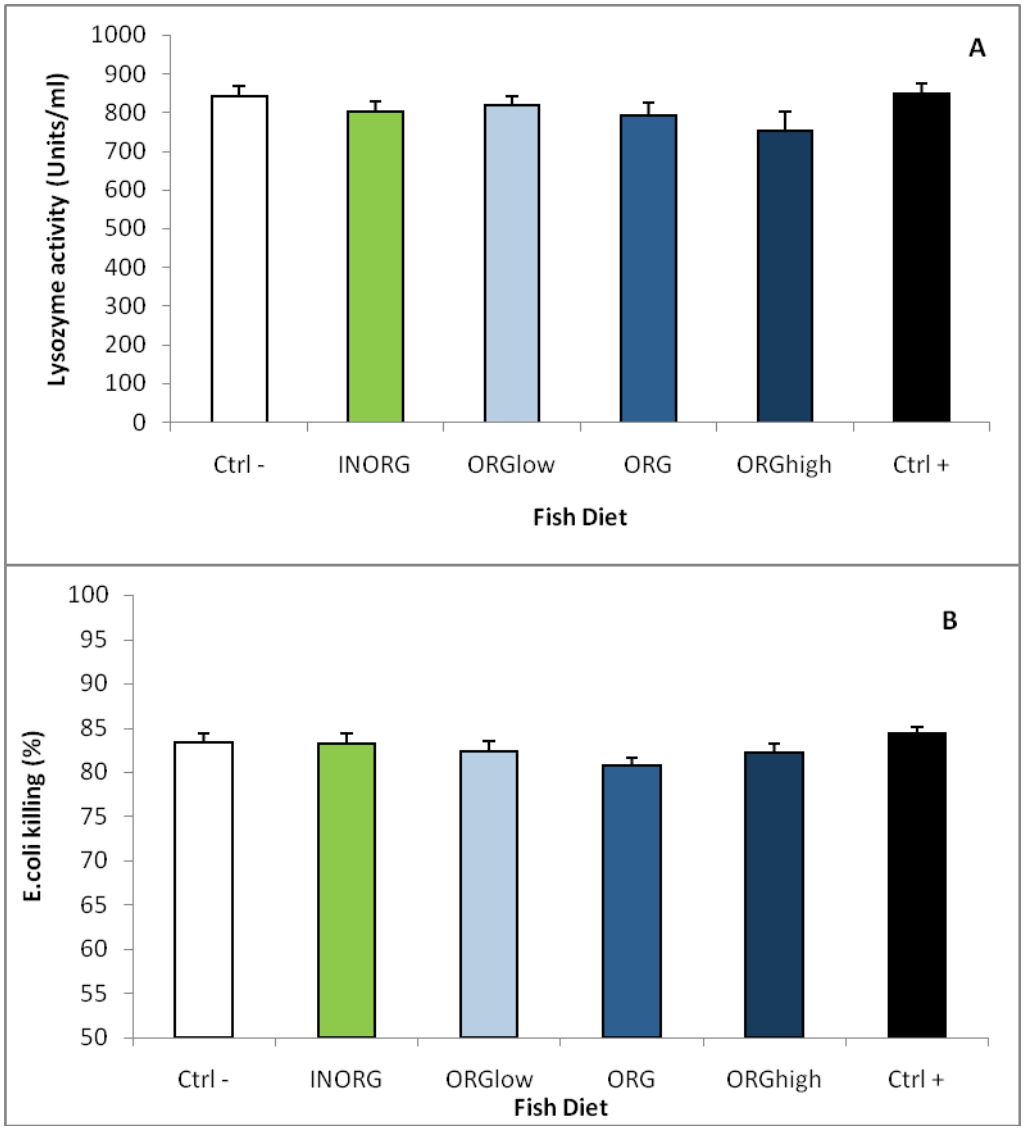


Figure 3.4. Antibacterial activity A) anti-Gram-positive bacterium Micrococcus luteus ; Lysozyme activity B) anti-Gram negative bacterium E. coli in the serum of European sea bass fed FM-based diet (Ctrl+) or a plant-meal-based diet (Ctrl-) enriched with inorganic (INORG) or organic (ORG) minerals at 100% of predefined requirement levels or at 65% (ORGlow) or at 150% (ORGhigh) of the organic minerals premix. Bars represent mean ± S.E.M. There was no significant difference between dietary treatments (P>0.05). n=16-23.

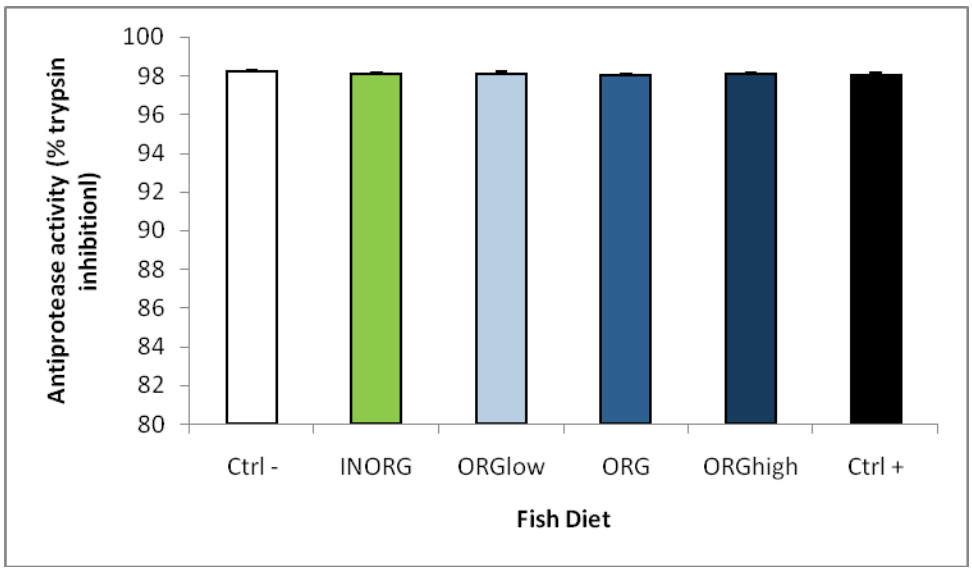


Figure 3.5. Antiprotease activity (Trypsin inhibition) in the serum of European sea bass fed FM-based diet (Ctrl+) or a plant-meal-based diet (Ctrl-) enriched with inorganic (INORG) or organic (ORG) minerals at 100% of predefined requirement levels or at 65% (ORGlow) or at 150% (ORGhigh) of the organic minerals premix. Bars represent mean ± S.E.M. There was no significant difference between dietary treatments (P>0.05). n=17-23.

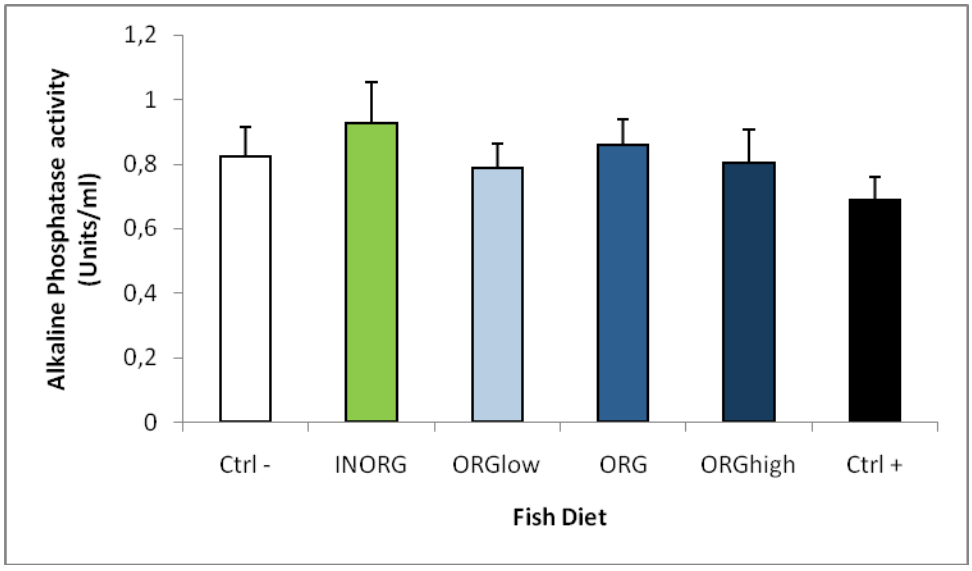


Figure 3.6. Alkaline phosphatase activity in the serum of European sea bass fed FM-based diet (Ctrl+) or a plant-meal-based diet (Ctrl-) enriched with inorganic (INORG) or organic (ORG) minerals at 100% of predefined requirement levels or at 65% (ORGlow) or at 150% (ORGhigh) of the organic minerals premix. Bars represent mean ± S.E.M. There was no significant difference between dietary treatments (P>0.05). n=18-24.

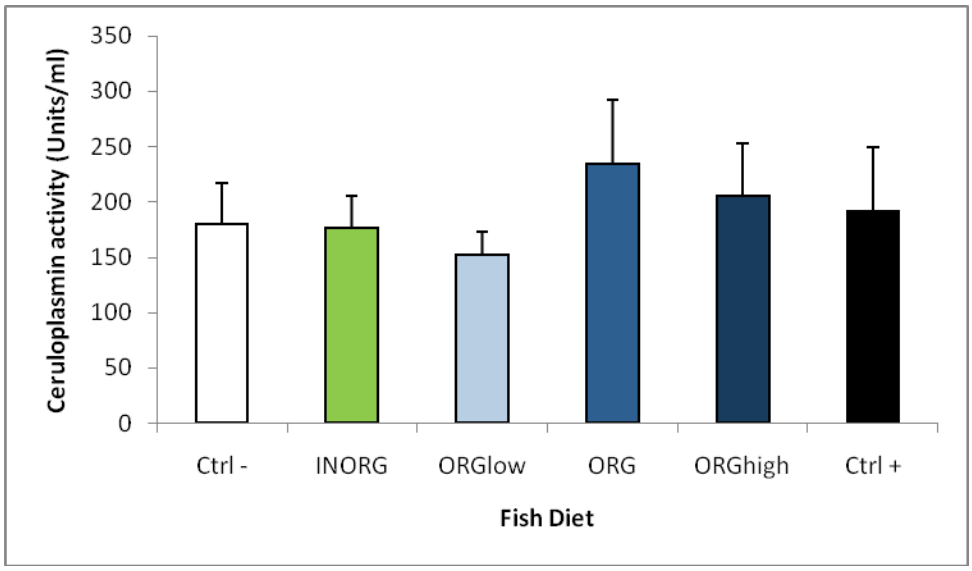


Figure 3.7. Ceruloplasmin activity in the serum of European sea bass fed FM-based diet (Ctrl +) or a plant-meal-based diet (Ctrl-) enriched with inorganic (INORG) or organic (ORG) minerals at 100% of predefined requirement levels or at 65% (ORGlow) or at 150% (ORGhigh) of the organic minerals premix. Bars represent mean ± S.E.M. There was no significant difference between dietary treatments (P>0.05). n=14-22.

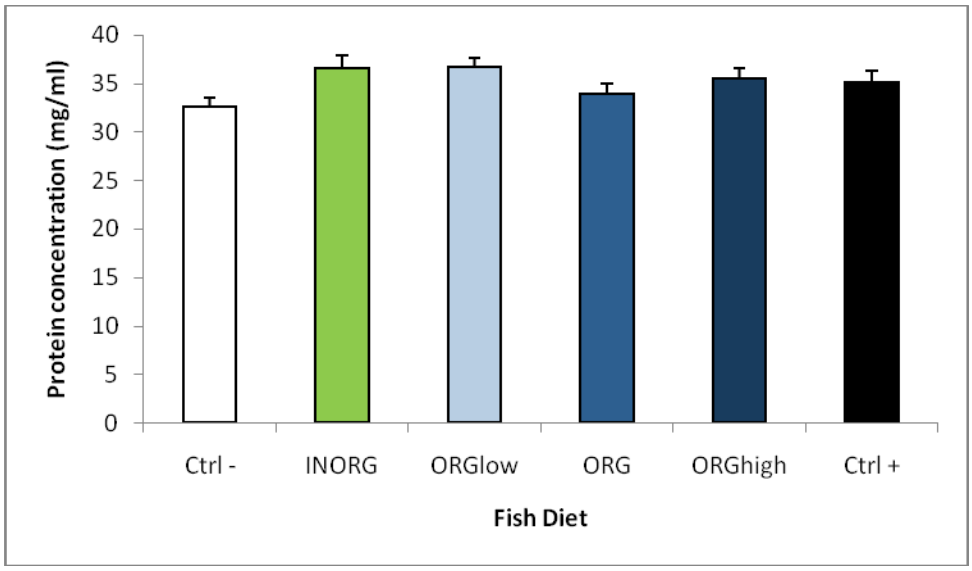
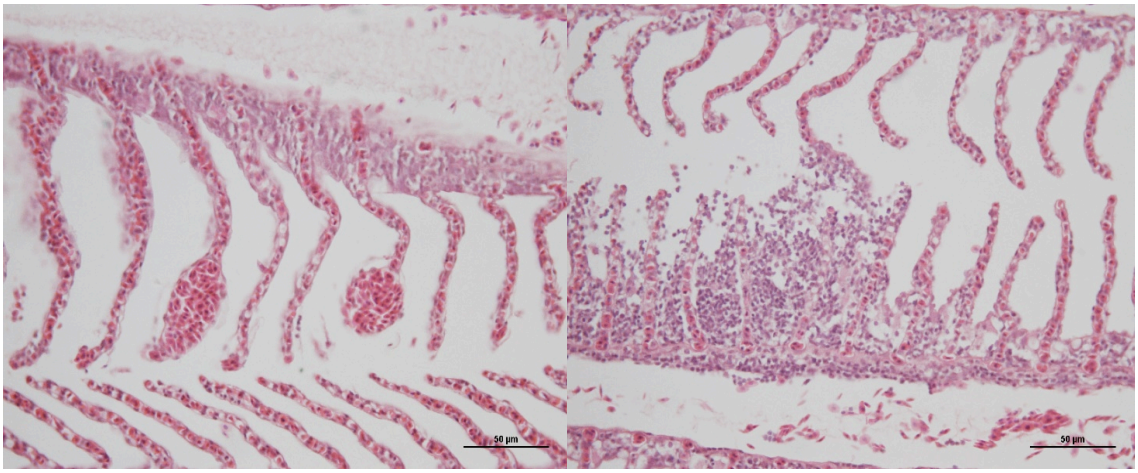
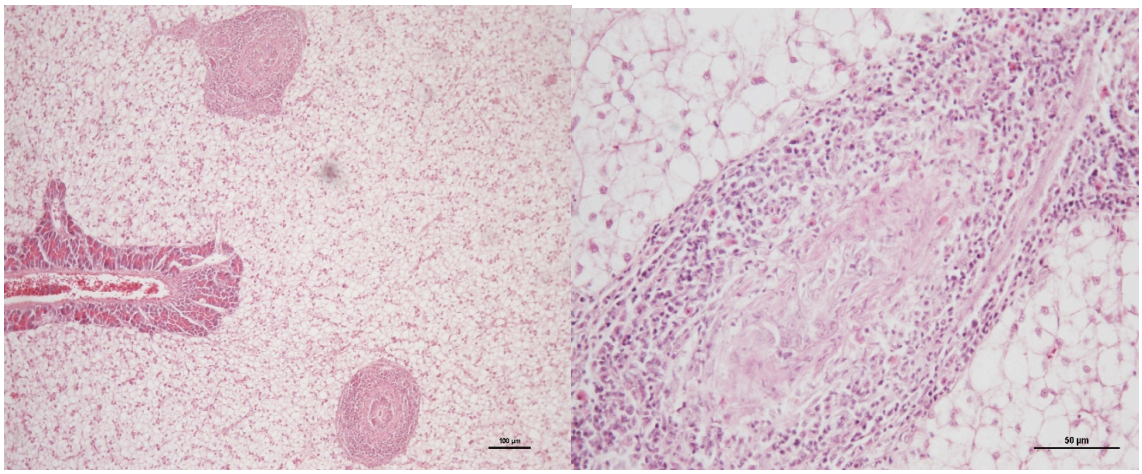


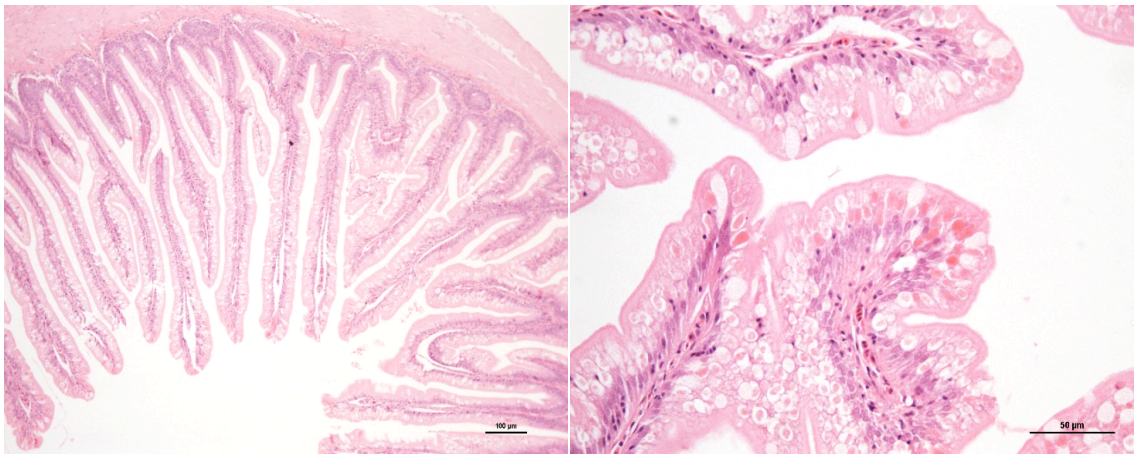
Figure 3.8. Protein concentration in the serum of European sea bass fed FM-based diet (Ctrl+) or a plant-meal-based diet (Ctrl-) enriched with inorganic (INORG) or organic (ORG) minerals at 100% of predefined requirement levels or at 65% (ORGlow) or at 150% (ORGhigh) of the organic minerals premix. Bars represent mean ± S.E.M. There was no significant difference between dietary treatments (P>0.05). n=18-24.



Figures 3.9. and 3.10. Multifocal sporadic capillary telangiectasia at the apex of secondary lamellae (Figure 9) and very mild multifocal epithelial hyperplasia at the base of secondary lamellae (Figure 10) in all tanks of all diets. No differences were found among diets.



Figures 3.11. and 3.12. Moderate to severe diffuse lymphoplasmacytic pericholangitis and cholangitis (Figures 11 and 12), and multifocal pancreatitis around pancreatic ducts. Diffuse high intracytoplasmic lipid storage into hepatocytes (Figure 11). No differences were found among diets.



Figures 3.13. and 3.14. An apparent goblet cells hyperplasia in the CTRL – (plant based) diet was observed. Diffusely the mucosa present numerous absorptive vacuoles referable to a good nutritional status and good absorptive functions in all the diets considered (Figures 13 and 14). No inflammatory reaction was seen in lamina propria or submucosa in all the diets examined. No other differences were found among diets.

Table 3.6. Growth performance parameters and feed utilization of sea bass commercial size trial 2

	CTRL +	ORG	ORGlow
Initial body weight (g)	218.2±2.6	217.5±1.8	217.3±4.4
Final body weight (g)	313.4±8.8	308.1±3.9	310.6±13.6
WI[1]	95.2±6.3	90.6±4.0	93.3±9.6
SGR[2]	0.39±0.02	0.37±0.01	0.38±0.03
DGI[3]	0.82±0.04	0.79±0.03	0.81±0.07
FCR[4]	1.65±0.09	1.74±0.1	1.68±0.15

[1], [2], [3], [4], as in table 3.3

Table 3.7. Whole body fillet gross composition & Fatty acids profile of sea bass commercial size, trial 2

	CTRL	ORG	ORGlow
Moisture	62.68±1.33	63.31±1.33	63.86±0.49
Ash	3.73±0.24	3.45±0.18	3.49±0.11
Protein	16.45±0.32	15.86±0.54	15.96±0.08
Fat	16.71±1.77	16.99±0.68	16.38±0.31
FILLET COMPOSITION			
Moisture	72.32±0.90	72.46±1.01	73.34±0.41
Ash	1.48±0.06	1.55±0.02	1.49±0.01
Protein	19.78±1.22	20.97±0.65	20.27±0.49
Fat	6.45±1.74^{ab}	5.16±1.6^{ab}	4.92±0.84^{b}
∑Saturates	24.67±0.22	25.09±0.61	24.55±0.42
∑Monounsaturates	46.41±1.38	45.32±2.51	46.01±0.42
∑ω9	34.29±1.11	33.23±2.8	33.93±0.78
∑ω6	13.06±0.19	13.04±0.68	13.45±0.42
∑ω3	15.50±1.25	16.48±0.97	15.79±0.34
EPA	4.10±0.3	4.43±0.54	4.21±0.01
DHA	7.73±1.1	7.01±0.97	7.45±0.04
ω3/ω6	1.19±0.09	1.27±0.27	1.18±0.06

3.1.4. Sea bass conclusions and recommendations

From the results of the present study and when feeding sea bass on a low fish meal based diet, enriched with an organic vs inorganic mineral premix consisting of Mn, Zn, Cu, Fe and Se, we can conclude the following:

Growth performance of juvenile sea bass was affected by the different forms of the mineral premix added in the diet. The addition of organic minerals at an optimum concentration (ORG diet) resulted in a better performance of the fish comparable to the FM based diet (CTRL+) which contained inorganic minerals and the INORG diet. At higher and lower addition levels of organic minerals growth was not affected.

Protein efficiency was better by the addition of organic minerals in the diet (ORG). CTRL+ diet showed lower values than the ORG, ORGlow and ORGhigh although due to high deviations in between replicates these differences were not significant.

Growth of commercial sized fish can be achieved with lower addition of organic minerals in the diet comparable with the CTRL diet (FM based diet).

Gills liver and intestine morphology didn't reveled differences between diets. The CTRL- diet showed a hyperplasia of the goblet cells in the intestine.

There was no significant effect of dietary supplementation with organic or inorganic minerals of the basal plant-based diet nor between the CTRL- and CTRL+ in any of the studied immune parameters.

4. Conclusions

As a summary of the former studies conducted in PerformFISH, a list has been prepared containing the optimum dietary levels determined by the project in tasks 2.1.1 (2017) and 2.1.2 (2020) in comparison with the data available in previous studies (Table 5.1). Besides, the information has been also plotted in several graphs for a better comparison of the results at a simple glance (Figures 5.1, 5.2 and 5.3)

Table 4.1. Recommended dietary levels of specific nutrients for gilthead sea bream based on the results obtained in PerformFISH and previous studies

Nutrient (mg kg^{-1})	Atlantic salmon	Gilthead sea bream			
	ARRAINA +NRC	NRC	ARRAINA	PERFORMFISH	
	2011-2015	2011	2015	2017	2020
Copper	5-8	-	16-17	10	5.5-9.5
Manganese	10-37	-	36.5	35	19-30
Selenium	1	-	-	0.77	0.94
Vitamin A (IU kg^{-1})	-	-	6,360-12,670	25,330	27,000
Vitamin D	0.04	-	-	0.06	0.15-0.30
Vitamin K	<10	-	-	9.8	10-13
Thiamin (B1)	>10	0.5	-	3.8	6
Folacin (B9)	2	-	-	3.12	3.5-5.5
Cobalamin (B12)	0.45	-	0.58	0.58	0.09-(0.40?)

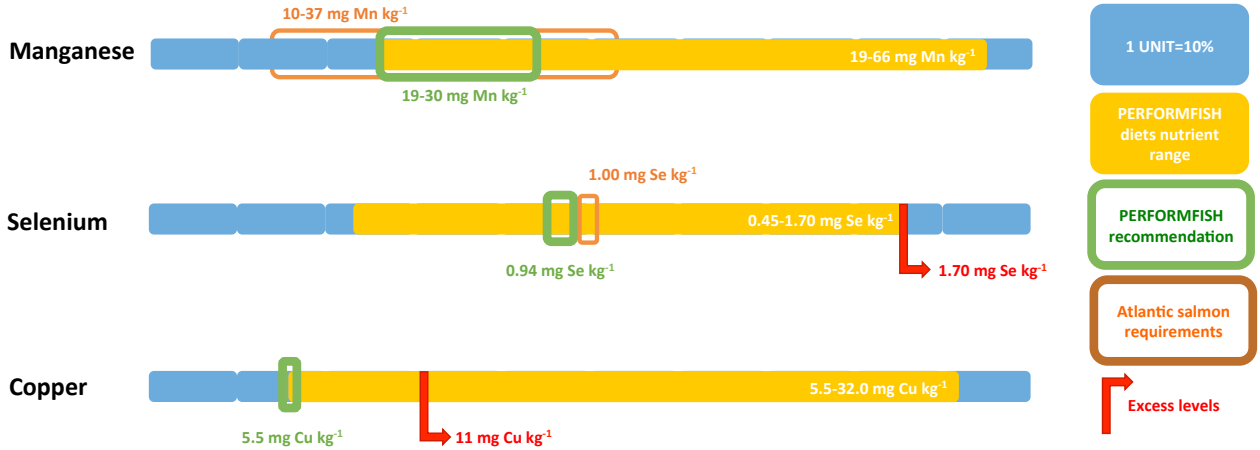


Figure 4.1. Summary of the dietary levels of minerals tested for gilthead sea bream in PerformFISH, the recommended and excess levels and the requirements for Atlantic salmon for comparison.

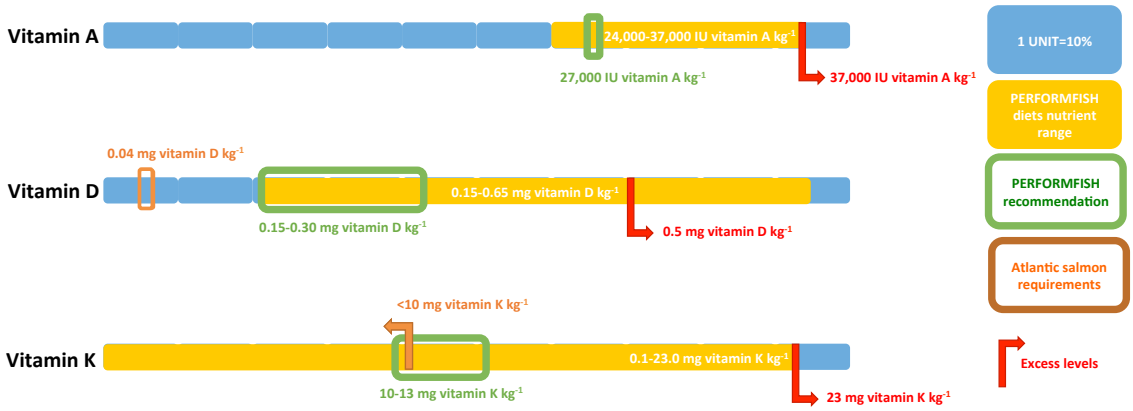


Figure 4.2. Summary of the dietary levels of fat-soluble vitamins tested for gilthead sea bream in PerformFISH, the recommended and excess levels and the requirements for Atlantic salmon for comparison.

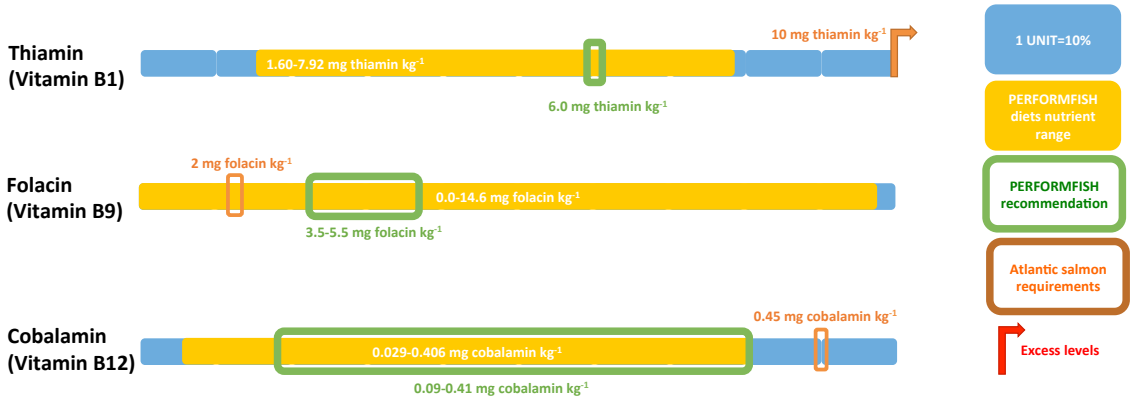


Figure 4.3. Summary of the dietary levels of the water-soluble vitamins tested for gilthead sea bream in PerformFISH, the recommended and excess levels and the requirements for Atlantic salmon for comparison.

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Domínguez, D., Montero, D., Zamorano, M.J., Fontanillas, R., Izquierdo, M., submitted b Effects of vitamin K supplementation in gilthead sea bream (*Sparus aurata*) fingerlings fed diets high in plant based feedstuffs.

Domínguez, D., Montero, D., Zamorano, M.J., Fontanillas, R., Izquierdo, M., submitted c. Effects of vitamin D supplementation in gilthead sea bream (*Sparus aurata*) fingerlings fed diets high in plant based feedstuffs.

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6. Annex 1. Publications derived from the deliverable with complete data

Aquaculture 507 (2019) 466–474

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Effects of copper levels in diets high in plant ingredients on gilthead sea bream (*Sparus aurata*) fingerlings

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ABSTRACT

Fish meal is increasingly substituted in diets for gilthead sea bream with ingredients of terrestrial origin which may affect the mineral content and availability. Among these minerals, copper (Cu) is an essential trace element whose excess may have a potential toxic effect. Since ingredients of terrestrial origin have higher Cu levels than marine ones it is important to define the optimal dietary supply of Cu. Therefore, the aim of this study was to evaluate optimal dietary inclusion level of Cu in low FM-FO diets for gilthead sea bream fingerlings.

Five practical diets with low FM (10%) and FO (6%) contents were respectively supplemented with 5 levels of $CuSO_4$ to provide 5.5, 7.4, 9.3, 11.0 and 32.0 mg Cu kg^{-1} diet. Sea bream fingerlings (12.6 ± 1.4 g, mean ± SD) were distributed in 15 tanks with 30 fish per tank in triplicates and randomly assigned one of the dietary treatments. The fish were fed three times a day until apparent visual satiation for 42 days. Growth was recorded at the end of the trial and samples were taken for biochemical, mineral, histology, X-ray and hepatic gene expression analyses.

The results obtained suggest that gilthead sea bream fed practical diets based on plant protein sources that provide at least 5.5 mg Cu kg^{-1} need no additional Cu supplementation, whereas dietary contents of 11–32 mg Cu kg^{-1} negatively affected gilthead sea bream performance by reducing growth, increasing oxidative risk and inducing hepatic damage and cholestasis.

Dietary Cu levels did not affect body weight, SGR, TGC or FCR, denoting that the level in the non-supplemented diet (5.5 mg Cu kg^{-1}) was enough to cover the requirements for growth. However, increasing dietary Cu levels from 5.5 to 9.3 mg/kg^{-1} up-regulated *cat* gene expression. On the contrary elevation of dietary Cu levels up to 11.0 and 32.0 mg Cu kg^{-1} tended to reduce growth and increased liver steatosis, broken cell margin, peripheral nuclei and sinusoid dilatation which are the markers of hepatic damage and cholestasis denoting potential toxic effects of Cu.

1. Introduction

In the context of feed-based aquaculture, fish derived ingredients, such as fish meal (FM) and oil (FO), are being widely substituted by those of plant origin. However, this replacement may alter dietary contents of certain minerals, requiring a revision of their optimum supplementation levels. Among these minerals, copper (Cu) is an essential metal that forms part of metalloenzymes involved in numerous physiological and structural functions in fish including antioxidant protection, such as CuZnSOD, cellular energy production, neurotransmitters metabolism or synthesis of collagen synthesis and melanin (Lall, 2002). Cu tends to accumulate in the liver, eyes, heart and brain (Halver and Hardy, 2002; Watanabe et al., 1997)

However, the concern with Cu has traditionally been related to its potential toxic effects on fish, which can vary from reduced growth, feed ingestion and productivity, to increased cell apoptosis, hepatic

Abbreviations: Copper, Cu; *CuZnSOD*, copper-zinc superoxide dismutase; FCR, Feed Conversion Ratio; FM, fish meal; FO, fish oil; SGR, Specific Growth Rate; TGC, Thermal Growth Coefficient

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lipid peroxidation, damage to gills and necrosis in liver and kidney (Clearwater et al., 2002; Tang et al., 2013; Watanabe et al., 1997; Woody and O'Neal, 2012). Freshwater species are particularly susceptible to Cu toxicity, as the lower levels of cations in the water increase the bioavailability of waterborne Cu to the gills, increasing the burden of total Cu uptake and reducing the margin for dietborne Cu intake ((Woody and O'Neal, 2012). In fact, effects of Cu toxicity can be seen in Channel catfish (*Ictalurus punctatus*) at daily Cu doses as low as 0.4–0.9 mg Cu kg^{-1} body $weight^{-1}$ (Murai et al., 1981). Nevertheless, dietary Cu requirements of most fish species range at concentrations of 3–13 mg Cu kg^{-1} dry diet, whereas this quantity may increase depending on the species or during rapid growth phases of their life cycle (Antony Jesu Prabhu et al., 2016; Clearwater et al., 2002). The highest optimum dietary levels reported (18.0 mg Cu kg^{-1}) are found for blunt snout bream (*Megalobrama amblycephala*). Furthermore, up to 28 mg Cu kg^{-1} can be found in certain plant ingredients, which is up to 5 fold the amount compared to FM (ARRAINA, 2015), thus, the amount of copper to be supplemented in feeds containing high levels of plant ingredients should be assessed.

Cu may lead to alterations in several physiological functions and markers when its level is inadequate. Growth and other productivity markers have been used to evaluate Cu requirements in several species including Atlantic salmon (*Salmo salar*, 8.5–13.7 mg Cu kg^{-1}, Lorentzen et al., 1998), large yellow croaker (*Larimichthys croceus*, 3.4 mg Cu kg^{-1}, Cao et al., 2014), malabar grouper (*Epinephelus malabaricus*, 2.0–6.0 mg Cu kg^{-1}, Lin et al., 2008, 2010), Russian sturgeon (*Acipenser gueldenstaedtii*, 5.0–8.0 mg Cu kg^{-1}, Wang et al., 2016, 2018) and tongue sole (*Cynoglossus semilaevis*, 11.0–12.0 mg Cu kg^{-1}, Wang et al., 2015). On the other hand, several molecular markers can be affected by Cu, such as regulators of oxidative damage like copper-zinc superoxide dismutase (*CuZnsod*, Antony Jesu Prabhu et al., 2016) and catalase (*cat*, Shao et al., 2012; Tang et al., 2013), and Cu transporters like copper transporter 1 (*ctp1*, Minghetti et al., 2008) and *atp7b* (Isani et al., 2011; Lanno et al., 1987). Cu deposition in tissues have also been used in Cu requirement determination studies (Antony Jesu Prabhu et al., 2016; Lall, 2002). High levels of Cu supplementation may lead to hepatic alterations related to liver damage (Handy et al., 1999; Shaw and Handy, 2006).

The effects of Cu on gilthead sea bream (*Sparus aurata*) have been evaluated focusing on Cu transporters (Minghetti et al., 2008), Cu proteins (Minghetti et al., 2010), seasonal Cu tissue changes (Carpenè et al., 1999) and the effects of toxic levels of Cu on metallothionein (Ghedira et al., 2010). However, none of these studies have addressed the Cu requirements in gilthead sea bream. Therefore, the aim of this study was to evaluate the effect of dietary Cu supplementation in gilthead sea bream growth, productive parameters and health status when fed diets low in FM-FO.

2. Material and methods

All the experimental conditions and sampling protocols have been approved by the Animal Welfare and Bioethical Committee from the University of Las Palmas de Gran Canaria.

2.1. Diets

In previous studies, gilthead sea bream fed graded levels of Cu and other micronutrients showed best Cu retention were fed at 10 mg Cu kg^{-1} (Dominguez et al., unpublished results), however the inclusion of several micronutrients simultaneously hindered a clear determination of requirements for Cu in this species. A basal diet closely mirroring practical gilthead sea bream feeds was formulated with low inclusion of FM (10%) and FO (6%). Five different experimental diets were produced by supplementing $CuSO_4$, to contain 5.5, 7.4, 9.3, 11.0 and 32.0 mg Cu kg^{-1} diet (Table 1). Diets were isoenergetic and isonitrogenous, and were designed to cover all known nutritional requirements for this species and were manufactured by extrusion process by Skretting Aquaculture Research Centre AS (Stavanger, Norway).

Table 1
Ingredients of the experimental diets supplemented with increasing levels of Cu.

Raw ingredient (%)	Cu5.5	Cu7.4	Cu9.3	Cu11.0	Cu32.0
Linseed oil	0.82	0.82	0.82	0.82	0.82
Wheat	11.69	11.69	11.69	11.69	11.69
Corn gluten	15.00	15.00	15.00	15.00	15.00
Wheat gluten	21.66	21.66	21.66	21.66	21.66
Soya concentrate	23.00	23.00	23.00	23.00	23.00
Faba beans	5.00	5.00	5.00	5.00	5.00
Fish meal	10.00	10.00	10.00	10.00	10.00
Rapeseed oil	3.00	3.00	3.00	3.00	3.00
Fish oil South American	6.00	6.00	6.00	6.00	6.00
Palm oil	1.64	1.64	1.64	1.64	1.64
Micronutrient premix[a]	2.19	2.19	2.19	2.19	2.19
Analysed Cu (mg/kg)	5.5	7.4	9.3	11.0	32.0

Proximal compostion (% fresh weight): lipids: 17.5, proteins: 50.4, ashes: 4.0.
[a] Micronutrient premix: methionine (10.6 g/kg), lysine (28.5 g/kg), phosphate (0.67%), vitamin premix (0.18%) and mineral premix excluding Cu (0.11%).

2.2. Feeding trial

The trial was carried out in the facilities of the Aquaculture Research Group (GIA) of the University of Las Palmas de Gran Canaria, Spain. Gilthead sea bream fingerlings, 12.6 ± 1.4 g (mean ± SD) were distributed in 15 tanks with 30 fish per tank in triplicate and randomly assigned one of the dietary treatments. The fish were fed until apparent satiation three times a day for 42 days. Water temperature (19.4 ± 0.4 °C, mean ± SD) and oxygen were monitored daily, while pH was registered weekly. Fish were kept under a natural photoperiod of approximately 12 h light. Growth was recorded and tissue samples were taken for biochemical, mineral, histology, X-ray and hepatic gene expression analyses at the end of the trial.

Growth, in terms of standard length (cm) and weight (g), was recorded at days 0, 18 and 42 of the trial by measuring and weighing all fish. Throughout the experiment, feed intake per tank was recorded. At the end of the trial productive parameters were calculated including Specific Growth Rate (SGR), Thermal Growth Coefficient (TGC) and Feed Conversion Ratio (FCR) using the following formulae:

SGR (%) = ((LnW2-LnW1))/days*100.
TGC = ((W2^1/3-W1^1/3))/(temp*days).
FCR = (Ingested food)/(generated biomass).
Where.
W1: initial body weight (g).
W2 final body weight (g).
Temp: Temperature (°C).

Previous to sampling, all fish were fasted for 24 h. During samplings fish were anesthetized with clove oil 50% (Guinama S.L.U., Valencia, Spain). Five fish per tank were sampled for biochemical, mineral and gene expression analysis. Samples were kept frozen at −80 °C until the analysis was conducted. Twenty fish per tank were sampled for radiographic assessment.

2.3. Gene expression

2.3.1. RNA extraction

Total RNA was extracted from 60 mg of liver using TRI Reagent Solution (Life Technologies, Carlsbad, CA, USA) and purified on RNeasy Mini Spin Columns (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Table 2
Sequences of primers used for gene expression analysis.

Gene	Nucleotide sequence (5′-3′)
bact	FW: TCTGTCTGGATCGGAGGCTC
	RV: AAGCATTTGCGGTGGACG
rpl27	FW: ACAACTCACTGCCCCACCAT
	RV: CTTGCCTTTGCCCAGAACTT
CuZnsod	FW: TTGGAGACCTGGGCAACGTGA
	RV: TCCTCGTTGCCTCCTTTTCCC
cat	FW: ATGGTGTGGGACTTCTGGAG
	RW: AGTGGAACTTGCAGTAGAAAC
ctr1	FW:CGGGTCTGCTCATCAACACCC
	RW: TGTGCGTCTCCATCAGCACCG
atp7b	FW: CGCTGGCCTCGTGCTTCAACC
	RW: CGACGACCGCAGGCTTCTCATTT

2.3.2. Reverse transcription

Reverse transcription of 1 μg total RNA from each experimental sample was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions with slight modifications. Briefly, 1 μg total RNA and nuclease-free water to a final volume of 15 μl were heated at 65 °C for 10 min and cooled in ice. Afterwards 1 μl of iScript reverse transcriptase and 4 μl of 5 × iScript reaction mix were added, reaching a final reaction volume of 20 μl. The complete reaction mix was incubated for 5 min at 25 °C, 30 min at 42 °C, and then 5 min at 85 °C to inactivate reverse transcriptase. For gene quantification, the reverse transcription reactions were diluted 1:10.

2.3.3. Quantitative PCR

The nucleotide sequences of primers used in this study are reported in Table 2. A total of 2 μl of diluted cDNA was used in real-time PCR for gene expression quantification using IQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Duplicate analyses were performed for each sample for both the housekeeping and the target gene in a final reaction volume of 20 μl. Beta actin (*bact*) and ribosomal protein 27a (*rpl-27a*) were used as housekeeping genes to normalize the expression of the target genes (*CuZnsod*, *cat*, *ctr1* and *atp7b*) in liver. Real-time quantitative PCR was performed using the iQ5 Multicolor Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The PCR conditions were as follows: 95 °C for 3 min and 30 s, followed by 40 cycles of 95 °C for 15 s, 58.1 °C for 30 s, and 72 °C for 30 s; 95 °C for 1 min, and a final denaturation step from 58 to 95 °C for 10 s. The $2^{-\Delta\Delta Ct}$ method was applied to analyse the relative changes in gene expression.

2.4. Chemical analyses

Chemical composition of fish was determined using near-infrared spectroscopy (FoodScan, Foss, Sweden). The evaluation of the mineral content was conducted by means of an inductively coupled plasma mass spectrometry (iCAPQ ICP-MS). Biochemical composition of diets and whole fish was determined following standard procedures (Association of Official Analytical Chemists, AOAC, 2000). Crude lipid was extracted according to the method of Folch et al. (1957) and ash by combustion in a muffle furnace at 600 °C for 12 h; protein content (N × 6.25) was determined by using the Kjeldahl method (AOAC, 2000) and dry matter content was determined after drying the sample in an oven at 105 °C until reaching constant weight. Fatty acid methyl esters were obtained by acid transmethylation of total lipid with 1% sulphuric acid in methanol following the method of Christie (1982).

Thiobarbituric acid-reactive substances (TBARs) were measured from triplicate samples following the method of Burk et al. (1980). Approximately 20–30 mg of tissue were homogenised in 1.5 ml of 20% trichloroacetic acid (*w/v*) containing 0.05 ml of 1% butylated hydroxytoluene in methanol. Then, 2.95 ml of freshly prepared 50 mM-thiobarbituric acid solution were added before mixing and heating for 10 min at 100 °C. After cooling, protein precipitates were removed by centrifugation at 2000*g* and the supernatant was read in a spectrophotometer (Evolution 300; Thermo Scientific) at 532 nm. Absorbance was recorded against a blank at the same wavelength. The concentration of thiobarbituric acid reactive substances (TBARs), expressed as mmol of acid-malonaldehyde (MDA)/g tissue, was calculated using an extinction coefficient of 0.156 cm/mM.

2.5. Histological studies

Four fish per tank were sampled for histological analysis of liver at the end of the trial. Tissues fixed in 10% buffered formaldehyde in a sample:formaldehyde ratio of 1:10.. The fixed tissue was placed in an automated tissue processor Histokinette 2000 (Leica, Nussloch, Germany) where it was treated with graded ethanol being the last two steps xylene and paraffin. Sections were cut at 3 μm thickness using a Leica RM 2135 microtome (Leica, Nussloch, Germany). Samples were then stained with haematoxylin – eosin staining (Martoja and Martoja-Pierson, 1970) for optical evaluation in search for signs of liver damage including steatosis, peripheral nuclei, broken cell margin and sinusoid dilatation and analysed by pair evaluators in a 0–3 scale, where 0 was absence of observation and 3 presence in most of the liver.

2.6. Skeletal anomalies

X-Ray analyses were conducted using a fixed X-ray apparatus (Bennett B-OTC, Bennett X-Ray Corp., Chicago, IL, USA) and a 35x43cm digital film (Fujifilm FDR D-EVO (Fujifilm Corporation, Tokyo, Japan). Radiographs were treated digitally (Onis 2.4, DigitalCore, Co.Ltd., Tokyo, Japan) and skeletal anomalies classified according to Boglione et al. (2001).

2.7. Statistics

All data were statistically analysed using. SPSS v21 (IBM Corp., Chicago, IL, USA) and means ± SD were calculated for every parameter measured. Data were tested for normality with the one-sample Kolmogorov– Smirnov test. For normally distributed data, one-way analysis of variance (ANOVA) was used to determine the effects of the different diets. Data were tested for homogeneity and post-hoc analysis was carried out using Tukey test if variances were homogeneous or Games-Howell test whenever variances were different. When data did not follow a normal distribution, logarithmic or arcsin transformation was carried out and the non-parametric tests of Kruskal-Wallis was used. Quadratic regressions and broken line analyses were conducted where possible. Significant differences were considered for $P < .05$.

3. Results

3.1. Feeding trial

All diets were readily accepted by fish, regardless the different dietary Cu contents. Mean survival throughout the trial was 98 ± 1% (mean ± SD). After 42 days of feeding fish had gained over 150% of their initial weight (Table 3). Final whole body weight was significantly ($P > .05$) affected by dietary Cu supplementation, where fish fed diets containing 5.5–9.3 mg Cu kg^{-1} presented the highest weight (Table 3). FCR and FE proved that fish fed the diet containing 11.0 mg Cu kg^{-1} presented a worse efficiency of the feed (Table 3). Other productive parameters including WG, SGR, or TGC proved not significantly different between treatments (Table 3).

3.2. Gene expression

Results from analyses of hepatic genes revealed that dietary Cu did

Table 3
Growth performance and feed utilization in gilthead sea bream fed increasing contents of Cu for 42 days.

Dietary Cu (mg/kg)	Cu5.5	Cu7.4	Cu9.3	Cu11.0	Cu32.0
IW (g)	12.5 ± 1.4	12.7 ± 1.4	12.7 ± 1.5	12.6 ± 1.3	12.7 ± 1.5
FW (g)	36.2 ± 1.4^{b}	36.0 ± 1.1^{b}	35.5 ± 1.5^{b}	31.5 ± 0.7^{a}	34.9 ± 0.3^{ab}
WG (%)	193 ± 10	182 ± 1	180 ± 19	152 ± 14	176 ± 8
SGR (%)	2.56 ± 0.08	2.47 ± 0.01	2.45 ± 0.16	2.20 ± 0.14	2.42 ± 0.07
TGC (g/days × t)	4.18 ± 0.73	4.18 ± 0.33	4.29 ± 0.34	3.86 ± 0.51	4.19 ± 0.05
FCR (g)	1.04 ± 0.02^{ab}	1.01 ± 0.02^{a}	1.05 ± 0.05^{ab}	1.15 ± 0.02^{b}	1.06 ± 0.03^{ab}
FE (g)	0.96 ± 0.02^{ab}	0.99 ± 0.02^{b}	0.95 ± 0.05^{ab}	0.87 ± 0.02^{a}	0.94 ± 0.03^{ab}

IW: initial weight. FW: Final weight. WG: Weight gain. SGR: Specific Growth Rate. TGC: Thermal Growth Coefficient. FCR: Feed Conversion Ratio. FE: Feed efficiency.
Different letters in the same row indicate significant differences, $P < .05$, n = 3.

Table 4
Hepatic gene expression analyses of gilthead sea bream fed increasing levels of dietary Cu for 42 days.

Dietary Cu (mg/kg)	Cu5.5	Cu7.4	Cu9.3	Cu11.0	Cu32.0
CuZnsod	1.01 ± 0.18	1.20 ± 0.18	1.06 ± 0.18	1.12 ± 0.01	1.21 ± 0.27
cat	1.40 ± 1.27^{ab}	3.31 ± 0.55^{b}	4.88 ± 0.19^{b}	1.08 ± 0.38^{a}	1.09 ± 0.68^{a}
ctr1	0.76 ± 0.22	0.72 ± 0.09	0.77 ± 0.17	0.55 ± 0.17	0.53 ± 0.13
atpb7	0.42 ± 0.04	0.41 ± 0.08	0.43 ± 0.10	0.31 ± 0.06	0.32 ± 0.05

Different letters in the same row indicate significant differences, $P < .05$, $n = 3$.

not have a significant effect on expression of *CuZnsod*. However, there was a significant ($P < .05$) up-regulation of the *cat* expression with the increase of dietary Cu levels up to 9.3 mg Cu kg^{-1}, whereas further inclusion of dietary Cu down-regulated the expression of this gene (Table 4). Despite the expression of genes related to Cu transport (*ctr1* and *atpb7*) was highest in fish fed 9.3 mg Cu kg^{-1}, there was no significant ($P > .05$) effect of dietary Cu levels on the expression of these genes (Table 4).

3.3. Chemical analyses

Whole body Cu content was not significantly affected by dietary Cu, independently of the level of Cu supplementation (Table 5). Similarly, no significant ($P > .05$) differences were found in liver Cu contents.

Biochemical composition in terms of whole body lipids, protein and ash of sea bream juveniles at the end of the trial was not significantly ($P > .05$) affected by dietary Cu supplementation (Table 6). On the contrary, TBARs contents in sea bream whole body at the end of the trial were significantly ($P < .05$) increased by the elevation of the dietary Cu levels from 5.5 to 32.0 mg Cu kg^{-1} diet (Table 6).

No significant differences were found in the fatty acid composition of whole body from fish fed different Cu levels (Table 7).

3.4. Histological studies

Increase in dietary Cu lead to increased liver steatosis and displacement of hepatocyte nucleus (Table 8, Figs. 1, 2, 3 and 4). At the end of the feeding trial fish fed the higher dietary Cu supplementation presented an increased prevalence of liver steatosis, broken cell margin, peripheral nuclei, and sinusoidal dilatation, symptoms of liver stress/injury (Figs. 1, 2, 3 and 4; Table 8).

3.5. Skeleton anomalies

At the end of the trial, dietary Cu supplementation did not have a significant effect on the prevalence of skeletal anomalies independent of the typology described (Table 9). However, fish fed the diet without Cu supplementation tended to present a higher prevalence of total skeletal anomalies.

4. Discussion

In studies conducted to determine optimum dietary levels of a nutrient, it is imperative to maximize the growth of the species studied to clearly determine the potential effects of nutritional deficiencies or excesses. This may be particularly important in determining optimum

Table 5
Whole body and liver Cu content of gilthead sea bream fed increasing dietary contents of Cu for 42 days.

Diet	Mineral	Cu5.5	Cu7.4	Cu9.3	Cu11.0	Cu32.0
Whole fish	Cu	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
	Mn	5.7 ± 0.3	6.3 ± 0.8	6.0 ± 0.6	6.2 ± 0.5	6.1 ± 1.0
	Fe	30 ± 3	31 ± 4	31 ± 3	37 ± 3	28 ± 2
	Zn	40 ± 5	40 ± 2	45 ± 3	44 ± 3	41 ± 3
	Se	0.65 ± 0.10	0.63 ± 0.09	0.65 ± 0.03	0.64 ± 0.05	0.65 ± 0.12
	Cd	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Liver	Cu	9.8 ± 1.1	9.1 ± 1.7	10.3 ± 0.6	9.1 ± 0.5	11.2 ± 1.3
	Mn	5.5 ± 0.2	5.2 ± 0.4	4.9 ± 0.7	5.6 ± 0.3	5.3 ± 0.2
	Fe	62 ± 11	66 ± 3	67 ± 8	51 ± 10	60 ± 12
	Zn	73 ± 2	73 ± 5	78 ± 2	71 ± 2	75 ± 2
	Se	2 ± 0.2	1.8 ± 0.1	1.9 ± 0.2	1.8 ± 0.2	1.7 ± 0.2
	Cd	0.11 ± 0.01	0.13 ± 0.01	0.11 ± 0.02	0.15 ± 0.02	0.13 ± 0.02

Different letters in the same row indicate significant differences, $P < .05$, $n = 3$.

Table 6
Whole body composition (% fresh weight) and TBARs (nMol MDA g^{-1} dw) contents of gilthead sea bream fed increasing contents of Cu for 42 days.

Dietary Cu (mg/kg)	Cu5.5	Cu7.4	Cu9.3	Cu11.0	Cu32.0
Lipids (%)	10.82 ± 1.39	9.42 ± 0.36	11.43 ± 0.18	8.22 ± 1.71	9.66 ± 0.93
Protein (%)	15.30 ± 0.77	14.21 ± 1.04	14.55 ± 1.11	14.84 ± 0.86	15.05 ± 0.48
Ash (%)	2.32 ± 0.50	2.66 ± 0.08	2.70 ± 0.22	2.56 ± 0.31	2.43 ± 0.81
TBARs (nMol MDA g^{-1} dw)	155 ± 92[a]	485 ± 143ab	371 ± 152ab	252 ± 26ab	506 ± 104b

Different letters in the same row indicate significant differences, $P < .05$, $n = 3$.

Cu dietary levels (Lin et al., 2010). Despite the diets in the present study contained only 10% FM and 6% FO, gilthead sea bream growth and feed utilization parameters were very good in comparison to previous studies (Benedito-Palos et al., 2007; Ballester-Lozano et al., 2015; Simó-Mirabet et al., 2018). Dietary supplementation with $CuSO_4$ above 9.3 mg/kg significantly reduced growth compared to the basal diet containing 5.5 mg Cu kg^{-1}. Therefore results of the present trial suggested that the Cu levels present in the basal diet (5.5 mg Cu kg^{-1}) were enough to cover gilthead sea bream fingerlings requirements for growth when fed a diet with only 10% FM and containing 75% terrestrial meals. In fact, several of the plant ingredients employed contain higher Cu levels than FM (5–6 mg Cu kg^{-1}), such as soya concentrate (23 mg Cu kg^{-1}) or corn gluten (12 mg Cu kg^{-1}) (ARRAINA, 2015).

Growth performance and other productive parameters have been used as a criteria to evaluate Cu requirements in other marine fish species such as large yellow croaker (*Larimichthys croceus*) (Cao et al., 2014) or malabar grouper (*Epinephelus malabaricus*) (Lin et al., 2008) and freshwater species including channel catfish (*Ictalurus punctatus*) (Wilson and Gatlin III, 1986), murrel (*Chana punctatus*) (Abdel-Hameid et al., 2017), Russian sturgeon (*Acipenser gueldenstaedtii*) (Wang et al., 2016), rainbow trout (*Oncorhynchus mykiss*) (Wilson and Gatlin III, 1986), common carp (*Cyprinus carpio*) (Ogino and Yang, 1980), tilapia (*Oreochromis niloticus* × *O. aureus*) (Antony Jesu Prabhu et al., 2016). In those trials, basal dietary Cu concentrations were lower than in the present trial, since diets were based on egg albumin or casein as main protein source. Nevertheless, the results of the present trial, suggesting a requirement around 5.5 mg Cu kg^{-1} to maintain gilthead sea bream juveniles growth, are close to those found for malabar grouper fed $CuSO_4$ (4–6 mg Cu kg^{-1} diet, Lin et al., 2008), channel catfish (1.5–5 mg Cu kg^{-1} diet, Murai et al., 1981; Gatlin and Wilson 1986), murrel (6.7 mg Cu kg^{-1}, Abdel-Hameid et al., 2017) or Russian sturgeon (6.6 mg Cu kg^{-1} fed $CuSO_4$, Wang et al., 2018). However, Cu requirements for maximum growth were lower for other feeding trials with lower growth rates such as rainbow trout and common carp (3 mg Cu kg^{-1} diet, Ogino and Yang, 1980), large yellow croaker (3.41 mg Cu kg^{-1} diet, Cao et al., 2014) or tilapia (4 mg Cu kg^{-1} diet, Shiau and Ning, 2003). In studies based on practical diets as the present one, the requirements stablished for maximum growth tend to be higher than in those conducted with purified diets (Antony Jesu Prabhu et al., 2016). Such is the case for crucian carp (*Carassius auratus gibelio*) with Cu requirements ranging from 6.43–9.47 mg/kg (Shao et al., 2010), Atlantic salmon (*Salmo salar*) from 8.5–13.7 mg Cu kg^{-1} (Lorentzen et al., 1998), tongue sole (*Cynoglossus semilaevis*) from 11 to 12 mg Cu kg^{-1} (Wang et al., 2015) or blunt snout bream (*Megalobrama amblycephala*) from 12 to 18 mg Cu kg^{-1} (Shao et al., 2012). Besides, dietary Cu requirements among different studies may also be affected by the dietary Cu source used, fish age, welfare status, water Cu levels and, most possibly, dietary levels of other minerals such as iron or zinc (Lall, 2002; Lin et al., 2010). Nevertheless, growth-related parameters may be an insufficient criteria to establish a defined requirement for micronutrients such as Cu (NRC, 2011; Antony Jesu Prabhu et al., 2016; Baker, 1986 and Cowey, 1992). Copper concentration in whole body or liver, as well as liver SOD activity, are among the most frequent parameters studied to determine Cu requirements (Lall, 2011; Antony Jesu Prabhu et al., 2016).

In the present trial, increase in dietary Cu up to 9.3 mg/kg did not affect Cu retention in whole body and only slightly, but not significantly, raised liver Cu contents. Liver is considered a main reservoir for Cu, particularly when Cu is fed in an inorganic form (Lin et al., 2010). In liver, expression of *CuZnsod* was neither affected by the elevation of dietary Cu levels. However, *cat* expression was significantly up-regulated by the increase in dietary Cu up to 9.3 mg/kg, in agreement with the increased CAT activity found in other fish species when dietary Cu levels are increased (Shao et al., 2012; Tang et al., 2013). This higher CAT activity or *cat* expression could be induced by a higher production of H_2O_2, a substrate for CAT activity and at the same time the product of Cu/ZnSOD activity, or could be due to a posttranscriptional effect of Cu. Therefore, the up-regulation of *cat* expression by increased dietary levels up to 9.3 mg/kg could be related to a higher SOD activity in liver, in agreement with the trends towards up-regulation of Cu transport related genes, *ctr1* and *atp7b,* and towards the increase in liver Cu contents in fish fed 9.3 mg Cu kg^{-1} diet.

On the contrary, further increase in dietary Cu up to 11.0 and, particularly, 32.0 mg/kg, which was reflected in slightly higher Cu contents in liver, significantly down-regulated hepatic *cat* expression. In malabar grouper, excessive dietary Cu levels (20 mg/kg diet) reduced hepatic SOD activity (Lin et al., 2008), what would lead to reduced H_2O_2 production and, consequently, to a reduced CAT activity. In the present trial, fish fed 11.0 and 32.0 mg Cu kg^{-1} showed a tendency to down-regulate *ctr1* and *atp7b*. In agreement, previous studies in gilthead sea bream have shown that elevation of dietary Cu from 7.7 mg Cu kg^{-1} to 12.6 or 130.0 mg Cu kg^{-1} down-regulates *ctr1* expression, as a protective response to excess dietary Cu (Minghetti et al., 2008). In mammals, it has been demonstrated that copper may initiate lipid peroxidation in biomembranes by generating peroxyl and alkoxyl radicals from the decomposition of lipid hydroperoxides (Murphy, 2001). In studies with high dietary (i.e. 32.0 mg/kg) or waterborne Cu levels, tissues lipid compositions are ultimately altered (Berntssen et al., 1999;

Table 7
Fatty acid composition in whole body of gilthead sea bream fed dietary Cu for 42 days (% total fatty acids).

Dietary Cu (mg/kg)	Cu5.5	Cu7.4	Cu9.3	Cu11.0	Cu32.0
n-3 PUFA	17.82 ± 3.15	16.95 ± 1.29	19.59 ± 1.77	16.44 ± 1.04	17.25 ± 1.61
n-6 PUFA	13.95 ± 0.16	13.92 ± 0.10	13.98 ± 0.29	14.22 ± 0.22	13.98 ± 0.07
20:5n-3	3.60 ± 0.45	3.50 ± 0.34	3.88 ± 0.33	3.44 ± 0.11	3.51 ± 0.24
22:6n-3	7.17 ± 1.99	6.55 ± 0.74	8.27 ± 1.04	6.16 ± 0.63	6.84 ± 1.09
20:4n-6/20:5n-3	0.16 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.00	0.17 ± 0.01
20:5n-3/22:6n-3	0.52 ± 0.07	0.54 ± 0.01	0.47 ± 0.02	0.56 ± 0.04	0.52 ± 0.05

Different letters in the same row indicate significant differences, $P < .05$, $n = 3$. PUFA: polyunsaturated fatty acids

Table 8
Hepatic histological analyses of gilthead sea bream fed increasing levels of dietary Cu for 42 days.

Dietary Cu (mg/kg)	Cu5.5	Cu7.4	Cu9.3	Cu11.0	Cu32.0
Steatosis	1.56 ± 0.1^{a}	1.93 ± 0.0^{ab}	1.95 ± 0.1^{ab}	2.11 ± 0.1^{ab}	2.78 ± 0.0^{b}
Peripheral nucleus	0.32 ± 0.0^{a}	0.72 ± 0.1^{ab}	1.02 ± 0.0^{bc}	1.40 ± 0.0^{c}	1.37 ± 0.1^{c}
Broken cell margin	0.50 ± 0.1^{a}	0.64 ± 0.0^{a}	0.99 ± 0.1^{b}	1.03 ± 0.1^{b}	1.30 ± 0.0^{b}
Sinusoids dilatation	0.35 ± 0.1^{a}	0.38 ± 0.0^{a}	0.56 ± 0.0^{a}	0.89 ± 0.0^{b}	1.00 ± 0.0^{b}

Different letters in the same row indicate significant differences, $P < .05$, $n = 3$.

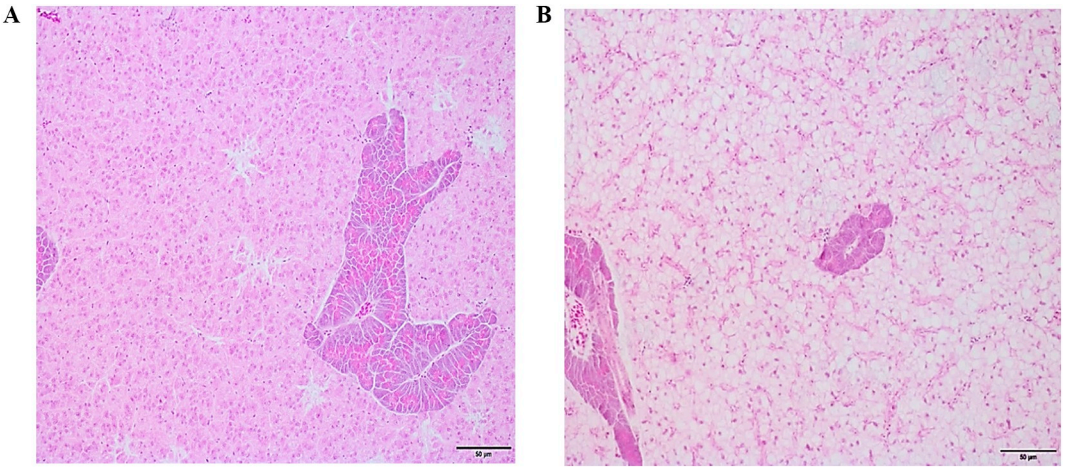

Fig. 1. Microscopic view of liver steatosis (20×). A) Low steatosis. B) High steatosis.

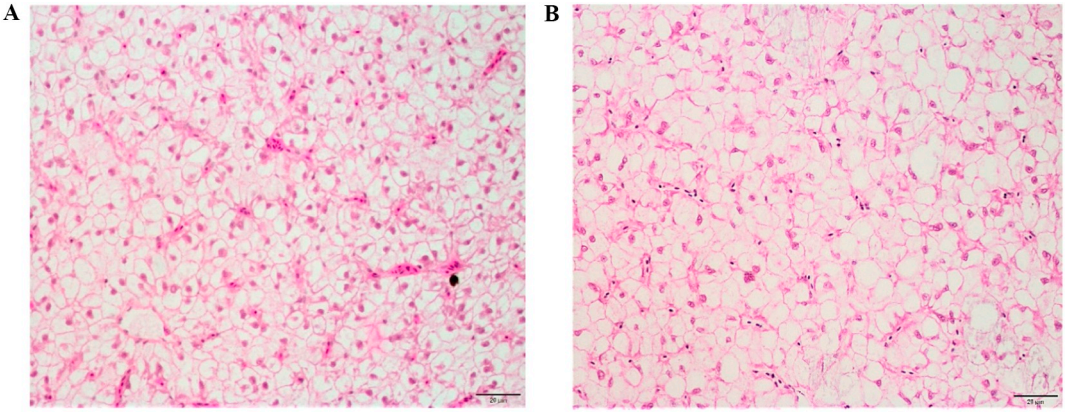

Fig. 2. Microscopic view of liver cell nucleus (40x). A) Central nucleus. B) Peripheral nucleus.

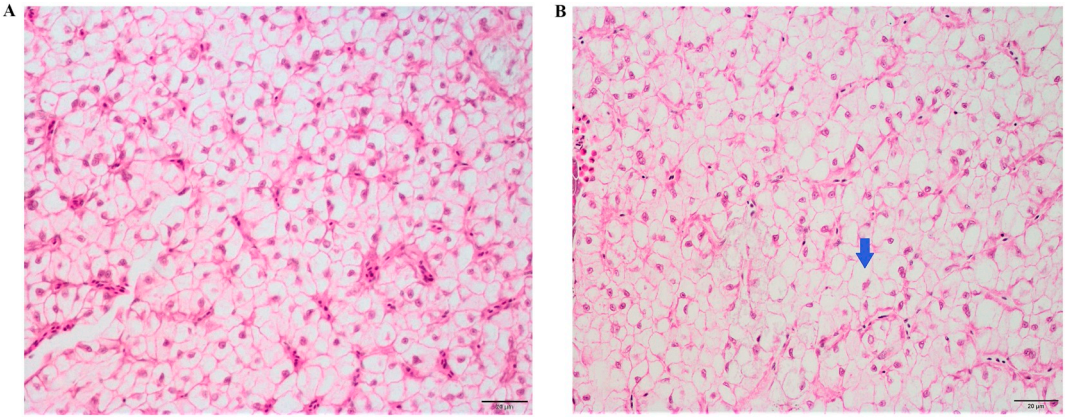

Fig. 3. Microscopic view of liver cell margin (40x). A) well-preserved cell margins. B) Broken cell margins.

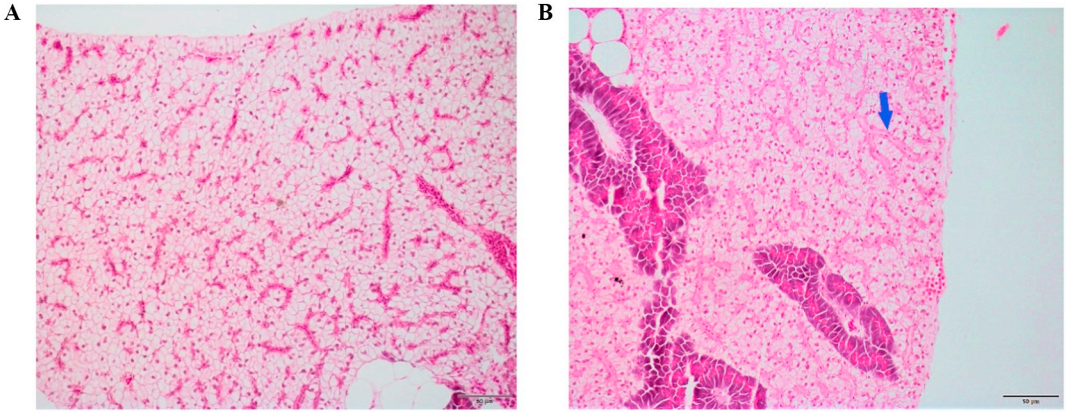

Fig. 4. Microscopic view of liver cell sinusoids (20x). A) Sinusoids with abundant erythrocytes. B) Sinusoids dilated with plasma and without erythrocytes.

Table 9
Prevalence of skeletal anomalies (%) in sea bream fed increasing levels of dietary Cu.

Dietary Cu (mg/kg)	Cu5.5	Cu7.4	Cu9.3	Cu11.0	Cu32.0
Total anomalies	73 ± 5	53 ± 6	67 ± 19	61 ± 9	63 ± 15
Pre-haemal lordosis	46 ± 10	38 ± 14	39 ± 17	43 ± 9	44 ± 18
Pre-haemal partial vertebral fusion	4 ± 6	4 ± 4	2 ± 4	4 ± 3	0 ± 0
Pre-haemal total vertebral fusion	0 ± 0	0 ± 0	2 ± 4	0 ± 0	2 ± 3
Pre-haemal vertebral anomaly	11 ± 9	8 ± 3	7 ± 7	4 ± 3	4 ± 3

Meng et al., 2016; Mustafa et al., 2012; Shaw and Handy, 2006). Moreover, under conditions of tissue unbalances between Cu and Mn, both minerals may compete for antioxidant proteins leading to malfunction and deleterious effects (Singh et al., 2010). In fact, increase in dietary Cu up to 32.0 mg/kg significantly increased TBARs values, one of the most commonly used indicator of tissue peroxidation (Rosmini et al., 1996), in agreement with the increased values found in malabar grouper fed excessive levels of dietary Cu (11 and 20 mg Cu kg^{-1} diet, Lin et al., 2008). In marine fish, excessive water borne Cu levels causing peroxidation of polyunsaturated membrane lipids or even proteins, damage cells and tissues in several organs (NRC, 2011; Watanabe et al., 1997; Roméo et al., 2000; Isani et al., 2011; Woody and O'Neal, 2012). In the present study, increase in dietary Cu, particularly up to 32.0 mg/kg, damaged hepatic tissue, leading to increased broken cell margin and sinusoids dilatation when compared to those fed 5.5–7.4 mg Cu kg^{-1}. Fat accumulation leading to steatosis and displacement of peripheral nucleus may be only a reversible form of energy storage in gilthead sea bream (Caballero et al., 2004). However, extreme steatosis may lead to lipid liver degeneration, which includes the break of the cellular margin, necrosis and macrophages infiltration. A large variety of toxic compounds including peroxides, aldehydes, and ketones are derived from the peroxidation processes, to which the most frequently reported clinical sign is lipoid liver degeneration (Roberts, 2002). Besides, excess of Cu in liver is discarded by hepatocytes through the bile by ATP7 protein (Isani et al., 2011; Lanno et al., 1987). Ultimately, this may lead to cholestasis (Diaz et al., 1998), causing the dilatation of the sinusoids due to the increase in plasma and a decrease in erythrocytes. Cholestasis could also produce necrosis and broken cell margin. Similarly, rainbow trout exposed to toxic Cu levels also showed increased dilatation of sinusoidal spaces (500 mg Cu kg^{-1} Handy et al., 1999). Nile tilapia presents similar alterations in liver morphology when fed toxic levels of Cu for 6 weeks, including lipidosis and loss of nuclei definition, however, sinusoidal spaces were reduced (Shaw and Handy, 2006). Moreover, as an accumulative contaminant heavy metal, at high concentrations Cu can be very toxic and even lethal (Tacon, 1992). Overall, results from hepatic histology indicate a detrimental effect of Cu on sea bream fingerlings fed dietary levels of 11.0 and 32.0 mg Cu kg^{-1}. Moreover, elevation of dietary Cu levels up to 11.0 and 32.0 mg/kg negatively reduced growth, a common indicator of Cu toxicity (Lall, 2002). Nevertheless, these toxicity signs would be more marked after a long chronic dietary exposure (Handy et al., 1999). In the present trial the contents in other minerals were not significantly affected by dietary Cu levels, despite excessive Cu levels (100–1000 mg Cu kg^{-1} diet) can alter liver contents in Se and Fe in other species (Damasceno et al., 2016; Lorentzen et al., 1998), but the levels tested in the present trial were much lower. Indeed, the highest level tested in the present trial was, at least, 3 times lower than those described to have effects on Fe levels for other species.

The effects of Cu supplementation on the prevalence of skeletal anomalies were mild, and overall not significant, despite the fact that fish fed the diet devoid of Cu supplementation tended to present a higher prevalence of skeletal anomalies. The role of Cu in collagen synthesis is essential due to its involvement in several enzymes. Collagen in turn, is fundamental in bone formation. In fact, Cu deficiency results in osteoporosis and spontaneous bone fractures in cattle and sheep (Hidiroglou, 1980), increased bone resorption, a decrease in bone formation and in the number and activity of osteoblasts in rats (Strause et al., 1986). On human patients with Menkes disease (caused by a mutation in *ATP7A* gene which regulates Cu levels in the body) several bone changes, including osteoporosis and fractures of long bones and ribs occur (Kodama et al., 1999).

To conclude, since Cu is frequently present in most animal and plant protein sources (5–30 mg Cu kg^{-1}), as well as in the aquatic environment, marked deficiency symptoms may only appear under extreme conditions (Lall, 2002). In the present study, as part of the EU-funded project PeformFish, a practical approach was targeted to better understand nutritional requirements of gilthead sea bream under semi-commercial conditions where FM and FO are being gradually substituted by plant ingredients. This approach lead to a basal dietary Cu content of 5.5 mg/kg, which was enough to cover Cu requirements for growth in gilthead sea bream, despite dietary supplementation up to 9.3 mg Cu kg^{-1} as $CuSO_4$ did not negatively affect any of the parameters studied. These results suggest that little or even non Cu supplementation is required in gilthead sea bream fed practical diets based on plant protein sources that provide at least 5.5 mg Cu kg^{-1}. Moreover, the present study demonstrated that, though the maximum dietary Cu content allowed by EFSA (2014) is 25 mg/kg, even dietary contents of 11–32 mg Cu kg^{-1} negatively affected gilthead sea bream performance by reducing fish growth, increasing oxidative risk and inducing hepatic damage and cholestasis.

Declaration of interest

R. Fontanillas is an employee of Skretting AS, Stavanger, Norway.

Author contributions

David Domínguez conceived and designed the experiments, performed the experiments, performed analyses, analysed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Paula Sarmiento performed analyses, analysed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Zakarya Sehnine performed the experiments, performed analyses, analysed the data.

Pedro Castro performed analyses, analysed the data, reviewed drafts of the paper.

Lidia Robaina contributed reagents/materials/analysis tools.

Ramón Fontanillas conceived and designed the experiments, contributed reagents/ma- terials/analysis tools, reviewed drafts of the paper.

P. Antony Jesu Prabhu performed analyses, analysed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Marisol Izquierdo conceived and designed the experiments, analysed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

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ORIGINAL ARTICLE

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Optimum selenium levels in diets high in plant-based feedstuffs for gilthead sea bream (*Sparus aurata*) fingerlings

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Abstract

Substitution of marine ingredients (FM-FO) by plant protein and oil sources can modify selenium (Se) levels in feeds. Se plays an important role in the antioxidative defence by forming part of selenoproteins. Se requirements of gilthead sea bream are not accurately determined; therefore, this study was conducted to define Se supplementation levels in low FM-FO practical diets for sea bream fingerlings. A plant-based diet containing 0.45 mg Se/kg diet was used as the basal diet. Four other diets were supplemented to contain 0.68, 0.86, 1.00 or 1.70 mg Se/kg diet, supplied as sodium selenite. Sea bream, weighing 12.6 ± 1.4 g, were distributed in triplicate groups per diet and fed for 42 days. Se supplementation up to 1.00 mg Se/kg significantly improved the growth of sea bream, whereas further increase up to 1.70 mg Se/kg diet reduced growth. The results of this study suggest that the optimum dietary levels of sodium selenite in diets with low FM-FO with basal levels of 0.45 mg Se/kg are around 0.94 mg Se/kg to promote growth of gilthead sea bream juveniles. On the contrary, dietary levels of 1.70 mg Se/kg were found to be excessive and caused growth reduction, increased catalase expression and hydropic degeneration in the liver.

KEYWORDS

fish mineral nutrition, gilthead sea bream, optimum levels, plant ingredients, selenium, selenium toxicity

1 | INTRODUCTION

Selenium (Se) is an essential element for fish and plays important roles in different biological processes including antioxidant protection or physiological responses to stress (Lall, 2002; Watanabe, Kiron, & Satoh, 1997). In commercial aquafeeds for carnivorous fish, such as gilthead sea bream (*Sparus aurata*), fish meal (FM) has traditionally been the main source of Se (Sissener et al., 2013). However, ingredients derived from fish captures have been greatly substituted by those of plant origin in the last decade. Se concentrations on plant ingredients vary greatly depending on plant species and soil, and certain areas have been observed to contain Se in levels toxic to livestock, while others are considered deficient for animal nutrition (Alfthan et al., 2015; Reis, El-Ramady, Ferreira Santos, Lupino Gratão, & Schomburg, 2017). Still, feeds formulated with ingredients with low levels of Se have little margin for Se supplementation since regulations in the European Union are strict and account for a maximum of 0.2 mg/kg for organic Se (Regulations [EU] No 427/2013; 445/2013; 121/2014; 847/2014 and 2015/489) and 0.5 mg/kg feed for total Se in animal feeds including fish (EC 1831/2003 and amendments).

Regulations in the use of Se as a supplement in feeds contribute to reducing discharges of Se to the environment and keeping its levels below toxic. In fact, the margin between requirement and toxicity

for this mineral is very narrow. Se in toxic levels associates to sulphur containing amino acids due to its similar properties, thus altering the functional enzyme. Furthermore, under stressful conditions, Se requirements are increased, whereas under normal conditions these levels would be considered toxic (Khan, Zuberi, Fernandes, Ullah, & Sarwar, 2017). Se toxicity curses with reduced growth and feed efficiency, increased oxidative stress mortality, increased skeletal anomalies, oedema, decreased egg viability, altered immunological functions, necrosis of renal tubules and renal calcinosis, while deficiencies can produce reduced growth, mortality, lethargy, diminished appetite, muscle dystrophy, reduced vitamin E levels and low haematocrit (Bell, Cowey, Adron, & Pirie, 1987; Berntssen et al., 2018; Betancor et al., 2012; Choi et al., 2015; Gatlin & Wilson, 1984; Lin & Shiau, 2005; Pacitti et al., 2015; Saleh et al., 2014; Schultz & Hermanutz, 1990; Tashjian, Teh, Sogomonyan, & Hung, 2006; Watanabe et al., 1997; Zee, Patterson, Gagnon, & Hecker, 2016).

Indeed, regulations for Se supplementation in feeds for certain fish species may be below the levels considered as required, such is the case of fast-growing species such as cobia (*Rachycentron canadum*), malabar grouper (*Epinephelus malabaricus*), meagre (*Argyrosomus regius*) or yellowtail kingfish (*Seriola lalandi*), fast-growing species that require 0.79–0.81, 0.90–0.98, 3.98 and 4.91–7.37 mg Se/kg, respectively (Le & Fotedar, 2014a, 2014b; Lin, 2014; Liu, Wang, Ai, Mai, & Zhang, 2010; Mansour, Goda, Omar, Khalil, & Esteban, 2017). Other species do not require Se supplementation, as cutthroat trout (*Oncorhynchus clarki bouvieri*; Hardy, Oram, & Möller, 2010), but most species typically require between 0.12 and 1.85 mg Se/kg. Catfishes belong to those species with the lowest requirements (*Clarias gariepinus* and *Ictalurus punctatus*; Abdel-Tawwab, Mousa, & Abbass, 2007; Wang & Lovell, 1997), while carps (*Carassius auratus gibelio*, *Ctenopharyngodon idellus* and *Cyrpinus carpio*; Ashouri, Keyvanshokooh, Salati, Johari, & Pasha-Zanoosi, 2015; Liu et al., 2018; Zhu et al., 2016), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*; Jaramillo, Peng, & Gatlin, 2009) and largemouth bass (*Micropterus salmoide*; Zhu et al., 2012) have the highest ones. As for sparids, requirements for red (*Pagrus major*) and black sea bream (*Acanthopagrus schlegelii*), species phylogenetically closely related to gilthead sea bream, were 1.34 mg Se/kg (Dawood et al., 2018) and 0.86 mg Se/kg (Wang et al., 2019), whereas optimum Se dietary levels for gilthead sea bream are not accurately determined yet.

Selenium requirements vary not only among different species, but also according to developmental stage, sources of supplementation and environmental factors that may cause stress (Khan et al., 2017). The main criteria to evaluate requirements for Se in fish are growth, feed efficiency, tissue retention, antioxidant activity/expression markers and immune response/haematology (Antony Jesu Prabhu, Schrama, & Kaushik, 2016; Khan et al., 2017).

Selenium forms part of selenoproteins, being the teleost fishes the organisms with the highest number of them, up to 38 in zebrafish (*Danio rerio*; Mariotti et al., 2012). The glutathione peroxidase (*gpx*) is among the most important selenoprotein family, as it forms part of one of two enzymatic systems present in vertebrates able to metabolise hydrogen peroxide to water (Di Giulio & Meyer, 2008; Holley, Bakthavatchalu, Velez-Roman, & St. Clair, 2011). The *gpx* homologues have been studied in gilthead sea bream (Malandrakis et al., 2014), and its activity and expression of glutathione peroxidase 1a (*gpx1a*) gene have been used to evaluate Se requirements in fish species (Antony Jesu Prabhu et al., 2016; Khan et al., 2017). The other enzymatic systems able to dispose of hydrogen peroxide are catalases. Unlike the more ubiquitous *gpx*, catalases are located in peroxisomes, where they protect these organelles from the hydrogen peroxide released as byproduct of the β-oxidation of fatty acids that takes place in peroxisomes (Di Giulio & Meyer, 2008). Expression of catalase (*cat*) can be used to evaluate oxidative status, and high doses of Se have been observed to increase its activity in several fish species (Ashouri et al., 2015; Elia, Prearo, Pacini, Dörr, & Abete, 2011; Mansour et al., 2017; Misra, Hamilton, & Niyogi, 2012; Penglase, Hamre, Rasinger, & Ellingsen, 2014). Oxidative stress may also alter transcription factors, such as glucocorticoid receptors (*gr*; Di Giulio & Meyer, 2008; Esposito, Cuccovillo, Morra, Russo, & Cimino, 1998; Olsvik, Torstensen, Hemre, Sanden, & Waagbø, 2011), which are zinc-finger-containing proteins, and as such are susceptible to inhibition by reducible Se compounds (with oxidation state of −I or higher) in which sodium selenite is included (oxidation state +IV; Blessing, Kraus, Heindl, Bal, & Hartwig, 2004). Furthermore, selenite catalyses the oxidation of SH groups, such as those present in the glucocorticoid receptor hormone binding sites (Tashima et al., 1989).

The scarce knowledge available for selenium nutrition in gilthead sea bream, the third major species produced in the EU (APROMAR, 2018), added to its essentiality and possible effects as contaminant highlights the importance of further research in this area. Thus, the aim of this study was to define optimum levels of selenium supplementation in gilthead sea bream fingerlings fed practical diets with high levels of plant-based feedstuffs.

2 | MATERIAL AND METHODS

All the experimental conditions and sampling protocols have been approved by the Animal Welfare and Bioethical Committee from the University of Las Palmas de Gran Canaria.

2.1 | Diets

In previous studies, gilthead sea bream fed graded levels of Se and other micronutrients showed best Se retention and enhanced antioxidant defences when fish was fed at 0.77 mg Se/kg (Dominguez et al., submitted); however, the inclusion of several micronutrients simultaneously hindered a clear determination of requirements for Se in this species. A basal diet closely mirroring practical sea bream feeds was formulated with low inclusion of FM (100 g/kg) and FO (60 g/kg). Five different experimental diets were then produced by supplementing sodium selenite (Na_2SeO_3; ANIMA Spółka z o.o.), which contained 0.45, 0.68, 0.86, 1.0 and 1.7 mg Se/kg diet (Table 1).

TABLE 1 Ingredient composition and analysed Se contents of the experimental diets supplemented with increasing levels of sodium selenite

Ingredient (g/kg)	Se-0.45	Se-0.68	Se-0.86	Se-1.00	Se-1.70
Soya concentrate; CJ Selecta S.A.	230.0	230.0	230.0	230.0	230.0
Wheat gluten; Cargill B.V.	216.6	216.6	216.6	216.6	216.7
Corn gluten; Cargill B.V.	150.0	150.0	150.0	150.0	150.0
Wheat; Lantmannen Ek. For. Lantbruk Handel	116.9	116.8	116.8	116.8	116.6
Fish meal; Norsildmel AS	100.0	100.0	100.0	100.0	100.0
Fish oil South American; Copeinca S.A.	60.0	60.0	60.0	60.0	60.0
Faba beans; Cefetra, B.V.	50.0	50.0	50.0	50.0	50.0
Rapeseed oil; Thywissen GmbH	30.0	30.0	30.0	30.0	30.0
Palm oil; Cargill B.V.	16.4	16.4	16.4	16.4	16.4
Linseed oil; Cargill B.V.	8.2	8.2	8.2	8.2	8.2
Micronutrient premix[a]; Trouw Nutrition	21.9	21.9	21.9	21.9	21.9
Analysed selenium (mg/kg)	0.45	0.68	0.86	1.00	1.70

[a]Micronutrient premix includes methionine (10.6 g/kg), lysine (28.5 g/kg), phosphate (6.7 g/kg), vitamin premix (1.8 g/kg) and mineral premix excluding Se (1.1 g/kg).

Diets were isoenergetic and isonitrogenous, were designed to cover all known nutritional requirements for this species and were manufactured by extrusion process by Skretting Aquaculture Research Centre AS.

2.2 | Experimental design and feeding trial

Sea bream fingerlings with an initial body weight of 12.6 ± 1.4 g (mean ± *SD*) were distributed in 15 tanks with 30 fish per tank and randomly assigned one of the dietary treatments in triplicates. The fish were fed three times a day until apparent visual satiation for 42 days. The trial was carried out in the facilities of the Aquaculture Research Group (GIA) of the University of Las Palmas de Gran Canaria, Spain. Seawater temperature and water-dissolved oxygen were daily recorded (19.4 ± 0.4°C, mean ± *SD*). Fish were kept under a natural photoperiod. Growth, in terms of standard length (cm) and weight (g), was recorded at days 0 and 42 of the feeding trial by measuring and weighing all the fish. Throughout the experiment, feed intake per tank was recorded. At the end of the trial, productive parameters were calculated including specific growth rate (SGR), feed conversion ratio (FCR) and thermal growth coefficient (TGC). Previous to sampling, all fish were submitted to 24-hr fasting. During samplings, fish were caught and introduced into an anaesthetic tank containing clove oil 4% (Guinama S.L.U.) to reduce stress and improve handling. Three pools of fish at the beginning of the experiment and five fish per tank at the end were sampled to conduct biochemical, mineral and gene expression analysis. Samples were kept frozen at −80°C until the analysis was conducted. Twenty fish per tank were sampled for radiographic assessment.

2.3 | Biochemical analyses

Biochemical analysis of diets and whole fish was conducted by following standard procedures (AOAC, 2000). Crude lipid was extracted according to Folch, Lees, and Stanley (1957), and ash was determined by combustion in a muffle furnace at 600°C for 12 hr; protein content (N × 6.25) was determined by using the Kjeldahl method (AOAC, 2000), and dry matter content was calculated after drying the sample in an oven at 105°C until reaching constant weight. The evaluation of the mineral content was conducted by means of an inductively coupled plasma mass spectrometry (iCAPQ ICP-MS).

2.4 | Gene expression

2.4.1 | RNA extraction

Total RNA was extracted from 60 mg of liver using TRI Reagent Solution (Life Technologies) and purified on RNeasy Mini Spin Columns (Qiagen) following the manufacturer's instructions.

2.4.2 | Reverse transcription

Reverse transcription of 1 μg total RNA from each experimental sample was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions with slight modifications. Briefly, 1 μg total RNA and nuclease-free water to a final volume of 15 μl were heated at 65°C for 10 min and cooled

in ice. Afterwards, 1 µl of iScript reverse transcriptase and 4 µl of 5× iScript reaction mix were added, reaching a final reaction volume of 20 µl. The complete reaction mix was incubated for 5 min at 25°C, 30 min at 42°C, and then 5 min at 85°C to inactivate reverse transcriptase. For gene quantification, the reverse transcription reactions were diluted 1:10.

2.4.3 | Quantitative PCR

The nucleotide sequences of primers used in this study are reported in Table 2. A total of 2 µl of diluted cDNA was used in real-time PCR for gene expression quantification using IQTM SYBR Green Supermix (Bio-Rad Laboratories). Duplicate analyses were performed for each sample for both the housekeeping and the target gene in a final reaction volume of 20 µl. Ribosomal protein 27a gene (*rpl-27a*) and beta actin (*bact*) were used as housekeeping genes to normalize the expression of genes (glutathione peroxidase 1a: *gpx*, catalase: *cat* and glucocorticoid receptor: *gr*) in liver. Real-time quantitative PCR was performed using the iQ5 Multicolor Real-Time PCR detection system (Bio-Rad Laboratories). The PCR conditions were as follows: 95°C for 3 min and 30 s, followed by 40 cycles of 95°C for 15 s, 58.1°C for 30 s, and 72°C for 30 s; 95°C for 1 min, and a final denaturation step from 58 to 95°C for 10 s. The 2−ΔΔC*t* method was applied to analyse the relative changes in gene expression.

2.5 | Histology

Four fish per tank were sampled for histological analysis of liver at the end of the trial. Tissues were stored in 10% buffered formaldehyde in a sample:formaldehyde ratio of 1:10 for several weeks prior to processing. Samples were further segmented to allow a better penetration of alcohol and introduced in histology cassettes. Dehydration of samples was carried out using a Histokinette 2000 (Leica) with gradually increasing alcohol grades beginning with 70° and ending with 100°, being the last two steps xylene and paraffin. Once the paraffin block was obtained, it was sliced at a thickness of 3 µm using a Leica RM 2135 microtome (Leica) and fixed to a slide including as much parts of the tissue as possible. Samples were then stained with haematoxylin-eosin staining (Martoja & Martoja-Pierson, 1970) for optical evaluation. Once the preparations were ready, they were subjected to optical analysis in search for signs of liver steatosis, including lipid accumulation, broken cell margin or nuclei displacement to cell periphery, as well as sinusoid dilatation. These morphological features were analysed by pair evaluators in a 0–3 scale, where 0 was absence of observation and 3 observation present in most of the liver. Additionally, macrophage aggregate, hyperaemia and hydropic degeneration were observed in some fish and its presence was used to determine the percentage of fish with this incidence in each tank.

TABLE 2 Sequences of primers used for gene expression analysis

Gene	Nucleotide sequence (5′–3′)	Accession numbers
rpl27	FW: ACAACTCACTGCCCCACCAT	DQ629167.1
	RV: CTTGCCTTTGCCCAGAACTT	
gpx1a	FW: GCTTTGAGCCAAAGATCCAG	KC201352.1
	RV: CTGACGGGACTCCAAATGAT	
cat	FW: ATGGTGTGGGACTTCTGGAG	JQ308823
	RW: AGTGGAACTTGCAGTAGAAAC	
gr	FW: GGGCTGGATGGAAGAACGACA	DQ486890.1
	RW: ACACCGAAAGCACTGAGGAGG	

2.6 | Statistics

All data were statistically analysed using STATGRAPHICS Centurion XVI (Version 16.2.04), STATGRAPHICS plus 5.1 (Statpoint Technologies), or SPSS v21 (IBM Corp.), and means ± *SD* were calculated for every parameter measured. Data were tested for normality with the one-sample Kolmogorov–Smirnov test. For normally distributed data, one-way analysis of variance (ANOVA) was used to determine the effects of the different diets. Data were tested for homogeneity, and post hoc analysis was carried out using Tukey test if variances were the same or Games–Howell test whenever variances were different. Significant differences were considered for $p < .05$. When data did not follow a normal distribution, logarithmic or arcsine transformation was carried out or non-parametric tests, such as Kruskal–Wallis, were used. Two different regression models (broken-line and quadratic regressions analysis; Lin, Shih, Kent, & Shiau, 2010; Robbins, Norton, & Baker, 1979) were applied to understand the effect of dietary Se on different parameters and estimate gilthead sea bream requirements for dietary Se. Survival, weight gain, specific growth rate (SGR) and thermal growth coefficient (TGC) were calculated using the following formulae:

$$\text{Survival}\,(\%) = 100 \times \frac{\text{final number of fish}}{\text{initial number of fish}}$$

$$\text{Weight gain}\,(\%) = 100 \times \frac{\text{final weight} - \text{initial weight}}{\text{initial weight}}$$

$$\text{SGR}\,(\%) = \frac{\text{Ln}_{W2} - \text{Ln}_{W1}}{\text{days}} \times 100$$

$$\text{FCR} = \frac{\text{Ingested food}}{\text{generated biomass}}$$

$$\text{TGC} = \frac{W2^{1/3} - W1^{1/3}}{\text{temperature°C} \times \text{days}}$$

where W_1 is initial body weight (g) and W_2 is final body weight (g).

3 | RESULTS

3.1 | Growth and productive parameters

All diets were readily accepted by sea bream juveniles and had no effect on mortality. Even though isolated deaths were recorded, they were accidental and had no significance to the overall result of the trial (Table 3). At the end of the trial, elevation of the dietary Se levels up to 1.0 mg Se/kg significantly improved standard length, whereas further increase significantly reduced this growth parameter (Table 3), where requirements were observed to be 0.91 mg Se/kg by means of broken-line analyses (data not shown). Equally, body weight was significantly increased by elevation of dietary Se levels up to 1.0 mg Se/kg, whereas further increase reduced body weight (p = .021; Table 3), an effect that could be observed by means of broken-line analyses to indicate the requirements were 0.91 mg Se/kg (Figure 1). Similar tendencies were found for other growth parameters studied (SGR, weight gain or TGC), despite no significant differences were detected (Table 3 and Figure 2). Dietary Se levels did not produce a significant effect on feed utilization and showed an average value of 0.88 ± 0.02 for all fish groups.

3.2 | Biochemical analyses

At the end of the trial, increase in dietary Se up to 0.86 mg Se/kg significantly increased whole body lipids whereas further Se elevation reduced this biochemical parameter (Table 4). Moisture content significantly increased in fish fed the highest dietary Se levels (1.7 mg Se/kg), particularly in relation to those fed 1.0 mg Se/kg (Table 4). Dietary Se supplementation did not cause a significant effect on whole body protein and ash content (Table 4).

Selenium content in whole fish increased not significantly with increasing Se supplementation for whole body that reached a plateau at 1.0 mg Se/kg (Table 5, Figure 3). On the contrary, liver Se content increased with increasing dietary Se following a linear regression (y = 1.8856x + 1.1904; R^2 = 0.96), significantly reaching the highest levels in fish fed 1.7 mg Se/kg and the lowest in those fed 0.45 mg Se/kg (Table 5). Results for copper, zinc, iron and manganese levels in the different tissues showed no significant differences between the treatments (data not shown).

3.3 | Gene expression

Dietary Se did not yield a significant effect on hepatic *gpx1a* expression. However, there was a significant increase in *cat* and *gr* expression in fish fed 1.7 mg Se/kg supplementation (Table 6).

3.4 | Histology

Dietary Se levels did not cause a statistically significant effect on the occurrence of hepatic steatosis in terms of intracellular fat accumulation, broken cell margin, peripheral nucleus or sinusoid dilatation (Table 7). However, increase of dietary Se contents up to 1.7 mg Se/kg tended to reduce sinusoid dilatation, a morphological characteristic associated with liver damage (Table 7). Furthermore, only these fish presented hydropic degeneration (Table 7, Figure 4). No differences were found in the occurrence of melanomacrophages (Figure 5) or hyperaemia.

4 | DISCUSSION

The essentiality of Se supplementation in fish has been discussed in several reviews (Antony Jesu Prabhu et al., 2016; Khan et al., 2017; Lall, 2002; NRC, 2011; Watanabe et al., 1997). In the present trial, an increase in dietary Se contents up to 1.00 mg Se/kg resulted in a significant growth increase in terms of standard length and body weight, in agreement with a proportional increase in other growth parameters such as SGR. Se requirements in farmed fish, based on growth as a criterion, range from 0.12 mg Se/kg in channel catfish (fed selenomethionine or yeast, Wang & Lovell, 1997) and common carp (fed sodium selenite, Gaber, 2009), up to 5.39–7.37 mg Se/kg in yellowtail kingfish (fed selenium yeast, Le & Fotedar, 2013, 2014a). In the present study, since excess Se is toxic and a narrow window exists between requirement and Se toxicity, the more conservative broken-line method was used to define Se requirements for gilthead sea bream. Thus, the broken-line model applied to growth parameters suggested optimum dietary levels of around 0.94 mg Se/kg for sea bream juveniles. In a trial conducted on black sea bream, a closely related species, the results were similar in terms of growth, where the optimum dietary Se level was 0.86 mg/kg (Wang et al., 2019). Nevertheless, increase of Se supplementation up to 1.70 mg/kg significantly reduced growth to levels comparable to those fish fed a diet without Se supplementation. These results are in agreement with the reduced growth obtained because of excessive Se levels in channel catfish fed different Se sources (Wang & Lovell, 1997).

Selenium deposition in liver increased linearly with increasing levels of dietary selenium. This linear increase has been described in multiple fish species, regardless of the source of Se, including Atlantic salmon (Berntssen et al., 2018), black sea bream (Wang et al., 2019), channel catfish (Wang & Lovell, 1997), cobia (Liu et al., 2010), gibel carp (Han et al., 2011), hybrid striped bass (Cotter, Craig, & Mclean, 2007), malabar grouper (Lin, 2014), Nile tilapia (*Oreochromis niloticus*, Lee, Nambi, Won, Katya, & Bai, 2016), olive flounder (*Paralichthys olivaceus*, Lee, Lee, Bai, & Hung, 2010), rainbow trout (Hilton & Hodson, 1983; Wang et al., 2018) and white sturgeon (Tashjian et al., 2006). Dietary Se may also affect the deposition of other minerals in liver, such as copper, which follows a positive correlation with Se deposition in Atlantic salmon (Poppe, Håstein, Frøslie, Koppang, & Norheim, 1986) or rainbow trout (Hilton & Hodson, 1983). However, neither copper nor zinc, iron or manganese contents in liver were affected in the present study.

TABLE 3 Survival (%), standard length (cm), body weight (g), weight gain, SGR (%) and TGC (‰) along the trial of gilthead sea bream fed increasing dietary Se levels for 42 days (means ± *SD*, *n* = 3)

Analysed dietary Se (mg/kg)	Days	Se-0.45	Se-0.68	Se-0.86	Se-1.00	Se-1.70
Survival rate (%)	42	98 ± 4	97 ± 3	100 ± 0	100 ± 0	98 ± 2
Standard length (cm)	42	11.3 ± 0.5^{a}	11.5 ± 0.5^{ab}	11.6 ± 0.5^{ab}	11.7 ± 0.4^{b}	11.3 ± 0.6^{a}
Body weight (g)	18	21.2 ± 2.9^{a}	21.8 ± 2.8^{a}	21.9 ± 2.8^{ab}	22.5 ± 2.9^{b}	21.8 ± 2.8^{a}
	42	31.6 ± 4.7^{a}	32.8 ± 4.3^{a}	33.4 ± 4.4^{ab}	35.7 ± 4.0^{b}	31.9 ± 4.8^{a}
Weight gain (%)	0–42	153 ± 13	160 ± 11	162 ± 4	183 ± 0	154 ± 12
SGR (%)	0–42	2.21 ± 0.12	2.27 ± 0.10	2.29 ± 0.04	2.48 ± 0.00	2.21 ± 0.12
TGC (‰)	0–42	0.81 ± 0.04	0.84 ± 0.04	0.85 ± 0.01	0.93 ± 0.00	0.81 ± 0.05

Note: Different superscript letters within a row indicate significant ($p < .05$).

Abbreviations and calculations: Survival = 100 × final number of fish/initial number of fish; Weight gain = 100 × (final weight − initial weight)/initial weight; SGR, specific growth rate = ($Ln_{final\ weight} - Ln_{initial\ weight}$)/days × 100; TGC, thermal growth coefficient = (Final weight$^{1/3}$ − Initial weight$^{1/3}$)/(temperature °C × days).

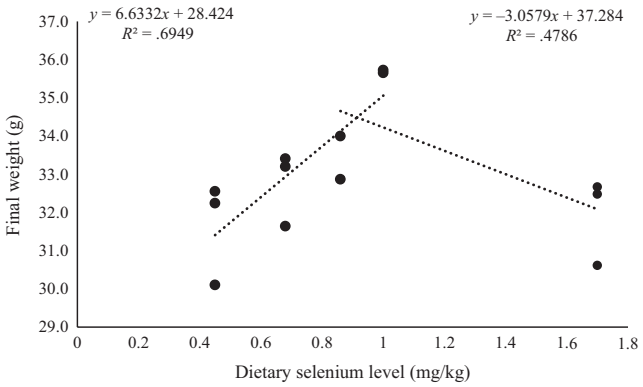

FIGURE 1 Relationship between dietary selenium level and final weight for gilthead sea bream fed increasing levels of dietary selenium for 42 days as described by a broken-line model. According to the plot, the optimal dietary level of selenium is 0.91 mg Se/kg. Values are for each of the three replicates per treatment. Formulae describe each of the two lines composing the broken-line model

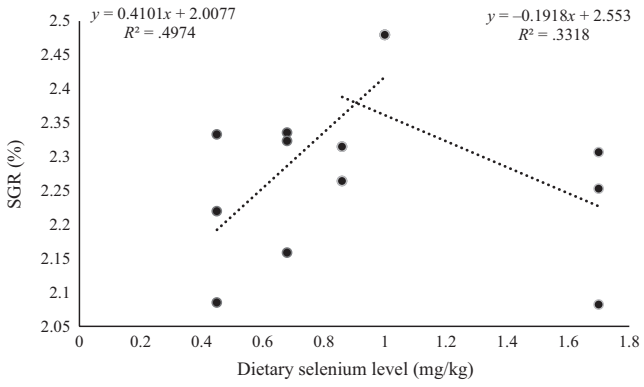

FIGURE 2 Relationship between dietary selenium level and SGR of gilthead sea bream fed increasing levels of dietary selenium for 42 days as described by a broken-line model. According to the plot, the optimal dietary level of selenium is 0.91 mg Se/kg. Values are for each of the three replicates per treatment. Formulae describe each of the two lines composing the broken-line model

Despite the whole body Se contents in whole fish were not significantly different among the treatments, a plateau was reached at 1.00 mg Se/kg. Similarly, in cobia, increasing selenomethionine dietary supplementation until 0.86 mg Se/kg increased whole body Se, reaching a plateau from this dietary level onwards (Liu et al., 2010). Using the broken-line model applied to Se body contents, the optimum dietary Se level was 0.94 mg/kg for sea bream juveniles.

Activity of *gpx* has been frequently used as a criterion to assess selenium requirements (Antony Jesu Prabhu et al., 2016; Khan et al., 2017). In fact, under semi-deficient conditions, there is a linear relationship between selenium concentrations in plasma and *gpx* activity (Daniels, 1996). In agreement, in vitro studies with rainbow trout hepatocytes show an elevation of *gpx* activity when exposed to increased levels of selenomethionine (Misra et al., 2012). However, only the increase from low dietary Se levels in fish larvae (Penglase et al., 2010) or high levels in juveniles (Pacitti et al., 2015; Penglase et al., 2014; Wang et al., 2018; Zee, Patterson, Gagnon, et al., 2016) up-regulates *gpx* expression. In larval gilthead sea bream, dietary increase from 1.73 to 6.41 mg Se/kg as selenium yeast is associated with best growth and significantly down-regulates *gpx* expression, whereas further increase in Se only tends to up-regulate this gene (Saleh et al., 2014). In agreement, in the present study the best growth was observed in fish supplemented up to 0.86 mg Se/kg, which was correlated to a reduction in *gpx1a* expression, while further increase 1.70 mg Se/kg tended to up-regulate the expression of this gene and reduced growth.

Elevation of selenium up to 1.70 mg Se/kg significantly up-regulated hepatic expression of *cat* denoting an increased oxidative stress associated with growth reduction in this fish. These results are in agreement with the increased *cat* activity associated with high Se levels in common carp (fed Se nanoparticles or a commercial diet, Ashouri et al., 2015; Elia et al., 2011), meagre (fed selenium yeast, Mansour et al., 2017), goldfish (*Carasius orates* exposed to Se, Choi et al., 2015) and in vitro rainbow trout hepatocytes (Misra et al., 2012). Indeed, oxidative stress is one of the main causes of

TABLE 4 Biochemical composition (g/kg fresh weight) in whole body of gilthead sea bream fed increasing contents of selenium for 42 days (means ± *SD*, *n* = 3)

Analysed dietary Se (mg/kg)	Se-0.45	Se-0.68	Se-0.86	Se-1.00	Se-1.70
Lipids	119 ± 9^{a}	123 ± 7^{a}	140 ± 7^{b}	118 ± 9^{a}	126 ± 9^{ab}
Protein	171 ± 6	172 ± 11	169 ± 7	174 ± 8	167 ± 6
Ash	35 ± 3	32 ± 6	33 ± 10	36 ± 21	40 ± 10
Moisture	663 ± 4.5^{b}	665 ± 7^{b}	664 ± 6^{b}	659 ± 11^{a}	674 ± 4^{c}

Note: Different letters in the same row indicate significant differences, $p < .05$.

TABLE 5 Whole body and liver Se content of gilthead sea bream fed increasing contents of selenium for 42 days (means ± *SD*, *n* = 3)

Analysed dietary Se (mg/kg)	Initial	Se-0.45	Se-0.68	Se-0.86	Se-1.00	Se-1.70
Whole body Se (mg/kg)		0.7 ± 0 0.1	0.8 ± 0.2	1.1 ± 0.4	1.1 ± 0.4	1.2 ± 0.4
Liver Se (mg/kg)	2.4	1.3 ± 0.1^{a}	1.9 ± 0.2^{b}	2.3 ± 0.1^{b}	2.5 ± 0.2^{b}	3.5 ± 0.4^{c}

Note: Different letters in the same row indicate significant differences, $p < .05$.

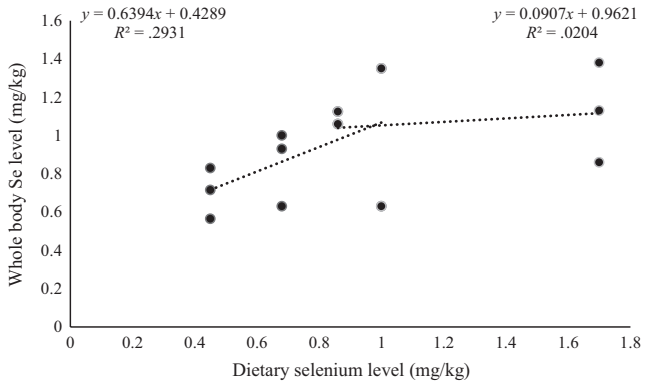

FIGURE 3 Relationship between dietary selenium level and whole body selenium content of gilthead sea bream fed increasing levels of dietary selenium for 42 days as described by a broken-line model. According to the plot, a plateau was reached for whole body selenium level when increasing dietary selenium levels above 1.0 mg Se/kg. Values are for each of the three replicates per treatment. Formulae describe each of the two lines composing the broken-line model

Se toxicity (Hauser-Davis et al., 2016) and has been related to its capacity to oxidize thiols in protein formation or create Se metabolites that originate reactive oxygen species (Berntssen et al., 2017). Therefore, supplementation with 1.70 mg Se/kg indicates a toxic effect on sea bream as denoted by the pronounced increase in *cat* expression.

Elevation of Se up to 1.70 mg Se/kg in the present study also markedly up-regulated hepatic expression of *gr*. Transcription factors can be affected by the redox status of the cell; thus, oxidative stress may alter their activity, and such is the case of *gr* (Di Giulio & Meyer, 2008; Esposito et al., 1998; Olsvik et al., 2011). In vitro studies have demonstrated a reduction of *gr* DNA binding in conditions of high oxidative stress (Esposito et al., 1998). Indeed, it seems that oxidation is a potent modulator of *gr*, but also of zinc-finger-containing proteins that seem to be inhibited by reducible Se compounds (with oxidation state of −I or higher) in which sodium selenite is included (oxidation state +IV; Blessing et al., 2004). In fact, sodium selenite compounds have been observed to inhibit the *gr* binding activity in rat livers (Tashima et al., 1989). The inhibition of *gr* binding activity may produce an increase in mRNA expression in an attempt to counteract this inhibition. However, this effect seems to be dose-dependent in vivo, as *gr* mRNA expression is increased with increasing selenium (3–4 mg/L Se) exposure in goldfish, whereas no effect was observed in lower levels (Choi et al., 2015). Furthermore, excess Se induces stress hormones in goldfish (Choi et al., 2015) and rainbow trout (Wiseman et al., 2011) or gilthead sea bream juveniles submitted to acute stress (Mechlaoui et al., 2019).

TABLE 6 Hepatic gene relative expression ($2^{-\Delta\Delta Ct}$) of gilthead sea bream fed increasing levels of dietary selenium for 42 days (means ± *SD*, *n* = 3)

Analysed dietary Se (mg/kg)	Se-0.45	Se-0.68	Se-0.86	Se-1.00	Se-1.70
gpx1a	1.1 ± 0.5	1.2 ± 0.38	0.7 ± 0.0	0.8 ± 0.2	1.2 ± 0.7
cat	1.0 ± 0.4^{a}	1.3 ± 0.9^{a}	0.5 ± 0.1^{a}	1.2 ± 0.2^{a}	19.2 ± 0.1^{b}
gr	1.0 ± 0.2^{a}	0.8 ± 0.2^{a}	0.6 ± 0.1^{a}	0.7 ± 0.1^{a}	5.1 ± 0.1 ^{b}

Note: Different letters in the same row indicate significant differences, $p < .05$.

TABLE 7 Hepatic histological analyses of gilthead sea bream fed increasing levels of dietary selenium for 42 days expressed in a 0–3 scale and as % based on absence or presence (means ± *SD*, *n* = 3)

	Analysed dietary Se (mg/kg)	Se-0.45	Se-0.68	Se-0.86	Se-1.00	Se-1.70
% of area affected	Lipid accumulation	73 ± 25	51 ± 14	56 ± 20	41 ± 16	38 ± 6
	Broken cell margin	52 ± 13	51 ± 19	70 ± 26	37 ± 23	49 ± 25
	Peripheral nucleus	41 ± 17	27 ± 10	31 ± 27	11 ± 19	7 ± 6
	Sinusoid dilatation	58 ± 28	43 ± 23	67 ± 34	44 ± 12	17 ± 16
% fish affected	Melanomacrophages	40 ± 43	33 ± 24	46 ± 42	27 ± 15	13 ± 30
	Hydropic degeneration	0 ± 0	0 ± 0	0 ± 0	0 ± 0	47 ± 45
	Hyperaemia	7 ± 15	33 ± 41	13 ± 25	7 ± 15	7 ± 15

Note: Different letters in the same row indicate significant differences, $p < .05$.

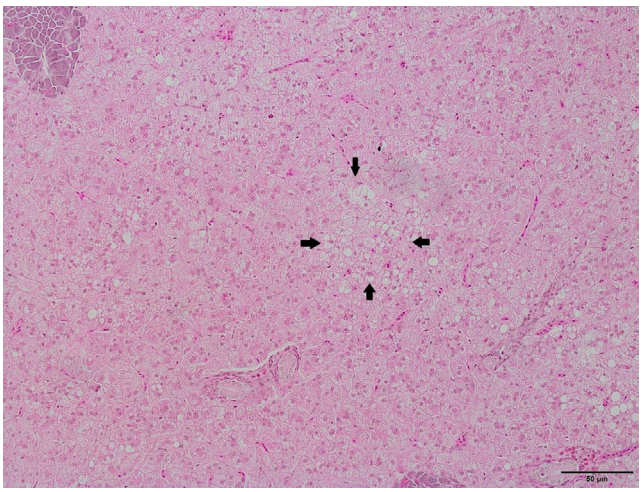

FIGURE 4 Microscopic view of liver cell nucleus (20×) presenting hydropic degeneration (black arrows delimit area affected)

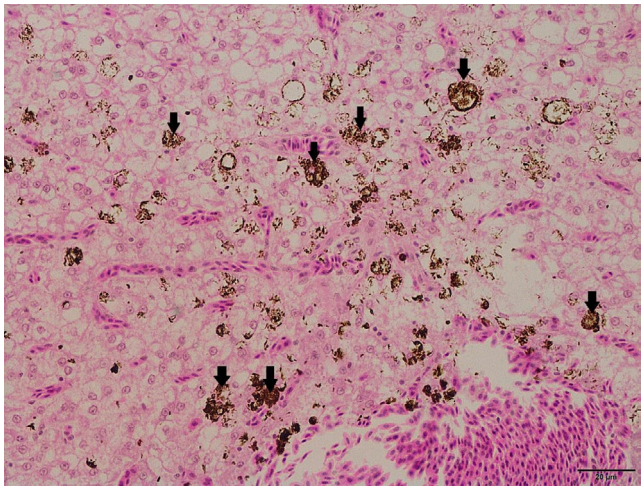

FIGURE 5 Microscopic view of liver cell nucleus (40×) presenting melanomacrophage aggregates (black arrows)

Excess of dietary Se also negatively affects hepatic tissue morphology, including pathological signs such as degeneration and focal necrosis (Berntssen et al., 2018). Other morphological alterations such as hydropic degeneration in kidney have also been associated with high levels of dietary Se in white and green sturgeon (*Acipenser medirostris*; De Riu, Lee, Huang, Moniello, & Hung, 2014). In the present study, hepatic hydropic degeneration was observed only on fish fed the diet containing 1.70 mg Se/kg, in agreement with the higher oxidative risk and the lower growth found in these fish. These results agree well with the hepatic damage caused by excess dietary Se levels in other species such as common carp (Ashouri et al., 2015). However, other histopathological signs of excess Se such as hepatocellular vacuolar degeneration and necrosis found in white sturgeon (Tashjian et al., 2006) were not observed in sea bream fed 1.70 mg Se/kg. This suggests that these levels were high enough to induce oxidative stress and reduce growth, but not sufficient to cause large damage in hepatic tissues. These results agree well with the lack of damages in the hepatic tissue of rainbow trout (Hilton & Hodson, 1983) or white sturgeon (Zee, Patterson, Gagnon, et al., 2016) fed high Se dietary levels. On the contrary, dietary Se levels of 11 mg Se/kg, 10 times higher than those tested in the present study, induce a wide range of pathological alterations in liver of Atlantic salmon (Berntssen et al., 2018).

Selenium is one of the elements with a smaller window between requirement and toxicity levels (Khan et al., 2017). Signs of Se toxicity in fish include reduced growth and feed intake, increased oxidative stress or disturbance of fatty acid metabolism (Berntssen et al., 2017). Whereas some species can even tolerate levels of up to 20.5 mg Se/kg (as selenomethionine) without presenting symptoms of toxicity, as is the case of white sturgeon (*Acipenser transmontanus*; Tashjian et al., 2006), others show toxic signs at dietary Se doses of 9 or 9.6 mg Se/kg as juvenile rainbow trout (as sodium selenite) or chinook salmon (*Oncorhynchus tshawytscha* fed selenomethionine; Hamilton, 2004), and 11 or 15 mg Se/kg (as sodium selenite or selenium yeast) as Atlantic salmon (*Salmo salar*, Berntssen et al., 2017, 2018). In the present study, inclusion of dietary Se at 1.70 mg Se/kg resulted in a reduced growth, oxidative status and altered liver morphology, symptoms found in fish affected by Se toxicity.

In conclusion, the results of this study suggest that the optimum dietary levels of total selenium in diets with 100 g/kg FM with basal levels of 0.45 mg Se/kg are around 0.94 mg Se/kg to promote growth of gilthead sea bream juveniles. Moreover, feed levels of 0.85–1.00 mg Se/kg supplemented as sodium selenite were safe and

did not produce a negative effect on growth, catalase expression or liver morphology. On the contrary, dietary levels of 1.70 mg Se/kg were found to be excessive and caused growth reduction, increased catalase expression and hydropic degeneration in the liver, thus should be avoided in gilthead sea bream formulated feeds containing low levels of marine ingredients.

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CONFLICT OF INTEREST

R. Fontanillas is an employee of Skretting AS, Stavanger, Norway.

AUTHOR CONTRIBUTIONS

David Domínguez conceived and designed the experiments, performed the experiments, performed analyses, analysed the data, wrote the paper, prepared figures and/or tables, and reviewed drafts of the paper. Zakarya Sehnine performed the experiments, performed analyses, analysed the data, and prepared figures and/or tables. Pedro Castro performed analyses, analysed the data, and reviewed drafts of the paper. Lidia Robaina contributed reagents/materials/analysis tools. Ramón Fontanillas conceived and designed the experiments, contributed reagents/materials/analysis tools, and reviewed drafts of the paper. Philip Antony Jesu Prabhu performed analyses, analysed the data, prepared figures and/or tables, and reviewed drafts of the paper. Marisol Izquierdo conceived and designed the experiments, analysed the data, contributed reagents/materials/analysis tools, wrote the paper, and reviewed drafts of the paper.

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DATA AVAILABILITY STATEMENT

All relevant data are within the paper.

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Dietary manganese levels for gilthead sea bream (Sparus aurata) fingerlings fed diets high in plant ingredients

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Dietary manganese levels for gilthead sea bream (*Sparus aurata*) fingerlings fed diets high in plant ingredients

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Suggested running title: **Manganese nutrition in gilthead sea bream**

Keywords

Manganese, Gilthead sea bream, Fish mineral nutrition, Plant ingredients

Abbreviations

CAT: Catalase; FCR: Feed Conversion Ratio; FM: fish meal; FO: fish oil; Mn: Manganese; *MnSOD*: manganese superoxide dismutase; SGR: Specific Growth Rate; TGC: Thermal Growth Coefficient

Abstract

Manganese (Mn) is an essential metal for fish and requirements have been established for several finfish but not for gilthead sea bream. Thus, the present study aims to establish the optimal dietary supplementation level of Mn in gilthead sea bream fingerlings fed vegetable based diets.

Gilthead sea bream fingerlings (weight 12.6 ± 1.5 g, mean ± S.D.) were fed five practical diets high in vegetable ingredients (fish meal: 10%, fish oil: 6%). The diets were supplemented to contain 19, 27, 30, 41 and 66 mg Mn kg^{-1} as $MnSO_4$. Four hundred and fifty sea bream fingerlings were randomly distributed in 15 tanks and fed one of the five diets until apparent satiation three times per day for 42 days. Growth parameters including feed intake, thermal growth coefficient and feed conversion ratio were calculated. At the end of the trial, samples were taken for biochemical, mineral, histological and gene expression analyses.

After the feeding trial, fish almost tripled their weight, but dietary Mn levels did not affect growth parameters or survival. The high fish meal substitution levels led to high Mn contents in the basal diet (19 mg Mn kg^{-1} diet), that seemed to be sufficient to promote sea bream growth. Body lipid composition, protein and ash were not affected by the dietary Mn. Similarly, whole body, liver and vertebrae mineral contents were not affected by Mn supplementation. Morphological characteristics of liver had no significant differences among dietary Mn levels. However, increase of Mn contents beyond 30 mg Mn kg^{-1} down-regulated *MnSOD* expression. Expression of *CAT* gene was not affected.

Overall, results suggest that the Mn content present in the basal diet (19 mg Mn kg^{-1}) was sufficient to cover the requirements in juvenile gilthead sea bream fed practical plant-based diets, although results from oxidative status markers might point out the

need to increase supplementation levels beyond this point when fish are under conditions that may affect their oxidative status.

Introduction

Substitution of fishmeal (FM) and fish oil (FO) by ingredients of terrestrial origin is of paramount importance for the sustainable production of gilthead sea bream. However, the mineral profile of plant ingredients considerably differs from that of marine ingredients (Antony Jesu Prabhu et al., 2018). Mn, one of the minerals whose content in marine or terrestrial plant ingredients markedly changes, is a transition metal essential for life and acts as a cofactor for metalloenzymes. Thus, Mn is involved in several enzyme complexes with antioxidant function including the Mn superoxide dismutase (MnSOD) (Holley et al., 2011), but also intervenes in carbohydrate, lipid, and protein metabolism (Lall, 2002). Mn can be found in high concentrations in bone, but other tissues with high levels of this mineral are liver, muscle, kidney, gonadal tissues, and skin, where it is more concentrated in the mitochondria (Lall, 2002). Despite water dissolved Mn can be absorbed by fish, diet borne Mn is the main source of uptake (Watanabe et al., 1997).

Mn deficiency may alter a wide range of biomarkers due to its ubiquity in the different tissues and its involvement in carbohydrate, lipid, and protein metabolism. The main criterion to assess Mn is considered to be vertebral Mn concentration (Antony Jesu Prabu et al., 2016). However, other markers can be strongly affected by a deficiency. For instance MnSOD activity is reduced in the heart and liver, as well as the level of Mn in the vertebrae when Mn deficiency is installed (Knox et al., 1981). Excess and deficiencies can affect the integrity of the intestinal immunity (Jiang et al., 2015). Other

deficiency symptoms include dwarfism, skeletal anomalies, cataracts, mortality, reduced growth and equilibrium disorders (Watanabe et al., 1997). Effects of Mn toxicity are rare when supplemented in the diet except for altered intestinal immunity (Jiang et al., 2015). In fact, EFSA establishes a maximum total content of Mn in complete feed for fish at 100 mg Mn kg^{-1}, but admits no maximum tolerable level were reached in the trials studied (EFSA, 2016). However, Mn intoxication through water is more common and can cause severe liver damage. Histopathological disorders caused by Mn intoxication include pycnotic degeneration of hepatocytes, congestion and dilatation in the sinusoids, mild necrosis, nuclear degeneration and hyper-vacuolization (Alm-Eldeen et al., 2018; Kaur et al., 2018; Krishnani et al., 2003).

Dietary manganese levels required to meet requirements have been described in several species including Atlantic salmon (4.9-3[illegible]0 mg Mn kg^{-1}, *Salmo salar*, Antony Jesu Prabu et al., 2019; Lorentzen et al., 1[illegible]; Maage et al., 2000), cobia (21.72-24.93 mg Mn kg^{-1}, *Rachycentron canadum*, Liu et al., 2013), hybrid grouper (12.70 mg Mn kg^{-1}, *Epinephelus lanceolatus* × *E. fuscoguttatus*, Liu et al., 2017) and rainbow trout (19 mg Mn kg^{-1}, *Oncorhynchus mykiss*, Satoh et al., 1991). On the other hand, manganese requirement based on purified diets differ according to the species, having been described for common carp (13-15 mg Mn kg^{-1}, *Cyprinus carpio*, Satoh et al., 1992), channel catfish (2.4 mg Mn kg^{-1}, *Ictalurus punctatus*, Gatlin and Wilson, 1984), gibel carp (13.77 mg Mn kg^{-1}, *Carassius auratus gibelio*, Pan et al., 2008), grass carp (20.6 mg Mn kg^{-1}, *Ctenopharyngodon idella*, Liang et al., 2015), hybrid tilapia (7 mg Mn kg^{-1}, *Oreochromis niloticus* × *O. aureus*, Lin et al., 2008), orange-spotted grouper (19 mg Mn kg^{-1}, *Epinephelus coioides*, Ye et al., 2009) and yellow catfish (5.5-6.4 mg Mn kg^{-1}, *Pelteobagrus fulvidraco*, Tan et al., 2012). However little is known about Mn nutrition in gilthead sea bream juveniles (Dominguez et al., 2017). For instance, the combined

supplementation of Mn, Zn and Se in inorganic rather than in organic forms in low FM diets for sea bream juveniles lead to an improved growth (Dominguez et al., 2017). However, Mn content in vertebrae, was not affected by dietary increase in Mn 22-52 mg Mn kg^{-1}, suggesting that such growth improvement was more related to dietary Zn or Se contents as it was later demonstrated (Dominguez et al., 2019a, 2020). In a second study, increase in dietary Mn from 30 to 60 mg Mn kg^{-1} together with amino acid chelated Zn did not affect gilthead sea bream growth, whole body or vertebrae Mn contents (Dominguez et al., 2019b). However, in comparison to amino acid chelated Mn, Mn oxide up-regulated the expression of superoxide dismutase gene (*MnSOD*) (Dominguez et al., 2019b). Also, dietary supplementation of Mn oxide from 21-73 mg Mn kg^{-1} as part of an increased nutrient package, did neither increased Mn contents in vertebrae, but affected *MnSOD* expression (Dominguez et al., 2020). In all these studies Mn was supplemented together with other minerals. Therefore, the main aim of this study was to determine the effect of different levels of Mn oxide in sea bream fingerlings fed vegetable based diets.

Material and methods

All the experimental conditions and sampling protocols have been approved by the Animal Welfare and Bioethical Committee from the University of Las Palmas de Gran Canaria.

2.1 Diets

A practical diet for gilthead sea bream was formulated with low inclusion of FM (10%) and FO (6%) following the trend of the aquafeed industry. Five different experimental diets were formulated by supplementing $MnSO_4$ to contain 19, 27, 30, 41 and 66 mg Mn kg^{-1} diet (Table 1). Diets were isoenergetic and isonitrogenous, and were designed to cover all known nutritional requirements for this species. The diets were manufactured by extrusion process by Skretting Aquaculture Research Centre AS (Stavanger, Norway).

Table 1. Ingredients and analyzed Mn content of the experimental diets supplemented with increasing levels of Mn and fed to gilthead sea bream juveniles for 42 days

Raw ingredient (%)	**Mn19**	**Mn27**	**Mn30**	**Mn41**	**Mn66**
Linseed oil	0.82	0.82	0.82	0.82	0.82
Wheat	11.69	11.69	11.69	11.69	11.69
Corn gluten	15.00	15.00	15.00	15.00	15.00
Wheat gluten	21.66	21.66	21.66	21.66	21.66
Soya concentrate	23.00	23.00	23.00	23.00	23.00
Faba beans	5.00	5.00	5.00	5.00	5.00
Fish meal	10.00	10.00	10.00	10.00	10.00
Rapeseed oil	3.00	3.00	3.00	3.00	3.00
Fish oil South American	6.00	6.00	6.00	6.00	6.00
Palm oil	1.64	1.64	1.64	1.64	1.64
Micronutrient premix*	2.19	2.19	2.19	2.19	2.19
Analyzed Mn (mg kg^{-1})	19.00	27.00	30.00	41.00	66.00

*Micronutrient premix: methionine (0.001%), lysine (1.235%), phosphate (0.67%), vitamin premix (0.18%) and mineral premix excluding Mn (0.11%).

2.2. Feeding trial

Gilthead sea bream fingerlings, 12.6 ± 1.4 g (mean ± SD) were distributed in 15 tanks with 30 fish per tank and randomly assigned one of the dietary treatments, in triplicates in the facilities of the Aquaculture Research Group (GIA) of the University of Las Palmas de Gran Canaria, Spain. Feeding was conducted until apparent satiation three times per day for 42 days and kept under a natural photoperiod of approximately 12 h light. Water parameters including, temperature (19.4 ± 0.4°C, mean ± SD) and oxygen, were monitored daily, while pH was registered weekly. Growth was recorded and tissue samples were taken for biochemical, mineral, histology, X-ray and liver gene expression analyses at the end of the trial.

Growth, in terms of standard length (cm) and weight (g), was recorded at days 0, 18 and 42 of the trial by measuring and weighing all fish. Throughout the experiment, feed intake per tank was recorded. At the end of the trial growth parameters were calculated including Specific Growth Rate (SGR), Thermal Growth Coefficient (TGC) and Feed Conversion Ratio (FCR) using the following formulae:

$$\text{SGR}\ (\%) = \frac{(\text{LnW2} - \text{LnW1})}{\text{days}} * 100$$

$$\text{TGC} = \frac{(\text{W2\^1/3} - \text{W1\^1/3})}{(\text{temp} * \text{days})}$$

$$\text{FCR} = \frac{\text{Ingested food}}{\text{generated biomass}}$$

Where

W1: initial body weight (g)

W2 final body weight (g)

Temp: Temperature (ºC)

Before sampling, fish were previously fasted for 24 h and, then, anesthetize with clove oil (Guinama S.L.U., Valencia, Spain). Tissues from five fish per tank were sampled for biochemical, mineral and gene expression analysis and kept frozen at -80ºC until the analysis was conducted. Twenty fish per tank were sampled for radiographic assessment.

2.3 Chemical analyses

Chemical composition of fish was determined using near-infrared spectroscopy (FoodScan, Foss, Sweden). Biochemical composition of diets and whole fish was determined following standard procedures (Association of Official Analytical Chemists (AOAC, 2000)). Crude lipid was extracted according to the method of Folch et al., (1957) and ash by combustion in a muffle furnace at 600 ºC for 12 h. Protein content (N×6.25) was determined by using the Kjeldahl method (AOAC, 2000) and dry matter content was determined after drying the sample in an oven at 105 ºC until reaching constant weight.

Thiobarbituric acid-reactive substances (TBARS) were measured in whole body of five fish pooled from each tank in triplicate samples according to Burk et al. (1980). Samples of 20–30mg of homogenized whole body were mixed with 1.5 ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% butylated hydroxytoluene in methanol. Afterwards, 2.95 ml of 50mM-thiobarbituric acid solution were added before mixing and heating for 10min at 100ºC. Protein precipitates were removed by centrifugation at 2000 g after cooling, and the supernatant was read in a spectrophotometer (Evolution 300; Thermo Scientific) at 532 nm. A blank at the same wavelength was used to record absorbance. The concentration of TBARS, expressed as

mmol of acid-malonaldehyde (MDA)/g tissue, was calculated using an extinction coefficient of 0.156 cm/mM.

The evaluation of the mineral content was conducted by means of an inductively coupled plasma mass spectrometry (iCAPQ ICP-MS), after submitting the sample to acid digestion.

2.4 Histological studies

Four fish per tank were sampled for histological analysis of liver at the end of the trial. Tissues were stored in 10% buffered formaldehyde in a sample:formaldehyde ratio of 1:10 for several weeks prior to processing. Samples were further segmented to allow a better penetration of the alcohol and introduced in histology cassettes. Dehydration of the samples was carried out using a Histokinette 2000 (Leica, Nussloch, Germany) with gradually increasing alcohol grades beginning with 70º and ending with 100º, being the last two steps xylene and paraffin. Once the paraffin block was obtained it was sliced at a thickness of 3µm using a Leica RM 2135 microtome (Leica, Nussloch, Germany) and fixed to a slide including as much parts of the tissue as possible. Samples were then stained with haematoxylin – eosin staining (Martoja and Martoja-Pearson, 1970) for optical evaluation. Once the preparations were ready they were subjected to optical analysis in search for signs of liver damage including steatosis, peripheral nuclei, broken cell margin and sinusoid dilatation and analyzed by pair evaluators in a 0-3 scale, where 0 was absence of observation and 3 presence in most of the liver.

2.5 Gene expression

2.5.1 RNA extraction

Total RNA was extracted from 60 mg of liver using TRI Reagent Solution (Life Technologies, Carlsbad, CA, USA) and purified on RNeasy Mini Spin Columns (Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.5.2 Reverse transcription

Reverse transcription of 1 µg total RNA from each experimental sample was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions with slight modifications. Briefly, 1µg total RNA and nuclease-free water to a final volume of 15 µl were heated at 65ºC for 10 min and cooled in ice. Afterwards 1 µl of iScript reverse transcriptase and 4 µl of 5 × iScript reaction mix were added, reaching a final reaction volume of 20 µl. The complete reaction mix was incubated for 5 min at 25 ºC, 30 min at 42 ºC, and then 5 min at 85 ºC to inactivate reverse transcriptase. For gene quantification, the reverse transcription reactions were diluted 1:10.

2.5.3. Quantitative PCR

The nucleotide sequences of primers used in this study are reported in Table 2. A total of 2 µl of diluted cDNA was used in real-time PCR for gene expression quantification using IQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Duplicate analyses were performed for each sample for both the housekeeping and the target gene in a final reaction volume of 20 µl. Beta actin (*bact*) and ribosomal protein 27a (*rpl-27a*) were used as housekeeping genes to normalize the expression of the target genes (*MnSOD* and *CAT*) in liver. Real-time quantitative PCR was performed using the iQ5 Multicolor Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The PCR conditions were as follows: 95 ºC for 3 min and 30 sec, followed by 40 cycles of 95 ºC for 15 sec, 58.1 ºC for 30 sec, and 72 ºC for 30 sec; 95 ºC for 1 min, and

a final denaturation step from 58 to 95 ºC for 10 sec. The 2−ΔΔCt method was applied to analyze the relative changes in gene expression.

Table 2. Sequences of primers used for gene expression analysis

Gene	**Nucleotide sequence (5'-3')**	**Accession number**
Beta-actin (*Bact*)	F: TCTGTCTGGATCGGAGGCTC	X89920
	R: AAGCATTTGCGGTGGACG	
Ribosomal Protein L27 (*rpl27*)	F: ACAACTCACTGCCCCACCAT	DQ629167.1
	R: CTTGCCTTTGCCCACAACTT	
Mn superoxide dismutase (*MnSOD*)	F: AGTGCCTCCTGATATTTCTCCTCTG	JQ3088331
	R: CCTGACCTGACCTACGACTATGG	
Catalase (*CAT*)	F: ATCGTGTGGGACTTCTGGAG	JQ308823
	R: AGTGGAACTTGCAGTAGAAAC	

2.6 Statistics

All data were statistically analyzed using STATGRAPHICS Centurion XVI (Version 16.2.04), STATGRAPHICS plus 5.1 (Statpoint Technologies, Warrenton, VA, USA), or

SPSS v21 (IBM Corp., Chicago, IL, USA) and means ± SD were calculated for every parameter measured. Data were tested for normality with the one-sample Kolmogorov–Smirnov test. For normally distributed data, one-way analysis of variance (ANOVA) was used to determine the effects of the different diets. Data were tested for

homogeneity and post-hoc analysis was carried out using Tukey test if variances were homogeneous or Games-Howell test whenever variances were different. When data did not follow a normal distribution, logarithmic or arcsin transformation was carried out and the non-parametric tests of Kruskal-Wallis was used. Quadratic regressions and broken line analyses were conducted where possible. Significant differences were considered for $P<0.05$.

3. Results

3.1 Feeding trial

Fish accepted well the feed from the beginning of the trial and the different dietary Mn levels did not alter significantly the survival rate at the end of the trial (99.3±1.4%). Dietary Mn did not have a significant effect on final weight, weight gain, SGR, FCR or FE (Table 3).

Table 3. Growth performance and feed utilization in gilthead sea bream fed increasing contents of Mn for 42 days

Growth Parameters	**Dietary Mn (mg kg^{-1})**				
	19	**27**	**30**	**41**	**66**
IW (g)	12.5±1.4	12.7±1.4	12.7±1.5	12.6±1.3	12.7±1.5
FW (g)	33.6 ± 1.2	33.1 ± 0.3	32.6 ± 0.3	32.2 ± 1.1	33.3 ± 1.3
WG (%)	166±11	161±3	156±3	157±10	165±13
SGR (%)	2.33±0.09	2.28±0.02	2.24±0.03	2.24±0.10	2.32±0.11

FCR (g)	1.08±0.01	1.15±0.02	1.16±0.04	1.12±0.03	1.13±0.08
FE (g)	0.93±0.01	0.87±0.02	0.87±0.03	0.89±0.02	0.88±0.06

Different letters in the same row indicate significant differences, P<0.05, n=3. IW: Initial Weight; FW: Final Weight; WG: Weight Gain; SGR: Specific Growth Rate; FCR: Feed Conversion Ratio; FE: Feed Efficiency.

3.2 Chemical analyses

Whole body biochemical composition in terms of lipids, proteins, and ash was not significantly affected by the inclusion of dietary Mn in diets for gilthead sea bream juveniles (Table 4). There was neither a significant difference in whole body TBARS contents among all treatments (Table 4), although TBARS values tend to be reduced by the increase in Mn contents in the liver ($y = 3.887^{e-0.413x}$, P=0.057 R^2= 0.75).

Table 4. Biochemical composition (% fresh weight) in whole body of gilthead sea bream fed increasing contents of Mn for 42 days

Parameter	**Dietary Mn (mg kg^{-1})**				
	19	**27**	**30**	**41**	**66**
Lipids (%)	10.5±0.9	11.9±2.0	11.0±1.2	11.5±0.1	12.0±0.6
Protein (%)	16.6±1.0	16.2±0.8	16.0±0.5	17.0±0.4	16.5±0.4
Ash (%)	3.5±0.9	3.2±0.4	3.1±0.6	3.1±0.2	3.1±0.4
TBARS (nmol MDA g^{-1} lipid)	2.44±0.64	3.81±2.16	2.83±0.46	3.16±1.12	2.20±0.24

Different letters in the same row indicate significant differences, P<0.05, n=3.

Mineral analyses of whole fish, liver, and vertebrae showed no significant effects of dietary Mn on the mineral concentrations on the different tissues (Table 5). However, vertebral Mn content followed a linear regression in relation to dietary Mn ($y = 0.06x + 23.90$, $R^2 = 0.81$, $P=0.037$).

Table 5. Whole body, liver and vertebrae mineral content of gilthead sea bream fed increasing dietary contents of Mn for 42 days

Tissue mineral concentration (mg kg^{-1} d.w.)		**Dietary Mn (mg kg^{-1})**				
		19	**27**	**39**	**41**	**66**
Whole fish	Mn	6.5±0.6	5.8±0.9	6.5±0.5	7.8±1.0	7.0±0.4
	Cu	1.53±0.12	1.57±0.12	1.55±0.07	1.67±0.06	1.50±0.10
	Fe	35±1	30±2	34±3	38±5	33±3
	Zn	44±2	42±3	41±3	44±3	41±4
	Se	2.06±1.17	1.70±0.50	1.45±0.49	1.43±0.06	1.70±0.56
Liver	Mn	6.0±0.9	5.6±0.6	6.1±1.7	5.6±0.7	6.7±1.5
	Cu	10.9±1.2	11.6±2.7	12.0±0.0	11.8±3.9	11.7±0.6
	Fe	80±4	79±18	68±8	82±9	75±15
	Zn	76±5	79±4	80±10	79±11	74±6
	Se	1.97±0.12	1.97±0.15	1.95±0.21	2.30±0.26	1.93±0.25
Vertebrae	Mn	25.0±1.7	26.3±1.5	25.0±0.0	26.3±1.2	28.5±1.0
	Cu	0.72±0.11	0.66±0.03	0.63±0.00	0.69±0.09	0.68±0.02
	Fe	39±10	34±14	27±2	27±5	26±2

Zn	66±3	70±8	70±1	67±10	67±6
Se	0.42±0.06	0.52±0.22	0.39±0.01	0.48±0.13	0.47±0.12

Different letters in the same row indicate significant differences, P<0.05, n=3.

3.3 Histological studies

Histological analyses of gilthead sea bream liver fed dietary Mn for 42 days showed no evidence of dietary Mn having a significant effect on the morphology of hepatocytes in terms of steatosis, peripheral nucleus, broken cell margin or sinusoid dilatation (Table 6).

Table 6. Liver histological analyses of gilthead sea bream fed increasing levels of dietary Mn for 42 days

Liver histological criteria (0-3)	**Dietary Mn (mg kg^{-1})**				
	19	**27**	**30**	**41**	**66**
Steatosis	1.25±0.25	1.75±0.43	2.00±0.25	1.58±0.76	1.75±0.43
Peripheral nucleus	0.33±0.14	0.83±0.76	1.08±0.63	0.67±0.29	0.92±0.52
Broken cell margin	1.42±0.38	1.75±0.25	1.42±0.38	1.58±0.29	1.58±0.38
Sinusoids dilatation	1.58±0.76	1.44±0.76	2.25±0.66	2.08±0.76	1.75±0.43

Different letters in the same row indicate significant differences, p<0.05, n=3.

3.4 Gene expression

Increase in dietary Mn levels over 30 mg Mn kg^{-1} down-regulated *mnsod* expression following a significant and negative exponential regression ($y = 1.5441e^{-0.02x}$, P=0.00,

R^2=0.74), being significantly lowest in fish fed 66 Mn kg^{-1} (Table 7). Broken-line analysis indicated an intersection point at 44.1 mg Mn kg^{-1}. Expression of *CAT* was not significantly different among fish fed the different Mn levels, although the expression of fish fed basal diet was more than double that of fish fed any of the other diets (Table 7).

Table 7. Liver gene expression analyses of gilthead sea bream fed increasing levels of dietary Mn for 42 days

Gene ($2^{-\Delta\Delta Ct}$)	**Dietary Mn (mg kg^{-1})**					**P-value**
	19	**27**	**30**	**41**	**66**	
MnSOD	1.02±0.26^{b}	0.84±0.02^{ab}	1.00±0.10	0.59±0.07^{ab}	0.41±0.14^{a}	0.006
CAT	1.08±0.47	0.55±0.02	0.33±0.01	0.43±0.06	0.40±0.14	n.s.

Different letters in the same row indicate significant differences, P<0.05, n=3.

4. Discussion

As an essential mineral, manganese function in the body has been amply described in several fish species (Antony Jesu Prabhu et al., 2014; NRC, 2011; Lall, 2002; Watanabe et al., 1997). However, the requirements for gilthead sea bream juveniles have not yet been addressed. Due to its essentiality, dietary manganese levels may exert an important effect on growth and feed utilization in several fish species. Based on these performing parameters, Mn requirements have been defined for yellow catfish (5.5 mg Mn kg^{-1}, Tan et al., 2012), cobia (21.72 mg Mn kg^{-1}, Liu et al., 2013), hybrid grouper (12.70 mg Mn kg^{-1}, Liu et al., 2017), rainbow trout (15 mg Mn kg^{-1}, Satoh et al., 1991) or common carp (10 mg Mn kg^{-1}, Satoh et al., 1992). Results from the present trial showed that increase in dietary manganese from 19 to 66 mg Mn kg^{-1} did not affect growth or feed utilization parameters in gilthead sea bream juveniles fed practical diets with high levels of plant ingredients. These results suggest that 19 mg Mn kg^{-1} are sufficient to cover

manganese requirements for growth in gilthead sea bream. These values are close to those described as optimum for other species such as rainbow trout (19 mg Mn kg^{-1}, Satoh et al., 1991) or cobia (21.72 mg Mn kg^{-1}, Liu et al., 2013), despite these studies were conducted with purified diets with very low basal levels of manganese. Even when semi-purified diets were used (Satoh et al., 2001), the main ingredient was fish meal, which has lower manganese concentration (8-12 mg Mn kg^{-1}) than several plant ingredients employed in the present practical diet (i.e. soy protein concentrate: 31 mg Mn kg^{-1} or wheat: 35 mg Mn kg^{-1}). The results indicate that in novel diets with high FM replacement by terrestrial plant ingredients basal levels of manganese may be enough to cover gilthead sea bream requirements for growth. Similar findings were observed in rainbow trout (Antony Jesu Prabhu et al., 2018) and carp (Antony Jesu Prabhu et al., 2017), where the basal diets already contained sufficient manganese to sustain growth.

Dietary manganese did not have a significant effect on whole body lipid, protein or ash content. Cobia fed purified diets and increasing levels of manganese supplementation (5.98-41.29 mg Mn kg^{-1} respectively) presented an increase in whole body protein and lipid deposition, and a reduction in ash content with increasing levels of supplementation (Liu et al., 2013). An opposite effect was observed in yellow catfish fed a semi-purified diet and levels of manganese (3.1-19.5 mg Mn kg^{-1}), where whole body lipid tended to decrease with increasing levels of manganese (Tan et al., 2012). The authors of both studies did not discuss these results, however, opposite to the results obtained in the present trial, these experiments proved that increasing dietary manganese resulted in an increased weight, thus altering the protein and lipid content of the whole body of these species.

Tissue contents in manganese for vertebrae and whole body have been also used as biomarkers to assess manganese requirements in different fish species including

Atlantic salmon (7.5-10.5 mg Mn kg^{-1}, Maage et al., 2000), cobia (22-25 mg Mn kg^{-1}, Liu et al., 2013), gibel carp (12.62 - 13.63 mg Mn kg^{-1}, Pan et al., 2008), grouper (*Epinephelus coioides*, 19 mg Mn kg^{-1}, Ye et al., 2009), hybrid grouper (12.7 mg Mn kg^{-1}, Liu et al., 2017), hybrid tilapia (7 mg Mn kg^{-1}, Lin et al., 2008), and rainbow trout (19 mg Mn kg^{-1}, Satoh et al., 1991). Interestingly, in the present study there was a positive significant correlation between dietary manganese and vertebrae Mn content. This denotes, as in other studies, that manganese deposition in vertebrae is highly responsive to dietary manganese (Antony Jesu Prabhu et al., 2016).

Increase in Mn contents in the liver was related to a decrease in TBARS values, which may be a reflection of the antioxidant status of gilthead sea bream tissues (Dominguez et al., 2019c). Indeed, oxidative status has been used as a criterion to assess Mn optimum level in other fish species. Therefore, despite the basal dietary Mn levels (19 mg Mn kg^{-1}) were sufficient to cover growth requirements, the overall reduction in oxidative risk in fish fed the higher Mn dietary levels suggest a higher requirement of this mineral for antioxidant protection under the conditions of the present trial. Further studies must be conducted in relation to other anti-oxidant and pro-oxidant components of the diet to determine the optimum dietary Mn levels to minimize oxidative risk in this species.

Interactions between manganese and other minerals have been observed in several species including cobia (Nie et al., 2016), grouper (Ye et al., 2009), hybrid grouper (Liu et al., 2017), large yellow croaker (Zhang et al., 2016), where increasing the dietary content of manganese tended to alter the mineral composition of whole body, liver and vertebrae for copper, iron and zinc. Iron and manganese interactions in these studies may be due to competition for the transporters that both minerals share, which include ferroportin and DMT1 (or DCT1) (Madejczyk and Ballatori, 2012; Rossander-Hultén et

al., 1991; Gunshin et al., 1997). However, this effect was not observed in the present trial perhaps due to the lower levels of iron supplementation compared to other studies, which in turn resulted in a lower iron tissue content, thus reducing the competition for the common transporters. Whatever the case may be, the mineral contents in gilthead sea bream tissues were not affected by manganese supplementation.

Dietary manganese did not alter liver morphology of gilthead sea bream juvenile. Fish submitted to large amounts of water-borne manganese and other heavy metals presented a series of liver alterations in morphology, those apparently more specific to manganese toxicity included pycnotic degeneration of hepatocytes in *Lates calcarifer* (Krishnani et al., 2003); congestion and dilatation in the sinusoids and cytoplasmic and nuclear degeneration with extensive cytoplasmic vacuolation and pyknotic nuclei in the liver tissue in Nile tilapia (Alm-Eldeen et al., 2018); cytoplasmic degeneration, severe cell death, melano-macrophage centres, presence of pyknotic nuclei, mild necrosis, nuclear degeneration and hyper-vacuolization in *Labeo rohita* (Kaur et al., 2018). Nevertheless, these observations were made in fish from highly polluted rivers, or submitted to very high manganese intoxications, while no liver alterations are mentioned in trials conducted on fish with dietary manganese nor in the present trial.

Manganese is an essential metal that forms part of *MnSOD*, and intervenes in preventing the initiation of the free radical chain reaction. It is mainly located in the mitochondria, where it protects the cell from the harmful reactive oxygen species produced as a by-product of vital cycles such as β-oxidation of fatty acids or ATP synthesis. Therefore, deficiencies of this mineral can reduce MnSOD activity and increase lipid peroxidation (Holley et al., 2011). MnSOD activity has been used as a criterion to assess manganese requirements in hybrid tilapia (Lin et al., 2008), yellow catfish (Tan et al., 2012) or stinging catfish (*Heteropneustes fossilis,* Zafar et al., 2019),

where fish fed diets without manganese supplementation (2.89, 3.1 or 1.85 mg Mn kg^{-1}, respectively) presented lower liver MnSOD activity than fish fed diets supplemented with manganese. In the present trial, *MnSOD* expression significantly decreased with increasing dietary Mn supplementation over 30 mg Mn kg^{-1}, suggesting a reduction in oxidative risk with the increase in dietary Mn. Besides, *CAT* expression was doubled in liver of sea bream fed the lowest Mn levels (19 mg Mn kg-^{1}), suggesting a higher oxidative risk in fish fed the non-supplemented diet.

In summary, the presence of manganese in higher concentrations in plant ingredients than animal sources suggests that practical diets based on plant ingredients may contain sufficient manganese to cover the requirements for gilthead sea bream fingerlings. In the present study, markers for growth, feed utilization, whole body chemical composition or liver morphology were not affected by manganese supplementation, suggesting that the manganese content present in the basal diet (19 mg Mn kg^{-1}) was sufficient to cover the requirement of juvenile gilthead sea bream fed practical plant-based diets, which remains lower than the maximum tolerable levels established by EFSA (2016) for fish (1000 mg Mn kg^{-1}). Nevertheless, an indication for reduction in oxidative risk in fish fed higher Mn dietary levels suggest a higher requirement for antioxidant protection. Further studies must be conducted to determine the optimum dietary Mn levels to minimize oxidative risk in this species, specifically under challenging conditions.

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6. Declaration of Interest

R. Fontanillas is an employee of Skretting AS, Stavanger, Norway

7. Author Contributions

David Dominguez conceived and designed the experiments, performed the experiments, performed analyses, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Zakarya Sehnine performed the experiments, performed analyses, analyzed the data.

Pedro Castro performed analyses, analyzed the data.

Lidia Robaina contributed reagents/materials/analysis tools, reviewed drafts of the paper.

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Marisol Izquierdo conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

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Author statements have been included in the text.

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Declaration of interests

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

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

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- The effects of dietary Mn in plant based feeds for gilthead sea bream was studied.
- The high Mn contents in the basal diet was sufficient to promote sea bream growth.
- Dietary Mn contents beyond 30 mg Mn kg^{-1} down-regulated *MnSOD* expression.
- Overall other parameters remained unaffected by the dietary Mn content.
- Under high oxidative stress, markers suggest the need to increase Mn levels.